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Streptococcus pyogenes

Basic Biology to Clinical Manifestations

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Streptococcal diseases have been recognized in recorded history for over two thousand years and remain today as a serious cause of worldwide health problems. Early research revealed that the streptococci were not only among the first organisms thought to be the cause of contagious diseases, but their presence initiated the introduction of cleanliness and the use of sterile procedures into hospital settings. More recent research on streptococci demonstrated that the hereditary material was DNA, paving the way to present day molecular and genomic studies. This book is focused on one of the streptococci, *Streptococcus pyogenes* (the group A Streptococcus), the bacteria responsible for diseases, such as scarlet fever, pharyngitis, impetigo, cellulitis, necrotizing fasciitis and toxic shock syndrome, as well as the sequelae of rheumatic fever and acute poststreptococcal glomerulonephritis. The goal of the 30 chapters in this volume is to present an up to date and comprehensive review of research on this organism, including its basic biology, epidemiology, genetics and pathways that facilitate group A streptococcal infections. Our intention is that this information will provide an important resource for the general public, students, researchers, and clinicians in future work towards an understanding of the mechanism of *Streptococcus pyogenes* disease, in hopes that it will lead to better methods of disease control.

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Table of Contents

Preface

Diseases caused by *Streptococcus pyogenes,* also known as the group A streptococcus, have been described since the early times of written history. While we have come to know a great deal about this human specific pathogen and its associated diseases, there is still much to learn. The aim of this volume is to present a current summary of every aspect of this microorganism: in other words, the A to Z of contemporary information about *Streptococcus pyogenes*.

The various topics covered in the following chapters are presented in a progression that ranges from basic biology to clinical manifestations. Early research on *Streptococcus pyogenes* during the last century was aimed at developing a vaccine to prevent its many associated diseases, and in the process, provided much of the basic biology, physiology, and immunology that has been essential for ongoing research. With the advent of genetic and molecular biology approaches, as well as the new tools of genetic engineering, DNA and protein sequencing, a new era of –omics appeared, including genomics, proteomics, and metabolomics. Information from these studies has been applied to the development of vaccines, understanding genetic regulation, and epidemiology.

Infections with *Streptococcus pyogenes* result in a wider variety of diseases than perhaps any other microorganism, ranging from throat and skin infections, scarlet fever, puerperal fever, and necrotizing fasciitis, to the post-infection sequelae of rheumatic fever and acute glomerulonephritis. More recent disease descriptions in the 1980s and 1990s include diseases such as streptococcal toxic shock syndrome and pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections (or PANDAS). Epidemiological studies have been important in defining these recent diseases, as well as providing a greater understanding of their transmission, control, and prevention. Moreover, a variety of model systems provide new information about mechanisms of pathogenesis, as well as insights into intracellular invasion and the carrier state. Finally, the development of a vaccine, as well as new and innovative methods of anti-infective control will be important areas of continued research.

This book represents a concerted effort by an international group of *S. pyogenes* researchers, each an expert in their own area of work, who have generously provided their time and energy to present the current status of work in their own field. In virtually all areas, their work was supported with financial assistance from various national and international research institutions, organizations, and foundations. In recognition of this support, these researchers pay forward their scientific knowledge to the general public, as well as to the next generation of students, scholars, and researchers. As a living document in an open access format, additions of new information and updates on each topic can be provided on a regular basis. Our hope is that this volume will serve not only as an important resource and standard reference for public information, but also an aid to stimulate further research that will lead to better methods of disease control and treatment.

Editors

Joseph J. Ferretti, Ph.D., is an emeritus professor in the Department of Microbiology and Immunology at the University of Oklahoma Health Sciences Center in Oklahoma City, Oklahoma. He has worked in the area of *Streptococcus pyogenes* research for over 50 years since his early days at the University of Minnesota, where he trained under the tutelage of Lewis Wannamaker, Elia Ayoub, and Ernest Gray. Following a postdoctoral fellowship at The Johns Hopkins University, where he worked with Philip E. Hartman in the study of microbial genetics, he joined the University of Oklahoma Health Sciences Center in 1969. His main research contributions, supported by years of support from NIH and other agencies, have been in the areas of streptococcal genetics, regulation of virulence factors, bacteriophages, and genomics. Dr. Ferretti is the author or co-editor of four books, including Basic Microbiology and Genetics; Streptococcal Genetics; Genetics of Streptococci, Enterococci, and Lactococci; and the ASM book on Gram-Positive Pathogens.

Dennis L. Stevens, Ph.D., M.D., is Chief of the Infectious Diseases Section, Director of Research and Development of the Veterans Affairs Medical Center in Boise, Idaho and Professor of Medicine at the University of Washington's School of Medicine in Seattle, Washington. Dr. Stevens obtained a Ph.D. in Microbiology from Montana State University and an MD from the University of Utah. Dr. Stevens' major research interests have been the pathogenesis of serious infections caused by toxin-producing Gram positive pathogens, including *Clostridium perfringens*, *Clostridium sordellii*, group A streptococcus, and methicillin-resistant *Staphylococcus aureus* (MRSA). He is a member of the CDC Working Group on Invasive Streptococcal Infections, a consultant to the WHO, and an invited participant to the NIH Workforce on severe group A streptococcal infections. Dr. Stevens is editor or co-editor of four books on skin and soft tissue infection, including co-editor with Dr. Edward Kaplan of the book Streptococcal Infections: Clinical Aspects, Microbiology, and Molecular Pathogenesis.

Vincent A. Fischetti, Ph.D., is Professor and Chairman of the Laboratory of Bacterial Pathogenesis and Immunology at the Rockefeller University in New York, and received his Ph.D. in Microbiology from New York University. He has over 50 years of experience in the field of anti-infectives, focusing on understanding the earliest events in Gram-positive bacterial infections. Over the years, one of his interests has been to examine the surface molecules on Gram-positive bacteria—particularly the surface M protein on *S. pyogenes* and its coiled-coil structure. His work with bacteriophages has led to the development of phage lysins as novel therapeutics. Dr. Fischetti is a fellow of the American Academy of Microbiology, and is the recipient of two NIH MERIT awards. He has been editor-in-chief of the ASM journal Infection and Immunity for 10 years, and is a co-editor of the ASM book Gram-Positive Pathogens.

Acknowledgments

We take this opportunity to first thank all the authors who have contributed and shared their expertise to make this book a reality. Additionally, our appreciation extends to the many international colleagues, who served as reviewers of each chapter, for their expertise, interest and encouragement.

The seed for this book has it origins from the many meetings of international collaborators who have attended the Lancefield International Symposia on Streptococci and Streptococcal Diseases and the ASM International Meetings on Streptococcal Genetics, which had their beginnings in the 1960s and 1980s, respectively. This forum of sharing and dissemination of information allowed collaborations to advance the field in many important ways. Although the proceedings of these meetings are no longer published, we hope that the present volume will serve as a resource and standard reference for the future.

We acknowledge the Presbyterian Health Foundation of Oklahoma City for its generous support of all the technical and editorial assistance involved in this work, and also the University of Oklahoma Health Sciences Center Library for serving as the book publisher. We are extremely grateful to our colleague and friend, Michael S. Gilmore, who advised us on almost every step of this effort, based on his experience in the publication of a similar volume on the Enterococci. A special thank you and appreciation goes to Mary Van Tyne, the copy editor of this volume, for her extraordinary expertise, efficiency, and timeliness in the completion of this book. It would not have been possible to complete this effort without her assistance. We also thank Manfred Rohde for his contribution of the electron micrograph image on the cover page, and Kyle Vogel for his improvement of figures within the work. Finally, a great deal of appreciation goes to Marilu A. Hoeppner and her staff at the National Center for Biotechnology Information Bookshelf, who provided us with guidance and assistance to bring this project to completion.

History of Streptococcal Research

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Introduction

Streptococcal diseases have been known for centuries, although their delineation into separate disease entities did not begin to occur until the 16th century AD. The original writings of Hippocrates from the 4th century BC describe the disease erysipelas (ἐρυσίπελας, red skin), as well as the symptoms of childbed fever, and "Galen remarks that not only erysipelas, but also inflammation, when it attacks the impregnated uterus, generally proves fatal" (Adams, 1849). Centuries later, after epidemic rates of mortality, this particular disease was recognized in 1716 as puerperal fever or childbed fever (Strother, 1716). Many theories about the origin of diseases appeared in the intervening centuries, including associating their occurrence with natural phenomena like comets and eclipses; the spread of disease by vapors and smells; "contagion," or the transferring of diseases from one to another; and the introduction of non-living matter into a patient (Karamanou, Panayiotakopoulos, Tsoucalas, Kousoulis, & Androutsos, 2012). It was not until the 18th century that further progress into the etiology of diseases was made. A major advancement was the invention of the microscope by Anton van Leeuwenhoek (1632-1723) as well as his descriptions of new life forms, including the shapes of cocci, bacilli, and spirochetes (Figure 1) (Dobell, 1932). However, as with a number of early scientific and medical discoveries or observations, the link between the microscopic discovery of these new life forms and diseases was not realized until almost two centuries later.

Scarlet Fever

Reviews of the writings of ancient scholars found many passages relating to sore throats or ulcerous tonsils, but lacked comment about an associated rash, which would be typical of what would later be known as scarlet fever. According to Rolleston (Rolleston, 1928) in his article on the history of scarlet fever, Giovanni Filippo Ingrassias, a Sicilian anatomist and practitioner, wrote in 1553 (Ingrassia, 1553) the first description of a disease termed "rossalia" that was characterized by "numerous spots, large and small, fiery and red, of universal distribution, so that the whole body appeared to be on fire." He also reported that this "rossalia" or rash was different than that observed in measles. (Rolleston, 1928). According to most scholars, Johann Weyer of the Netherlands was the first to describe a sore throat occurring during epidemics of *scarlatina anginosa*, which he did in 1565. In 1578, Jean Cottyar of Poitiers gave the first definitive description of scarlet fever in France as a "general weariness, headache, redness of the eyes, sore throat, and fever. Purpura appeared on the second or third day, accompanied by delirium and soreness of throat". Daniel Sennert (Sennert, 1628) described an epidemic in Wittenberg in the beginning of the seventeenth century and was the first to describe scarlatinal desquamation, arthritis, and post-scarlatinal dropsy and ascites. The term "scarlatina" was first introduced into the medical literature in 1675 by Sydenham (Sydenham, 1676), who identified it as a separate disease entity from other exanthemas, especially measles (Rolleston, 1928).

Epidemics of scarlet fever were reported throughout Europe and North America during the 17th and 18th centuries, some of which were associated with high mortalities. It was not until the 1920s that George and Gladys Dick showed that scarlet fever was associated with a sore throat caused by hemolytic streptococci that

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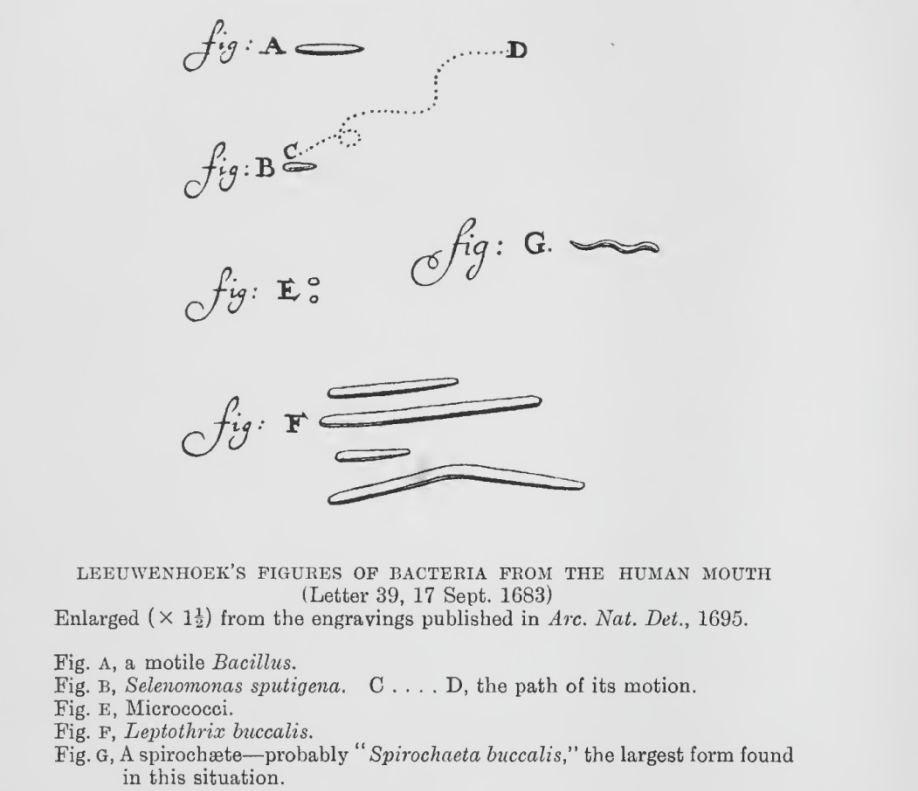

LEEUWENHOEK'S FIGURES OF BACTERIA FROM THE HUMAN MOUTH
(Letter 39, 17 Sept. 1683)
Enlarged (× 1½) from the engravings published in *Arc. Nat. Det.*, 1695.

Fig. A, a motile *Bacillus*.
Fig. B, *Selenomonas sputigena*. C . . . . D, the path of its motion.
Fig. E, Micrococci.
Fig. F, *Leptothrix buccalis*.
Fig. G, A spirochæte—probably "*Spirochaeta buccalis*," the largest form found in this situation.

Figure 1. Leeuwenhoek's drawings of bacteria from the human mouth.

produced a secreted toxin known as scarlet fever toxin, or Dick toxin (Dick & Dick, 1924a; Dick & Dick, 1924b; Dick & Dick, 1924c). Scarlet fever remained an important infectious disease until the advent of antibiotic therapy in the 1940s, and prior to that time, a brightly colored quarantine sign was placed on the door of the house of diseased individuals to isolate the patient and prevent the further spread of this feared disease (Figure 2).

Puerperal Fever

Although streptococcal diseases were not recognized as a cause of puerperal fever at the time, epidemics associated with high mortality rates during childbirth were reported across Europe and North America in the 18th and 19th centuries. In 1843, the American surgeon Oliver Wendel Holmes, Sr. (Figure 3), published a paper on "*The Contagiousness of Puerperal fever*" (Holmes, 1843). Holmes wrote, "This disease seized such women only as they were visited or delivered by a practitioner, or taken care of by a nurse, who had previously attended patients affected with the disease" (Holmes, 1843). His observations were the subject of more debate than application, and many of his contemporaries responded to his report with more ridicule than acceptance.

In 1842, Ignac Semmelweis, a young Hungarian physician (Figure 4) working in the obstetric wards of the Vienna Lying-in Hospital (Semmelweis, 1983; Semmelweis Society, 2009), independently monitored hospital mortality rates and observed that the mortality rates of women giving birth attended by physicians in Clinic 1 were almost ten times higher than when midwives attended the deliveries in Clinic 2. He observed that physicians and medical students who arrived at the hospital in the morning first visited the autopsy rooms before seeing patients in other wards. Because physicians and medical students did not wash their hands before going to the obstetric ward, Semmelweis surmised they were physically carrying the disease entity from cadaverous material directly to patients. In 1847, he ordered all medical attendants to scrub their hands in a chlorinated lime solution before entering the obstetric ward. After seven months, the mortality from puerperal

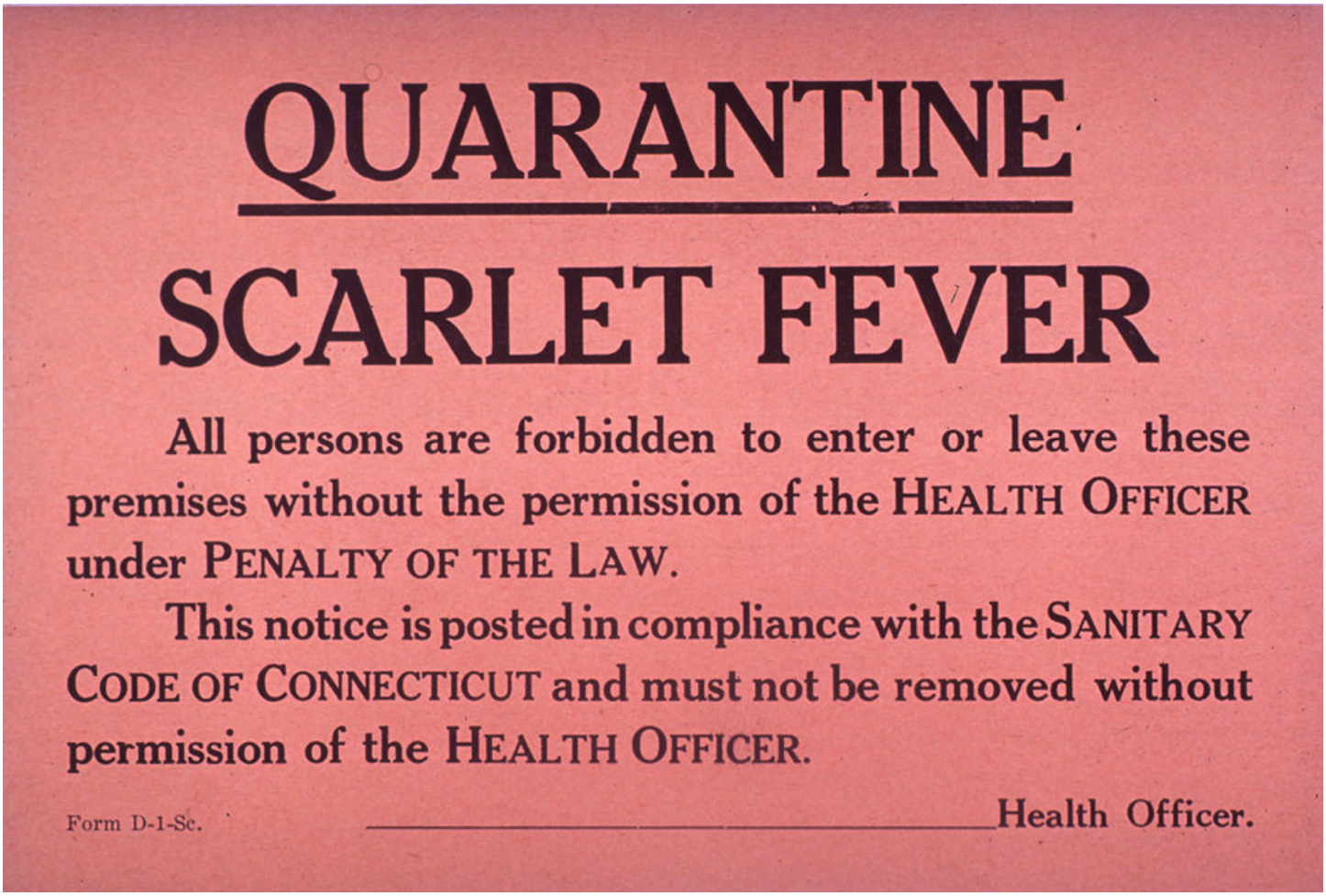

Figure 2. Scarlet Fever Quarantine sign.

fever in Clinic 1 decreased dramatically, from 11.4% to 2.7%. Additionally, he noted that soiled bed sheets containing blood or other bodily materials were not changed between patients. In 1848, chlorine solution washings were carried out during the year, and Semmelweis also ordered the changing of sheets between patients. His recordings showed that the mortality rate that year in Clinic 1 was 1.27% and in Clinic 2 was 1.3%. Though the results were profound, Semmelweis had no scientific evidence to explain the cause of the disease—except cleanliness. Nevertheless, his immediate superiors resented Semmelweis's *Lehre* (teaching) methods and treated his findings with skepticism and ridicule. In 1849, Semmelweis returned to Budapest, because his contract was not renewed at the Vienna Hospital. In 1860, Semmelweis published his detailed observations in a treatise, *The Etiology, Concept and Prophylaxis of Childbed Fever* (Semmelweis, 1983). He was an early revolutionary in the medical sciences in the use of statistical methods to prove his hypothesis that childbed fever was caused by medical personnel failing to wash their hands and who then transmitted the disease to patients.

A full recognition of Semmelweis's brilliant work did not come until 14 years after his death in 1865. In a discussion on puerperal fever at the French Academy of Sciences in Paris in 1879, a physician named Hervieux elaborated on the causes of epidemics in lying-in hospitals, ascribing them to an undefined "puerperal miasma. Louis Pasteur interrupted him: 'None of those things cause the epidemic; it is the nursing and medical staff who carry the microbe from an infected woman to a healthy one.' And when the speaker replied that he feared the microbe would never be found, Pasteur went to the blackboard and drew a diagram of the dangerous chain-forming microbe, saying, 'There: This is what it looks like'" (Pasteur, 1879; Schwartz, 1997). A complete and

detailed history of puerperal fever can be found in two books by Irvine Louden: *Childbed Fever, A Documentary History*; 1995 (Loudon, 1995) and *The Tragedy of Childbed Fever*, 2000 (Loudon, 2000).

Discovery of Streptococci

The first description of streptococcal infection is attributed to the Austrian surgeon, Theodor Billroth (Figure 5), in 1874, when he described the organism in cases of erysipelas and wound infections (Billroth, 1874; Billroth, 1877). He described these "small organisms (Kettenkokken) as found in either isolated or arranged in pairs, sometimes in chains of four to twenty or more links (Streptococcus; Gr. strepto, a chain, and coccus, a berry)." The real importance and formal entry of streptococci to history came in 1879 when Louis Pasteur (Figure 6) isolated the microorganism from the uteruses and blood of women with puerperal fever (Alouf & Horaud, 1997). He further demonstrated that the streptococcus was the etiological agent responsible for the disease that caused the highest mortality rates of women and newborns at that time. Additional refinement of the name streptococcus came from Friedrich Julius Rosenbach in 1884, who examined bacteria isolated from suppurative lesions, and the species was named *Streptococcus pyogenes* (Gr., pyo, pus, and genes, forming) (Evans, 1936). Previously, Fehleisen isolated streptococci from a patient with erysipelas and Rosenbach named the organism *S. erysepaltis* (Evans, 1936; Rosenbach, 1884). However, later reviews showed that there was no particular characteristic associated with organisms isolated from specific diseases, and Andrewes and Christie suggested that the proposed species names of pyogenes, eryespaltis, scarlatinae, and puerperalis all be included in the single name *Streptococcus pyogenes* (Evans, 1936; Andrewes & Christie, 1932).

Differentiation and Classification of Streptococci

The introduction of blood agar plates by Hugo Schottmuller in 1903 was an important step forward for the differentiation of streptococci (Schottmuller, 1903; Becker, 1916). When mixtures of streptococci were streaked on blood agar plates, two types of hemolysis were observed: a clear zone surrounding a colony containing organisms in long chains, termed *Streptococcus haemolyticus*; and a green zone surrounding a colony with short chains, termed *Streptococcus viridans*. Further elaboration by Brown in 1919 (Brown, 1919) resulted in three classes of hemolytic patterns surrounding a colony on a blood agar plate: 1) "Alpha"—a green zone of discoloration, which is characteristic of the viridans streptococci; 2) "Beta"—a clear zone of complete hemolysis, which is found in the *S. haemolyticus* type organisms; and 3) "Gamma"—no change in the medium, which is characteristic of the enterococcus and *S. faecalis* organisms. At the same time, Dochez, Avery, and Lancefield began using immunological approaches to demonstrate that differences could be demonstrated between strains of *S. haemolyticus* (Dochez, Avery, & Lancefield, 1919). In 1933, Lancefield used surface antigen differences among various streptococci to further subdivide them into groups designated by the letters A through X (Köhler, 2008). The strains from human diseases were classified as Group A; the strains from bovine and dairy sources as Group B; the strains from a variety of animal sources as Group C; and so forth. For epidemiological studies, the determination of the T-antigens by slide agglutination was introduced by Fred Griffith of London in 1934 (Griffith, 1934) and was widely used, even in International Type Distributions Surveys of *S. pyogenes* at the end of the 1960s (Kohler, 1974). The Group A strains, *Streptococcus pyogenes*, were further subdivided according to the presence of a surface protein named M protein (due to its matte appearance in colony formations) into different antigenic types. Lancefield was able to demonstrate over 50 different M-types of group A streptococci during her career (Lancefield, 1962; National Academy of Sciences, 1987), and since then, more than 200 M-types have been identified using a combination of serological and molecular typing methods. Lancefield also identified the M protein as the major virulence factor of *Streptococcus pyogenes* due to its antiphagocytic property (Lancefield, 1962). Maclyn McCarty considered Lancefield's monumental work as "the opening chapter in the modern investigation of hemolytic streptococci" (Figure 7) (National Academy of Sciences, 1987). The importance of this classification system is underscored by its continued development and use in clinical, bacteriologic, and epidemiologic studies. Facklam has provided a summary of the nomenclature and taxonomy

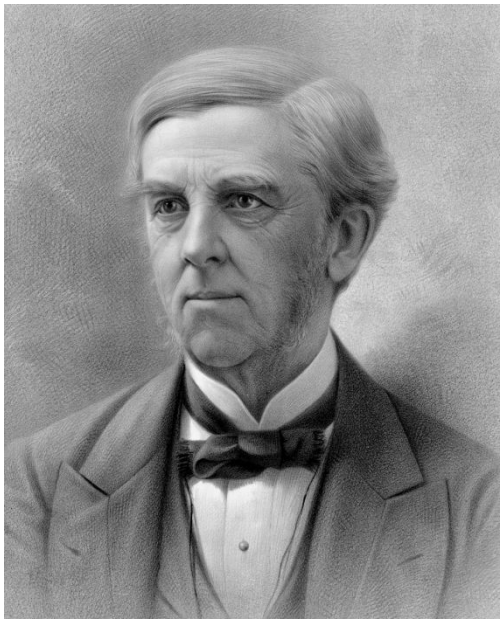

Figure 3. Oliver Wendell Holmes Sr., 1809–1894.

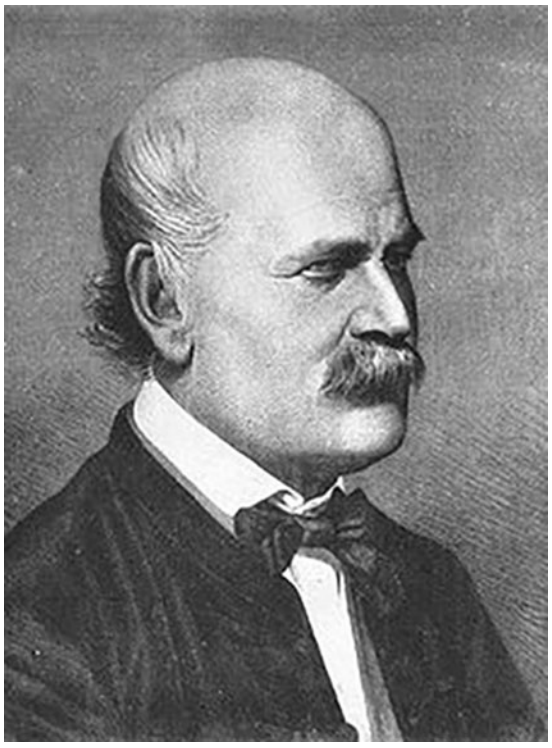

Figure 4. Ignaz Semmelweis, 1818–1865.

changes of the Streptococcus genus that have occurred, up to the year 2002 (Facklam, 2002). More recent descriptions of the "present state of species within the genus Streptococcus and Enterococcus" by Köhler (Köhler, 2007), and a "phylogenomic analysis of genome evolution in the genus Streptococcus" by Richards et al. (Richards, et al., 2014) provide important new overviews of the genus Streptococcus.

Figure 5. Theodor Billroth, 1829–1894.

Figure 6. Louis Pasteur, 1822–1895.

Figure 7. Walter Bauer, Rebecca Lancefield, and Maclyn McCarty at the Rockefeller Institute.

Biology of the Group A Streptococci

Early research in the first part of the 20th century on the biology of the hemolytic streptococci was perhaps more intensive than the research conducted on any other species of pathogenic bacteria. These studies focused on the basic biology and physiology of the organism, with an emphasis on the relationship of the organism to the important diseases of the day, scarlet fever and rheumatic fever. As mentioned earlier, one of the most important advances was the study by Lancefield that classified the organisms into various serological groups and identified the group A streptococcus, *Streptococcus pyogenes*, as the organism responsible for most of the hemolytic streptococcal infections in humans (Lancefield, 1941). An overview of many of the early studies on the biology of the group A streptococci is presented in the following sections; however, more detailed and comprehensive reviews can be found in McCarty (McCarty, 1952); Wannamaker and Matsen (Wannamaker & Matsen, 1972); Stollerman (Stollerman, 1975); and Köhler (Köhler, 2008).

An essay about collaborative research by Ferretti (Ferretti, 2008) describes "events during the early 1960s in which streptococcal research flourished and was propelled by interactions at many levels, particularly international conferences and symposia. The World Health Organization sponsored an international symposium on streptococci and streptococcal diseases in 1960 in Prague, Czechoslovakia. Two of the twenty-six participants in attendance were Werner Köhler from the German Democratic Republic and Dennis Watson from the United States, both technically non-members of the WHO. Following this conference, Köhler suggested continuation of these meetings, and three years later in 1963, the second conference was held in Jena, GDR, with a three-fold increase in attendance. The third conference was held in 1966 in Paris, and a fourth was again held in Jena in 1969. Thus, the first meeting in Jena was a starting point for what is now known as the Lancefield International Symposium on Streptococci and Streptococcal Diseases." This symposium has been held every three years in various cities in the world (as recorded in Table 1) and continues to be an important forum for discussion, learning, and collaboration about current and new topics in streptococcal research.

Table 1. Lancefield International Symposium on Streptococci and Streptococcal Diseases

Symposium	Year	City	Country	Organizer	Reference
I	1960	Prague	Czech Republic	Raska, K	
II	1963	Jena	East Germany	Köhler, W	(Köhler, 1964)
III	1966	Paris	France	Caravano, R	(Caravano, 1968)
IV	1969	Jena	East Germany	Köhler, W	
V	1972	Amsterdam	Netherlands	Haverkorn, MJ	(Haverkorn, 1974)
VI	1975	Liblice	Czech Republic	Rotta, J	
VII	1978	Oxford	England	Williams, REO	(Parker, 1979)
VIII	1981	Lund	Sweden	Holm, SE	(Holm & Christensen, 1982)
IX	1984	Yamanaka	Japan	Kimura, Y	(Kimura, Kotani, & Shiokawa, 1985)
X	1987	Cologne	West Germany	Lütticken, R	
XI	1990	Siena	Italy	Orefici, G	(Orefici, 1992)
XII	1993	St. Petersburg	Russia	Totolian, A	(Totolian, 1994)
XIII	1996	Paris	France	Horaud, T	(Horaud, Bouvet, Leclerq, de Montclos, & Sicard, 1997)
XIV	1999	Auckland	New Zealand	Martin, D	(Martin & Tagg, 2000)
XV	2002	Goa	India	Ganguly, NK	(Chhatwal, 2004)
XVI	2005	Palm Cove	Australia	Good, MF	(Sriprakash, 2006)
XVII	2008	Porto Heli	Greece	Legakis, NJ	
XVIII	2011	Palermo	Italy	Orefici, G	
XIX	2014	Buenos Aires	Argentina	Lopardo, H	
XX	2017		Fiji		

Cultural Conditions and Growth

The group A streptococci are fastidious organisms that have complex growth requirements. A highly nutritious growth medium that provides optimal growth was described in 1932 by Todd and Hewitt that consisted of a broth of meat extract, peptones, dextrose, and salts (Todd & Hewitt, 1932). This medium was subsequently prepared in a dehydrated form according to a formula by Updyke and Nickle in 1954, and is still used in present times for the growth of group A streptococci and other fastidious organisms (Updyke & Nickle, 1954). In order to further study the nature of extracellular toxins, such as streptolysin O, Bernheimer and Pappenheimer developed a defined protein-free medium that produced a large amount of growth (Bernheimer & Pappenheimer, Jr., 1942). Later studies showed that the alteration of environmental conditions could increase the amounts of various extracellular products. For example, the addition of a digested RNA fraction increased streptolysin S production (Bernheimer & Rodbart, 1948); while the addition of reducing agents (such as glutathione) increased streptolysin O production (Slade & Knox, 1950); maintaining a slightly acidic pH enhanced the production of the cysteine proteinase precursor (Elliott, 1950); and the addition of hyaluronate to the growth medium increased the production of hyaluronidase (Rogers, 1945). Various formulations of a chemically defined medium were described thereafter; however, most of these have suffered from the inability to provide adequate growth yields and/or the production of different virulence factors. A new chemically defined medium, described by van de Rijn and Kessler in 1980, overcame many of the previous deficiencies and provided high culture densities as well as adequate amounts of virulence factors, such as M protein and extracellular

products (van de Rijn & Kessler, 1980). A more detailed description of the metabolism of the group A streptococci can be found in a subsequent chapter.

Ultrastructure and Cellular Components

In initial studies of the biology of hemolytic streptococci, immunological differences were found to exist among strains isolated from human disease. These organisms were identified as being strongly Gram-positive and growing in chains of varying length (Dochez, Avery, & Lancefield, 1919). Colonies formed on agar plates were identified as small, smooth, and moist in appearance, but capsule formation was not identified at that time. Almost 20 years later, a polysaccharide composed of N-acetylglucosamine and glucuronic acid was isolated from encapsulated group A streptococci and was found to be identical to hyaluronic acid found in mammalian tissue (Kendall, Heidelberger, & Dawson, 1937). This capsular material did not stimulate antibody production, an observation consistent with the fact that this substance occurs naturally in mammalian tissues. A comprehensive review of the capsular polysaccharide of group A streptococci and its biochemistry, genetics, and role in virulence has been reported by Wessels (Wessels, 2006), and in a subsequent chapter in this volume.

The studies of Lancefield and colleagues have provided a great deal of information about the antigenic structure of the streptococcal cell, particularly about the M protein and its importance as the major virulence factor of the group A streptococci (Lancefield, 1962). In addition to the 1962 review by Lancefield (Loudon, 2000), detailed reviews on the streptococcal M protein have been presented by Fox in 1974 (Fox, 1974), Fischetti in 1989 (Fischetti, 1989), and Fischetti et al. in another chapter in this volume.

Besides the M protein, many more proteins have been found on the cell surface by their ability to bind mammalian serum or secretory proteins, such as immunoglobulins, albumin, fibrinogen, and fibronectin, or by enzymatic digestion. These include T-protein, serum opacity factor, C5a peptidase, collagen-like protein Scl1, GRAB, and protein F, among others. Fischetti and colleagues have shown that the C-terminal region of these anchored proteins contains a highly conserved heptapeptide consensus sequence of LPXTG, which is an anchor signal found in most surface proteins from Gram-positive bacteria for covalent attachment to the peptidoglycans (Mazmanian, Ton-That, & Schneewind, 2001). Finally, Mora and colleagues reported that group A streptococci possess pilus-like structures that contain protective antigens and Lancefield T antigens (Mora, et al., 2005). A recent publication that used microarray analysis identified more than 80 different proteins on the surface of an M1 strain of group A streptococci (Galeotti, et al., 2012).

A major component of the streptococcal cell wall, making up approximately 50 percent of its weight, was found to be carbohydrate in nature and was identified as the group-specific carbohydrate (Lancefield, 1941). Eighteen groups of specific antigens were established by Lancefield, and the group A carbohydrate was subsequently shown to be composed of a polymer of rhamnose and *N*-acetylglucosamine (Krause & McCarty, 1961). The next several chapters of this volume present a more detailed and comprehensive view of the ultrastructure of the group A streptococci.

Streptococcal Bacteriophages

Bacteriophages were first independently identified by Twort in 1915 and were later confirmed by d'Herelle in 1917. Frederick Twort, an English bacteriologist, discovered agents that he termed "filter-passing viruses" which required bacteria for growth (Twort, 1915). Twort's studies were interrupted by World War I, and as a result, he did not return to research until several years later. Felix d'Herelle, a French-Canadian microbiologist, first applied the name "bacteriophage" (from bacteria and the Greek word *phagein*, "to devour") to a phage that was able to kill a number of pathogenic bacteria, including streptococci (d'Herelle, 1915). Subsequent work relating to these two investigators resulted in the "Twort-d'Herelle Controversy," a topic covered in detail in other historical reviews (Ackermann, Martin, Vieu, & Nicolle, 1982; Duckworth, 1976; Fruciano & Bourne, 2007; Summers, 1999).

In 1924, a report by Dick and Dick (Dick & Dick, 1924a) about the presence of a rash-producing substance in the culture filtrates of certain hemolytic streptococci stimulated further research on the substance, and was variously termed as scarlet fever toxin, Dick toxin (Dick & Dick, 1924b), scarlatinal toxin (Trask, 1926), or erythrogenic toxin (Stock, 1939). Shortly after, the role of a filterable factor (bacteriophage) that was able to change a non-toxigenic streptococcal strain to a toxigenic strain was reported by Cantacuzène and Bonciu (Cantacuzène, 1926) in 1926, and Frobisher and Brown in 1927 (Brown, 1927), and was later confirmed by Bingel in 1947 (Bingel, 1949).

In the intervening years, various investigators found both virulent and temperate phages in streptococci. Clark and Clark (Clark & Clark, 1927) and Evans (Evans, 1934a; Evans, 1934b; Evans, 1934c), identified virulent phages isolated from sewage, and Kjems (Kjems, 1955) later described methods to isolate temperate phages from *S. pyogenes*. Kjems further observed that the virulent phage obtained from sewage were unable to penetrate the hyaluronic capsule of *S. pyogenes*, whereas the temperate phage possessed its own hyaluronidase (Maxted, 1955) to penetrate the capsule (Kjems, 1958). In a following study, Kjems found that lysogeny was a common occurrence in group A streptococci and could be found in up to 83% of strains tested (Kjems, 1960).

Transduction by streptococcal bacteriophages was first demonstrated by Leonard et al. of streptomycin resistance by several group A streptococcal phages (Leonard, Colón, & Cole, 1968). Malke demonstrated the phage A25-mediated transfer induction of a prophage in *S. pyogenes*, as well as the transfer of antibiotic resistance markers in strains of 30 different representative group A streptococcal M types (Malke, 1972; Malke, 1973; Malke & Köhler, 1973). Tagg was able to transduce bacteriocin production and host cell immunity into three different group A strains (Tagg, Skjold, & Wannamaker, 1976). The advent of molecular cloning and successful artificial transformation procedures in group A streptococci transcended the further use of transduction as a tool in the genetic analysis of these organisms, but nevertheless, the importance of this form of horizontal gene transfer to streptococcal evolution remains an important consideration (McLaughlin & Ferretti, 1995).

In 1964, Zabriskie reported on the conversion of a non-toxigenic *S. pyogenes* strain $T25_3$ to toxin production by phage T12 isolated from toxigenic strains (Zabriskie, 1964). The new toxigenic strains were $T25_3$(T12) lysogens that produced erythrogenic toxin; or, as later termed, streptococcal pyrogenic exotoxin A(Watson, 1960). These observations were later confirmed by Nida and Ferretti (Nida, Houston, & Ferretti, 1979; Nida & Ferretti, 1982), Johnson et al (Johnson & Schlievert, 1984; Johnson, Schlievert, & Watson, 1980), and McKane and Ferretti (McKane & Ferretti, 1981) employing the same $T25_3$ non-lysogen and T12 phage system that Zabriskie reported. The demonstration that T12 phage contained the structural gene for the streptococcal pyrogenic exotoxin A (*speA*) was confirmed independently by Weeks and Ferretti (Weeks & Ferretti, 1984) and Johnson and Schlievert (Johnson & Schlievert, 1984). In subsequent experiments, the *speA* containing T12 bacteriophage was shown to integrate into a gene that encodes a serine tRNA in the host chromosome (McShan, Tang, & Ferretti, 1997). Further information about bacteriophages can be found in the bacteriophage chapter of this volume.

Extracellular Products

The group A streptococci produce a large number of proteins that are secreted into the extracellular fluid during growth. Many of these proteins were originally detected by their biological activities or by immunological detection with human antiserum. Historically, the first extracellular products studied were those thought to be directly related to diseases; namely, erythrogenic toxin, streptolysin O, streptolysin S, proteinase, streptokinase, DNase, RNase, and hyaluronidase (McCarty, 1952). As research in the area of genetics and genomics has increased in recent years, additional secreted extracellular products have been discovered, such as CAMP factor, streptococcal inhibitor of complement, immunogenic secreted protein, and a number of superantigens. Additionally, these secreted proteins may be encoded by either chromosomal or bacteriophage-associated genes. Many of these secreted proteins are known virulence factors and play important roles in colonization, invasion,

spreading, and pathogenesis. Comprehensive reviews on the topic of group A streptococcus extracellular products have been published by Alouf (Proft & Fraser, 2006; Alouf, 1980), Ginsburg (Ginsburg, 1972), Hynes (Hynes, 2004), McCormick et al (McCormick, Peterson, & Schlievert, 2006), and in another chapter in this volume.

Streptococcal Diseases

Streptococcus pyogenes is a strict human pathogen responsible for a wider variety of human diseases than perhaps any other microorganism. The classical diseases of the group A streptococci are now known by newer names. For example, streptococcal pharyngitis is a "strep sore throat," and if accompanied by a rash, it is known as scarlet fever. Skin infections categorized as pyoderma or impetigo may include erysipelas and cellulitis, which infect deeper layers of the skin. If these infections spread to the fascia, the disease is known as necrotizing fasciitis, which is sometimes referred to as a "flesh-eating disease" by the popular press. This is a severely invasive disease that, unless given immediate surgical and medical intervention, results in a high mortality rate. Another severe invasive disease is streptococcal toxic shock syndrome (STSS), a systemic disease entity recognized in the early 1990s (The Working Group on Severe Streptococcal Infections, 1993; Stevens, 1995) and is described further in the chapter on toxic shock in the present volume.

Additional diseases that occur as a result of a previous streptococcal infection are referred to as sequelae, including rheumatic fever (RF) and acute post-streptococcal glomerulonephritis (APSGN). Although the symptoms of these diseases were recognized in earlier times, the pathogenesis and association with group A streptococci were not clearly defined until the early 1900s. Excellent historical accounts of rheumatic fever are presented by Benedek (Benedek, 2006) and Stollerman (Stollerman, 1975), as well as in a subsequent chapter. Historical descriptions of APSGN are found in a review by Rodríguez-Iturbe and Batsford (Rodríguez-Iturbe & Batsford, 2007) in this volume.

An illness described in 1998 that may follow a streptococcal infection in children is Pediatric Autoimmune Neuropsychiatric Disorders Associated with Streptococcal infections (PANDAS) (Swedo, et al., 1998). This disorder may arise from multiple aetiologies, and its neuropsychiatric symptoms, such as tic disorders and obsessive-compulsive disorder (OCD), perhaps do not fit any known criteria of association with a streptococcal infection. However, PANDAS symptoms do seem to have similarities to neuropsychiatric symptoms that are associated with Sydenham's chorea, which is a disease associated with a prior group A streptococcal infection. The interest in this topic is both important to many and controversial to others, but it is nevertheless worthy of presentation in this volume as presented in a following chapter.

Treatment of Streptococcal Diseases

Treatments of diseases resembling streptococcal infections were described from the earliest writings in the 5th century BC, e.g., "when erysipelas supervenes, emetics, or purgatives are to be administered. When parts around the sore are swollen, a cataplasm (poultice) is to be applied" (Adams, 1849). Treatments over the centuries have also included bloodletting, as well as drugs derived from herbs with emetic, purgative, diaphoretic, or narcotic properties. Additional cures and remedies prescribed during the Middle Ages for all kinds of diseases can be found in the book "Fasciculus Medicinae," a collection of medical treatises compiled in 1491 by the German physician Johannes de Ketham (Ketham, 1988).

It was not until the 1930s that streptococcal infections were treated with sulphur antibacterials—they were among the first infectious disease organisms to be treated in this way. Domagk found that certain azo dyes coupled with sulphur resulted in an antibacterial agent, named Prontosil, which was inhibitory to a highly virulent strain of *Streptococcus hemolyticus* (Domagk, 1936; Domagk, 1957). Later modifications of the azo dyes resulted in the sulphonamides, which were marketed as early as 1937 for the treatment of pneumonia and other bacterial infections (Bentley, 2009).

Prior to the discovery of sulphonamides, the Scottish physician Alexander Fleming began searching for antibacterial agents that killed invading bacteria. In 1921, he accidentally found that a substance in his own nasal mucosa was able to dissolve certain bacteria, but not streptococci or staphylococci. This substance was later found to be lysozyme (Bentley, 2009), an enzyme that cleaves the peptidoglycan found in the cell walls of many bacteria. In 1928, Fleming made another accidental discovery that a Petri dish containing Staphylococci also contained a contaminating fungal colony that lysed the bacteria in the surrounding areas. Fleming named the substance causing the bacterial lysis penicillin, after the *Penicillium* fungus found on the plate (Fleming, 1929). He continued work with attempts to isolate the penicillin from culture filtrates, but made little progress in ensuing years. It was not until 1939 that significant progress was made in the production and isolation of a stable form of penicillin by the Australian physiologist Howard Walter Florey and his German colleague, Ernst Boris Chain (Ligon, 2004). They were able to establish large-scale fermentation conditions to produce penicillin in sufficient quantities to begin effective treatment in the early 1940s. These important discoveries were not only the beginning of the antibiotic era, but also a new industry, based on fermentation, for the production of antibiotics and pharmaceuticals. For these outstanding achievements, Fleming, Chain, and Florey were awarded the 1945 Nobel Prize in physiology or medicine. *Streptococcus pyogenes* has been and is still extremely sensitive to penicillin-based antibiotics. In fact, despite being the treatment of choice for decades because of its efficacy and low toxicity, streptococci resistant to penicillin have not evolved. The reason for this is still unknown, but may be related to the inability of naked DNA to enter the streptococcal cell because of the presence of DNases in the cytoplasmic membrane, or that beta-lactamase may not be expressed or may be toxic to the organism (Horn, et al., 1998).

Genetics and Genomics

Maclyn McCarty is well known for his many contributions to streptococcal research, and his career of distinguished scientific achievements was documented in an essay by Gotschlich and Fischetti (Gotschlich & Fischetti, 2005). Along with Avery and MacCleod, McCarty is also known as one of the pioneers of molecular genetic research. The 1944 demonstration that DNA is the carrier of genetic information (Tagg, Skjold, & Wannamaker, 1976) was a truly revolutionary finding that transformed and propelled biological research into the next century (Avery, MacLeod, & McCarty, 1944). An interesting personal account of this historical discovery can be found in the book, "The Transforming Principle, Discovering that Genes are Made of DNA" (McCarty, 1986).

A genetic approach to the study of group A streptococci and other factors related to disease had a somewhat slower start, compared to other microorganisms, due to the lack of a convenient gene transfer mechanism, as well as the fastidious growth requirements of the organism. Although a bacteriophage transduction system was available, the lack of convenient selection markers limited the transduction analysis to positive selection markers, such as antibiotic resistance genes (Malke, 1972; Stuart & Ferretti, 1978), or to the analysis of transposon-Tn916–generated mutations (Caparon & Scott, 1991). Additionally, although many attempts were made to effect gene transfer by conjugation or natural transformation, no successful reports were recorded until artificial transformation was achieved by electroporation and other methods (McLaughlin & Ferretti, 1995).

The introduction of genetic engineering technology in the 1970s provided a new approach to analyze virulence factors at the gene level, but the power of this technique was not realized until appropriate streptococcal cloning vectors were made available. The first gene to be cloned in streptococci was an erythromycin resistance determinant (Behnke & Ferretti, 1980), and shortly thereafter, the genes for streptokinase (Malke & Ferretti, 1984), streptococcal pyrogenic exotoxin A(Johnson & Schlievert, 1984; Weeks & Ferretti, 1984), M protein (Scott & Fischetti, 1983), and many others were cloned. The availability of these genes allowed sequence analysis to be completed, as well as overexpression studies for further studies of the individual proteins. Outgrowths of these molecular studies were the area of the "genome," the genetic material (DNA) of an organism; the "transcriptome," the set of RNAs in an organism; and the "proteome," the structure and function of the proteins

of an organism. As they relate to the group A streptococci, these areas are presented in several other chapters of this volume.

The genome era began with the first complete genome sequence of *Hemophilus influenza* in 1995 (Fleischmann, et al., 1995), and was followed by the sequencing of several other microbial genomes. The first complete sequence of a group A streptococcal genome was reported for an M1 strain in 2001 (Ferretti, et al., 2001), and since then, numerous complete genomes of other M-type strains have been reported. Their sequences are available in the NIH genebank at https://www.ncbi.nlm.nih.gov/genome/. Additionally, as the cost of DNA sequencing has decreased over time, hundreds of draft genomes of group A streptococcal strains were produced and analyzed, either by public institutions or by industry. One of the more impressive recent genome studies used the sequences of over 3,600 genomes to delineate the step-wise genetic events that led to the worldwide epidemic of an M1 serotype strain with increased virulence (Nasser, et al., 2014). A subsequent chapter presents an overview of comparative genomics of the group A streptococci.

Another important discovery from genome analyses was the identification of clustered regularly interspaced short palindromic repeats (or CRISPRs), and the associated DNA-cutting enzyme, Cas9 (Koonin & Makarova, 2009; Sorek, Kunin, & Hugenholz, 2008). The CRISPR-Cas modules found in many bacteria are adaptive immunity systems that defend the organisms against invading genetic elements (Makarova, et al., 2011). This system also contains elements useful for directly editing or modulating the function of DNA sequences and provides a trailblazing new technology for genome engineering (Jinek, Chylinski, Fonfara, Hauer, & Doudna, 2012). A complete description of this system is presented in another chapter of this volume.

Conclusion

Diseases caused by *Streptococcus pyogenes* have been well documented through the ages, and the overall importance of streptococci in medical history has been appropriately summarized by Schwartz: "not only were streptococci among the first organisms to be presumed to be the cause of contagious diseases, but also their existence forced the introduction of hygiene and asepsis into hospital wards" (Schwartz, 1997). Medical research using streptococci have also played a role in understanding the pathogenesis of infectious diseases, the development of antibiotics, the role of DNA as a carrier of genetic information, and in genetic engineering. Future research will be directed toward employing the tools of systems biology: namely, "investigating the behavior and relationships of all the elements in a particular biological system while it is functioning" (Ideker, Winslow, & Lauffenburger, 2006); understanding the role of epigenetic gene regulation, especially DNA methylation resulting in alterations in gene expression (Casadesús & Low, 2006; Euler, Ryan, Martin, & Fischetti, 2007); and deciphering the signals that influence transcriptional regulatory networks and control of virulence (as in the chapter on this topic). Additional research will focus on understanding the mechanisms of *Streptococcus pyogenes* disease, identifying ways to eliminate the carrier state, and better methods of disease control. Finally, further identification of the CRISPR-Cas9 system may provide revolutionary new tools for genome engineering that will have broad applications for basic biology and medicine.

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M Protein and Other Surface Proteins on Streptococci

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Preface

Molecules on the surface of *S. pyogenes* range from a complex coiled coil modular structure, like the M protein, to conventional globular proteins and polysaccharides. All of the molecules on the bacterial surface have specifically evolved to enable survival in its human environment like the throat, blood, or skin. In particular, the M protein molecule has been finely tuned to allow the streptococcus to persist in infected tissues while skillfully avoiding human immune cells. As the first molecule on *S. pyogenes* to be completely sequenced, it is considered the archetypical surface molecule for these organisms and other Gram-positive bacteria; and as a result, in this chapter, it will be used as a comparator for the other surface proteins on streptococci.

Introduction

Streptococcus pyogenes (or group A streptococcus) is responsible for a number of suppurative human infections, of which acute pharyngitis and impetigo are the most common. As a consequence of ineffective antibiotic therapy or no therapy, as many as 3 to 5% of individuals who suffer a group A streptococcal pharyngeal infection may develop acute rheumatic fever (Wannamaker, 1973; Breese, 1978), a disease that often results in cardiac damage, particularly to the mitral valve. Rheumatic fever is not currently a major problem in the developed world; however, it is the leading cause of heart disease in school-age children in developing nations (Agarwal, 1981). Recent estimates indicate that there are at least 517,000 deaths worldwide each year due to group A streptococcal diseases (such as acute rheumatic fever, rheumatic heart disease, acute post-streptococcal glomerulonephritis, and invasive infections) (Carapetis, Steer, Mulholland, & Weber, 2005). Rheumatic heart disease, with a prevalence of at least 15.6 million cases and 233,000 deaths each year, has the greatest disease burden (Rammelkamp & Weaver, 1953).

The ability of *S. pyogenes* to persist in infected tissues can be primarily attributed to the cell surface M protein, a molecule that gives the streptococcus the ability to resist phagocytosis by polymorphonuclear leukocytes in the absence of type-specific antibodies. The importance of the M molecule is seen in an M-knockout mutant, which has all the other surface and secretory molecules of the wild-type organism, yet will not survive in human blood that contains phagocytes (Perez-Casal, Caparon, & Scott, 1992). Resistance to a group A streptococcal infection is directly related to the presence of type-specific antibodies to the M molecule (Lancefield, 1959; Lancefield, 1962). Since there are >200 different serotypes of M protein (i.e., M6, M12, M18, M24, and so on), an individual may become infected by more than one group A streptococcal type during a lifetime (Lancefield, 1962). Rebecca Lancefield identified the streptococcal M protein nearly 90 years ago (Lancefield, 1928). A review by Lancefield in 1962 (Lancefield, 1962) clearly describes the studies carried out over a 35-year period that defined this molecule as a major virulence factor for the *Streptococcus pyogenes* bacterium.

The streptococcal M protein is now probably one of the best-defined molecules among the Gram-positive bacterial virulence determinants. Its structure, function, immunochemistry, and method of antigenic variation are unique among known virulence molecules, and may serve as a model for certain microbial systems (Fischetti, 1989).

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Streptococcal Cell Wall

Like the cell wall of other Gram-positive bacteria, the streptococcal cell wall contains a thick peptidoglycan, which is intercalated and covered with protein, teichoic acid, and lipoteichoic acid. The group-specific antigenic determinant for the group A streptococcus, which differentiates it from other streptococci, is the *β*-linked *N*-acetylglucosamine, which caps the polyrhamnose chains that extend from the *N*-acetylmuramic acids of the peptidoglycan (Coligan, Schnute, & Kindt, 1975). In contrast, group C streptococci (which cause human and animal infections) has an α-linked *N*-acetylgalactosamine as its group-specific determinant (Coligan, Schnute, & Kindt, 1975; Coligan, Kindt, & Krause, 1978). Surface proteins on streptococci contain an N-terminal signal peptide for Sec-dependent secretion and may be separated into three main categories: those that anchor at their C-terminal end (through an LPXTG motif); those that bind by way of charge or hydrophobic interactions; and those that bind via their N-terminal region (or lipoproteins, which are characterized by a cysteine-containing 'lipobox' within their signal peptide sequence) (Sutcliffe & Harrington, 2002) (Figure 1).

M Protein

M protein is the major virulence determinant for *S. pyogenes*, since M^- mutants are unable to survive in phagocyte-containing human blood (Maxted, 1956), thus all human isolates have surface M-protein, which appears like the fuzz on a tennis ball when viewed by transmission electron microscopy (Figure 2). It was subsequently found that protection against infection by these organisms was dependent on the presence of antibodies directed to the N-terminal region of the M protein (Cunningham & Beachey, 1974; Beachey, Campbell, & Ofek, 1974), the type-specific segment of the molecule (Beachey, Seyer, Dale, Simpson, & Kang, 1981; Dale, Seyer, & Beachey, 1983). It is known that the region of the M molecule responsible for this type specificity is limited to 50 or so amino acids on the N-terminus. However, the mechanism by which the sequence in this region changes to produce a new serotype is unknown.

M protein structure

M protein may be considered the archetypical molecule for those surface proteins that anchor via their C-terminal region on the Gram-positive bacterial cell wall. A feature of the M molecule, as with many surface proteins on bacteria, is its multi-domain structure. As Figure 3 shows, the M6 protein is composed of four sequence repeat domains, with each differing in size and sequence (Hollingshead, Fischetti, & Scott, 1986). The A-repeats are each composed of 14 amino acids, where the central blocks are identical and the end blocks slightly diverge from the central consensus repeats. The B-repeats, composed of 25 amino acids each, are arranged similar to the A-repeats. The C-repeats, composed of 2.5 blocks of 42 amino acids each, are not as identical to each other as the A and B repeats. There are also 4 short D-repeats, which show some homology among each other. These repeat segments make up the central helical rod region of the M6 and other M molecules, because of the high helical potential ascribed to the amino acids found within this region, as determined by conformational analysis (Fischetti, et al., 1988; Phillips, Flicker, Cohen, Manjula, & Fischetti, 1981). Recombination within the repeats has been shown to cause size variation among and within M proteins of the same and different serotypes, even in strains isolated from the same outbreak (Fischetti, Jones, & Scott, 1985; Fischetti, Jarymowycz, Jones, & Scott, 1986; Hollingshead, Fischetti, & Scott, 1987). It has been suggested that this could be a strategy by which the organism could escape immune recognition.

Examination of the sequence within the helical rod region of the M6 molecule revealed a repeating 7-residue periodicity of non-polar amino acids, a characteristic of α-helical coiled-coil proteins like mammalian tropomyosin and myosin. Generally, α-helical coiled-coil molecules are constructed from a reiterating 7-residue pattern of amino acids $(a\text{-}b\text{-}c\text{-}d\text{-}e\text{-}f\text{-}g)_n$ where residues in positions 'a' and 'd' are hydrophobic and form the 'core' residues in the coiled-coil, while the intervening residues are primarily helix-promoting. Irregularities in the heptad pattern, which are found especially in the B repeat region and other coiled-coil molecules, probably

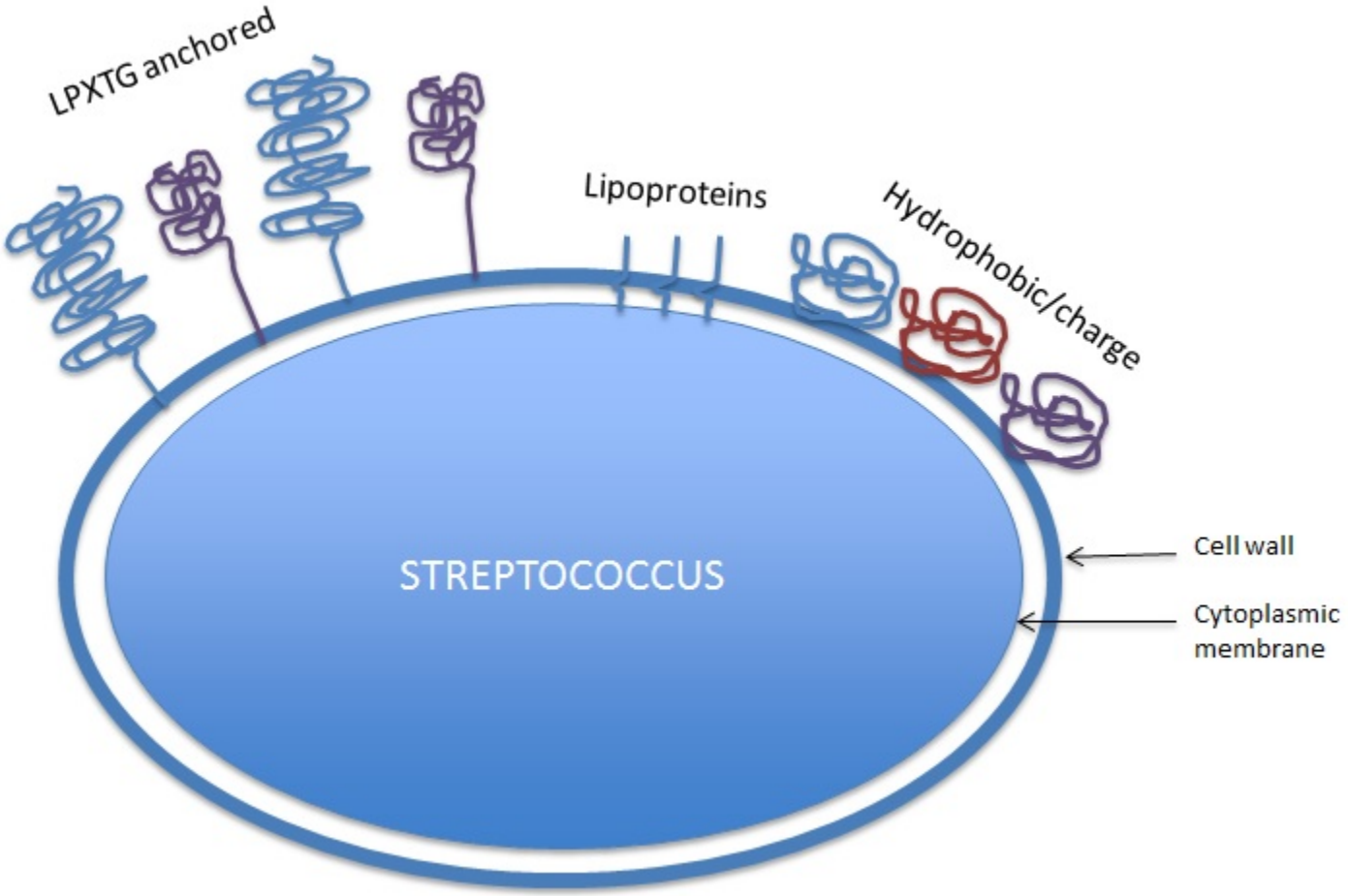

Figure 1. Proteins on the surface of the streptococcal cell wall. Surface proteins are linked by three mechanisms: i) lipoproteins have a lipid at the N-terminus linked through a cysteine; ii) C-terminal cell wall anchored proteins are attached and stabilized in the peptidoglycan through a C-terminal complex containing an LPXTG motif (the majority of surface proteins are anchored in this way); and iii) certain surface proteins are attached through hydrophobic and/or charge interactions to the cell surface (some proteins are ionically bound to the lipoteichoic acid).

account for the flexibility of the M molecules that has been observed in electron micrographs (Phillips, Flicker, Cohen, Manjula, & Fischetti, 1981). Based on these discontinuities in the heptad pattern, the central rod region is divided into three sub-regions, which correlate to the A-, B-, and C-repeat blocks (Fischetti, 1989).

Whereas M6 and other M molecules conform to this general arrangement of amino acids, other M proteins diverge somewhat from this configuration. A recent analysis of 1086 streptococcal isolates from 31 countries, representing 175 M protein types, revealed differences in the central rod region of the molecule showing fewer repeats, in some cases, and a shorter rod as a result (McMillan, et al., 2013). It was found that the A-repeats, in particular, were missing from the majority of the M proteins, which belonged to pattern D and E M proteins (see the chapter in this book on the molecular basis of stereotyping for more information).

Despite several attempts to crystallize the whole M molecule, only an N-terminal fragment that contains both the A and B repeats of the M1 protein was successfully accomplished (McNamara, et al., 2008). The structure confirmed the presence of significant irregularities in the coiled-coil structure also found in other mammalian coiled-coil proteins, like tropomyosin and myosin. The authors found that if these irregularities were corrected in the B-repeat region (a region responsible for fibrinogen binding), a loss in fibrinogen binding occurred, which suggests that these irregularities are biologically important. The high structural similarity between the M and host α-helical coiled-coil proteins has led researchers to hypothesize the elicitation of anti-M cross-reactive

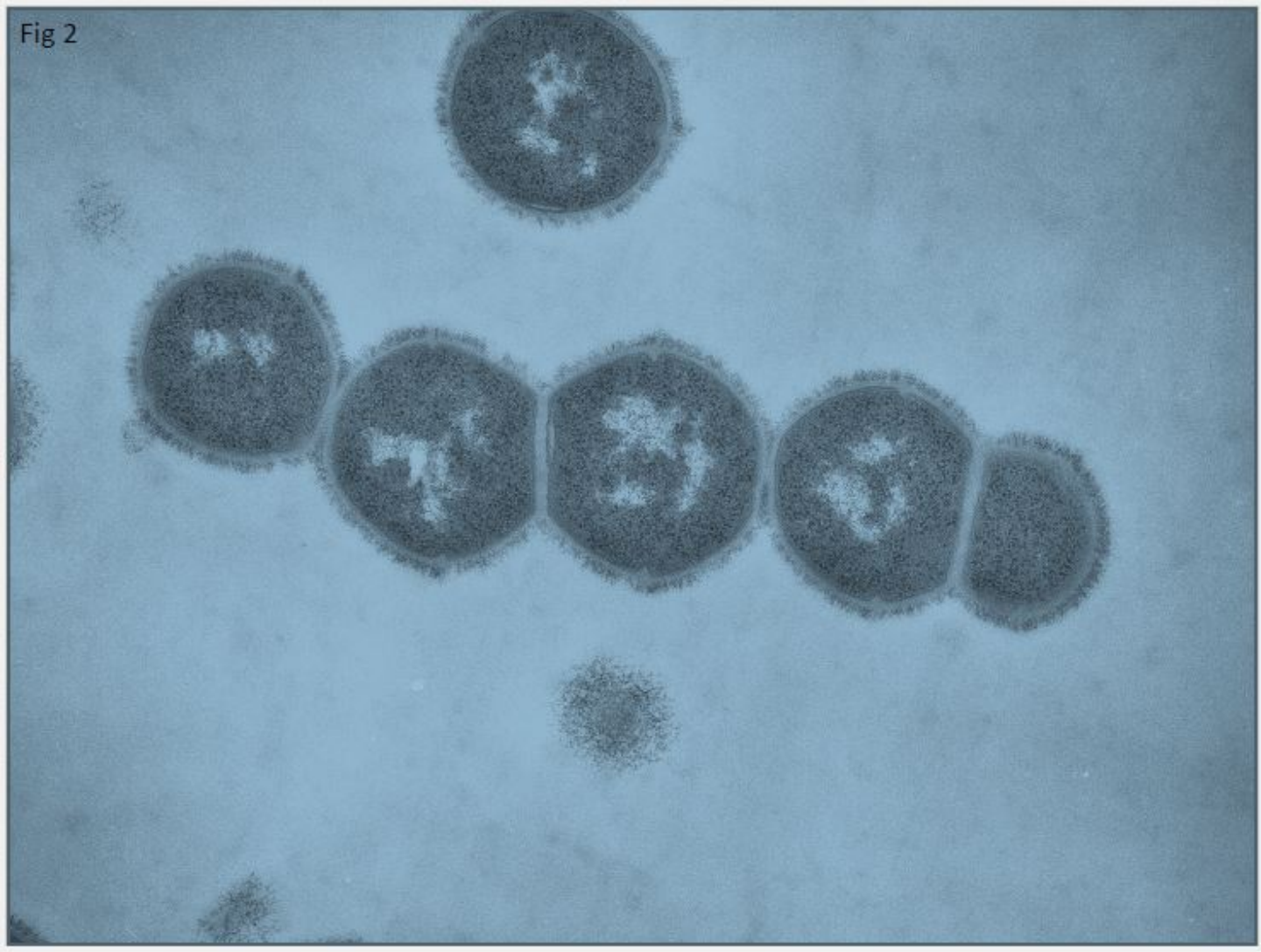

Figure 2. Thin section electron micrograph of a chain of streptococci showing the surface M protein. Magnification 50,000x.

antibodies and T cell receptors directed against these human proteins as a possible cause for *S. pyogenes*-associated autoimmune sequelae (Cunningham, 2000).

Swanson et al. (Swanson, Hsu, & Gotschlich, 1969) published the first electron micrographs (EM) of the M protein on the surface of *S. pyogenes*. This was the first evidence to show that the M molecule was an elongated structure. Although at the time, other proteins were reported to also be on the streptococcal surface, they were not apparent in these EM preparations. Thus, direct visualization of surface molecules is limited to those with certain physicochemical characteristics. In the case of the M protein, it is the α-helical coiled-coil structure that allowed for this visualization (Figure 2 & 4).

In experiments designed to answer questions about the synthesis and placement of M protein on the cell wall, Swanson et al. (Swanson, Hsu, & Gotschlich, 1969) found that after trypsinization to remove existing M protein on living streptococci, newly synthesized M protein was first seen by EM on the cell in the location of the newly forming septum. In similar experiments designed after the classical experiments of Cole and Hahn (Cole & Hahn, 1962), trypsinized streptococci placed in fresh media for ten minutes revealed M protein first at the newly forming septum (Raz, Talay, & Fischetti, 2012); whereas Sfb (a fibronectin binding protein on streptococci) first appears at the poles and increases gradually in concentration, as compared to its rapid appearance of the M molecule. On organisms examined after 40 minutes or more of incubation, M protein was not observed in the

position of the "old wall," which suggests that the M molecule is produced only where new cell wall is synthesized, and confirms the observations of Swanson et al. (Swanson, Hsu, & Gotschlich, 1969). These results demonstrate a close relationship between the regulation of cell division and protein anchoring (for more information, refer to the chapter on the spatial regulation of protein sorting in this book).

C-terminal anchor region

Hundreds of proteins from Gram-positive bacteria have now been reported that anchor to the cell wall through their C-terminal region (Table 1 shows a representative list of streptococcal surface proteins). From sequence alignments of a variety of surface proteins on Gram-positive bacteria, it became obvious that there was a common theme within the C-terminal region of these molecules, without exception: all had a similar arrangement of amino acids (Figure 5). Up to seven charged amino acids are found at the C-terminus, which are composed of a mixture of both negatively and positively charged residues. Immediately N-terminal to this short-charged region is a segment of 15-22 predominately hydrophobic amino acids that are sufficient to span the cytoplasmic membrane of the bacterium. In all these proteins, the sequences found in the hydrophobic and charged regions are not necessarily identical, but the chemical characteristics of the amino acids used to compose them are conserved. About eight amino acids N-terminal from the hydrophobic domain is a heptapeptide with the consensus sequence LPXTG, which is extraordinarily conserved among all the C-terminal-anchored proteins examined (Fischetti, Pancholi, & Schneewind, 1990). While several amino acid substitutions are seen in position 3 of the heptapeptide (predominantly A, Q, E, T, N, D, K, and L), positions 1, 2, 4, and 5 are nearly 100% conserved (see Table 1). This conservation is also maintained at the DNA level. The preservation of this hexapeptide and the high homology within the hydrophobic and charged regions suggest that the mechanism of anchoring these molecules within the bacterial cell is also highly conserved.

The secretion and anchoring of surface proteins are highly regulated in *S. pyogenes*. For example, as previously mentioned, anchoring M-protein to the cell wall occurs exclusively at the septum (Cole & Hahn, 1962; Swanson, Hsu, & Gotschlich, 1969), along with the biosynthesis of the cell wall (Raz & Fischetti, 2008). The resulting coupling of M-protein anchoring and cell wall synthesis leads to the coating of the entire cell surface with M protein. In contrast to M-protein, SfbI (also known as protein F, or PrtF), a major fibronectin-binding protein in certain streptococcal strains (Talay, Valentin-Weigand, Jerlström, Timmis, & Chhatwal, 1992a; Hanski & Caparon, 1992), is anchored at the old poles (Ozeri, et al., 2001; Raz & Fischetti, 2008). Secretion of M-protein and SfbI at their respective cellular locations is directed by information found in their signal sequences (Carlsson, et al., 2006). Proteins that contain a YSIRK/GS motif in their signal sequence are directed for secretion at the septum, while proteins that do not contain this motif are secreted at the poles, or in a hemispherical distribution. The N-terminal signal sequence directs these molecules for translocation through the secretion channel, and translocation is halted when the C-terminal LPXTG sorting signal reaches the channel. At this point, the LPXTG motif is exposed on the extracellular side of the membrane, the hydrophobic stretch spans the membrane, and the C-terminus positively-charged residues are within the cytoplasm (Figure 6) (Schneewind, Mihaylova-Petkov, & Model, 1993; Schneewind, Pancholi, & Fischetti, 1992). At this time, the membrane-associated transpeptidase sortase then cleaves the LPXTG motif between the threonine and glycine residues, and connects the freed threonine to lipid II (Marraffini & Schneewind, 2006; Mazmanian, Ton-That, & Schneewind, 2001a; Perry, Ton-That, Mazmanian, & Schneewind, 2002). The lipid II–protein complex is then processed by penicillin binding proteins and finally attached to the cell wall. The C-terminal portion of the cleaved sorting signal, which contains the hydrophobic region and positively charged residues, is released back into the cytoplasm (Navarre & Schneewind, 1994).

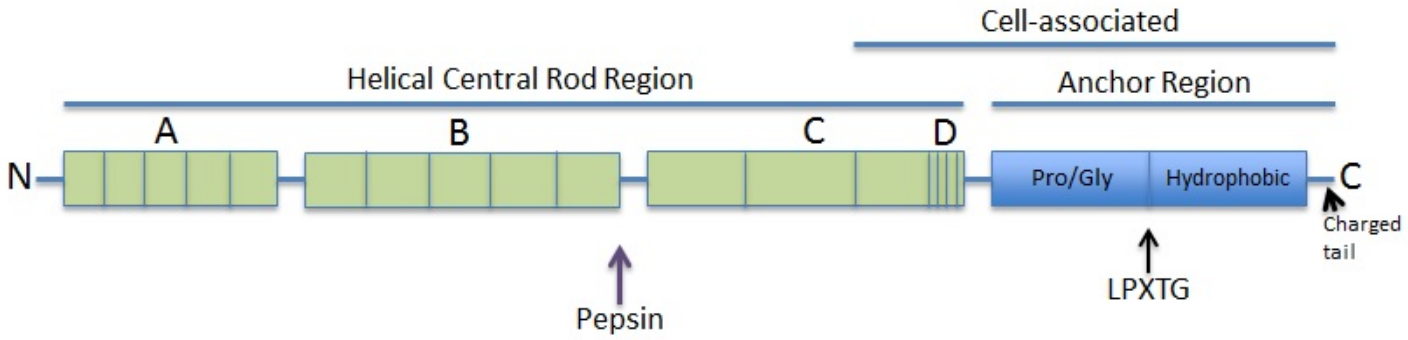

Figure 3. Characteristics of the complete M6 protein sequence. Blocks A, B, C, and D designate the location of the sequence repeat blocks. Pro/Gly denotes the proline- and glycine-rich region that is likely located in the peptidoglycan. The hydrophobic region is composed of a 19 hydrophobic amino acid region, adjacent to a 6 amino acid charged tail. The LPXTG motif is located N-terminal to the hydrophobic region, which places it outside of the cytoplasmic membrane. The cell-associated region would be imbedded from the inner side of the cytoplasmic membrane to the top of the surface carbohydrates (this begins at Ala-298). Pepsin identifies the position of the pepsin-sensitive site after amino acid 228. The helical rod region is essentially the coiled-coil segment of the M molecule.

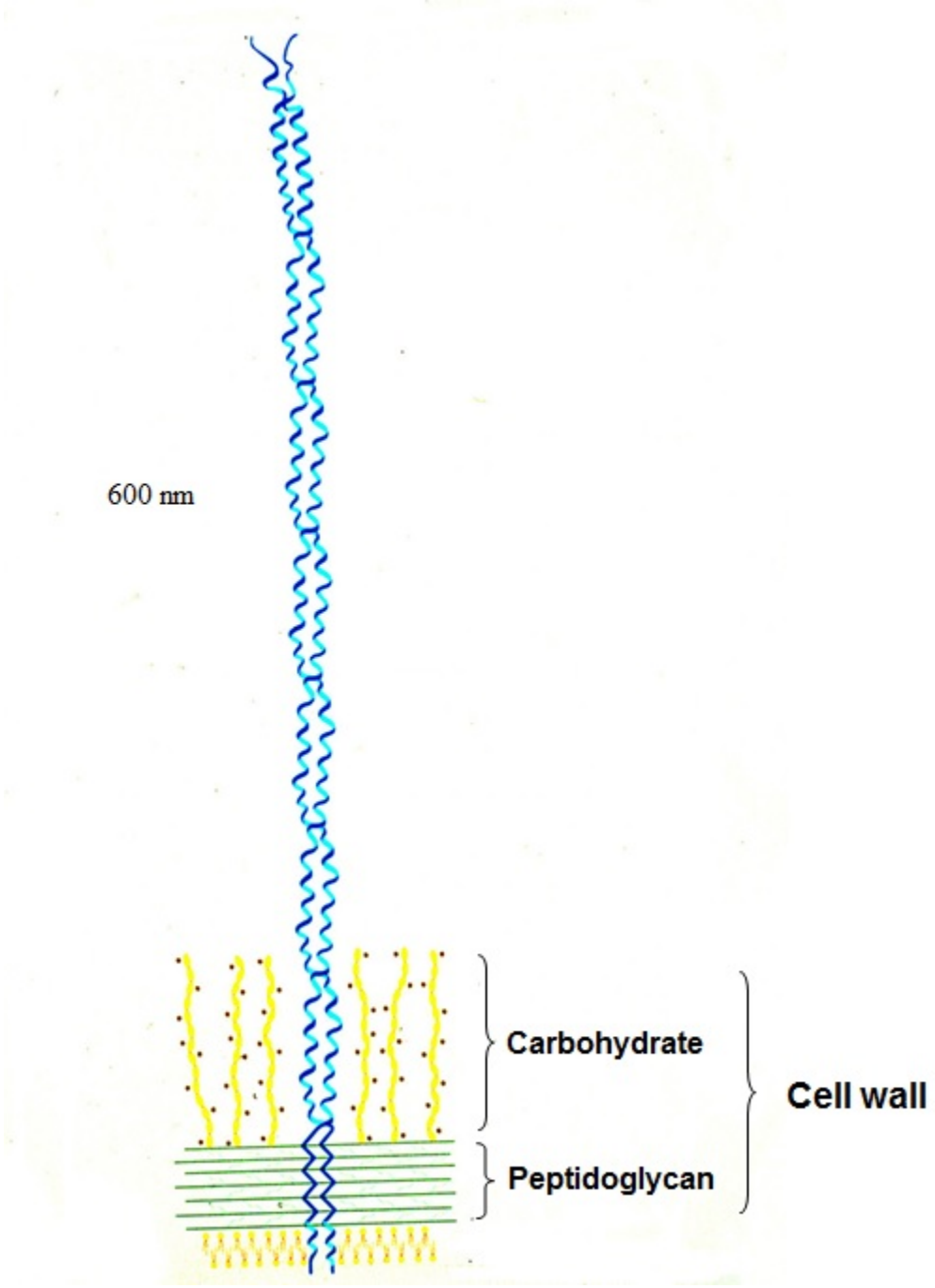

Figure 4. The appearance of the M molecule on the surface of the streptococcal cell wall. See caption of Figure 3 for more details.

Figure 5. Sequence alignment of the C-terminal end of M protein and other streptococcal surface proteins, depicting the charged tail (blue), hydrophobic domain (green), and LPXTG motif (red). Sequences are aligned at the LPXTG motif.

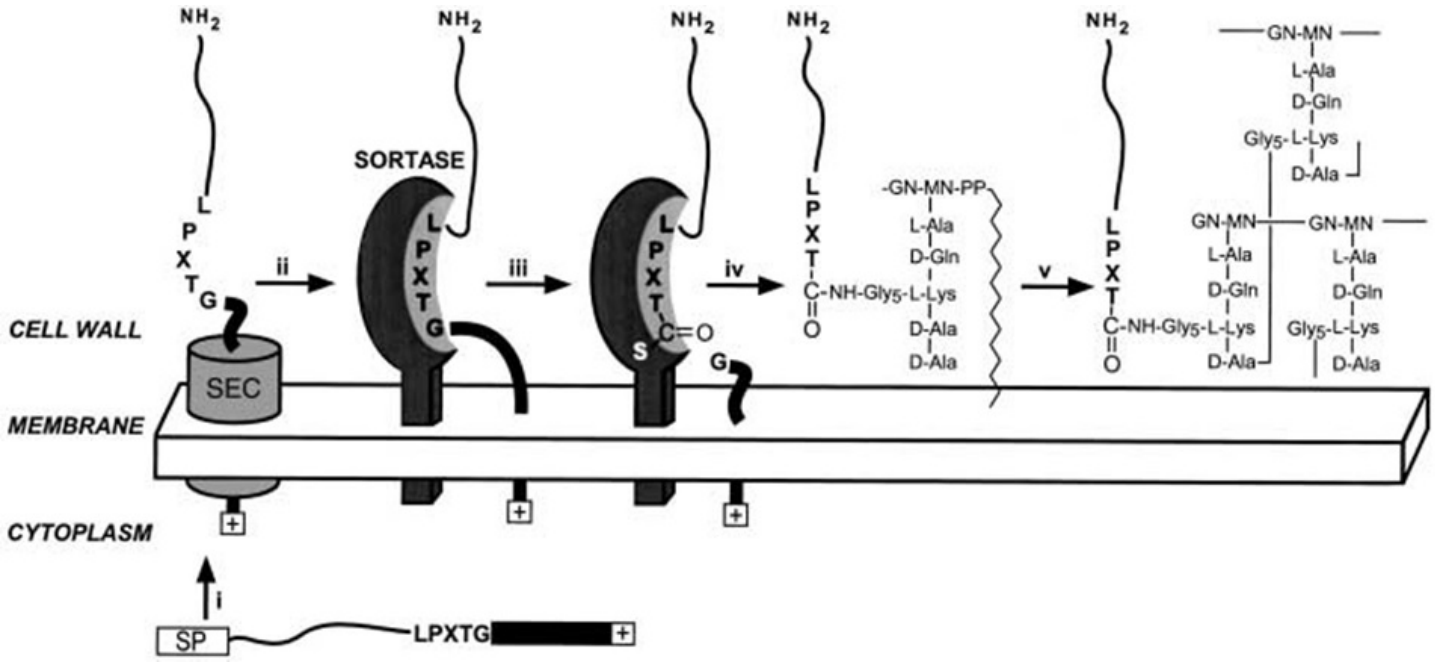

Figure 6. Translocation of proteins with an LPXTG motif to the surface of the cell wall for anchoring (Mazmanian, Ton-That, & Schneewind, 2001b).

Table 1: C-terminal LPXTG-Linked Surface Proteins on Streptococci

NAME / GENE	FUNCTION / NAME	ORGANISM	LPXTG	REFERENCE	ACCESS#
emm6	M protein	*S. pyogenes*	**LPSTG**	(Hollingshead, Fischetti, & Scott, 1986)	M11338
emm5	M protein	*S. pyogenes*	**LPSTG**	(Miller, Gray, Beachey, & Kehoe, 1988)	M20374

Table 1 continued from previous page.

NAME / GENE	FUNCTION / NAME	ORGANISM	LPXTG	REFERENCE	ACCESS#
emm12	M protein	*S. pyogenes*	**LPSTG**	(Robbins, Spanier, Jones, Simpson, & Cleary, 1987)	U02342
emm24	M protein	*S. pyogenes*	**LPSTG**	(Mouw, Beachey, & Burdett, 1988)	M19031
emm49	M protein	*S. pyogenes*	**LPSTG**	(Haanes & Cleary, 1989)	M23689
emm57	M protein	*S. pyogenes*	**LPSTG**	(Manjula, Khandke, Fairwell, Relf, & Sriprakash, 1991)	X60959
emm2	M protein	*S. pyogenes*	**LPSTG**	(Bessen & Fischetti, 1992)	X61276
emm3	M protein	*S. pyogenes*	**LPSTG**	(Katsukawa, 1994)	Z21845
ARP2	IgA binding protein	*S. pyogenes*	**LPSTG**	(Bessen & Fischetti, 1992)	X61276
ARP4	IgA binding protein	*S. pyogenes*	**LPSTG**	(Frithz, Hedén, & Lindahl, 1989)	X15198
Mrp4	IgG/fibrinogen binding	*S. pyogenes*	**LPSTG**	(O'Toole, Stenberg, Rissler, & Lindahl, 1992)	M87831
FcRA	Fc binding protein	*S. pyogenes*	**LPSTG**	(Heath, Boyle, & Cleary, 1990)	M22532
Prot H	Human IgG Fc binding	*S. pyogenes*	**LPSTG**	(Gomi, et al., 1990)	M29398
SCP	C5a peptidase	*S. pyogenes*	**LPTTN**	(Chen & Cleary, 1990)	J05229
T6	Protease resistant protein	*S. pyogenes*	**LPSTG**	(Schneewind, Jones, & Fischetti, 1990)	M32978
sof22	Serum opacity factor	*S. pyogenes*	**LPASG**	(Rakonjac, Robbins, & Fischetti, 1995)	U02290
Sfb	Fibronectin binding	*S. pyogenes*	**LPATG**	(Talay, Valentin-Weigand, Jerlström, Timmis, & Chhatwal, 1992b)	X67947
ZAG	Binds α_2M, Alb, IgG	*S. zooepidemicus*	**LPTTG**	(Jonsson, Burtsoff-Asp, & Guss, 1995)	U02290
PrtF	Fibronectin binding	*S. pyogenes*	**LPATG**	(Sela, et al., 1993)	L10919
PAM	Plasmin binding	*S. pyogenes*	**LPSTG**	(Berge & Sjöbring, 1993)	Z22219
bac	IgA binding protein	Gr. B strep	**LPYTG**	(Hedén, Frithz, & Lindahl, 1991)	X58470
bca	alpha C antigen	Gr. B strep	**LPATG**	(Michel, et al., 1992)	M97256
fnbA	Fibronectin binding	*S. dysgalactiae*	**LPQTG**	(Lindgren, et al., 1993)	Z22150
fnbB	Fibronectin binding	*S. dysgalactiae*	**LPAAG**	(Lindgren, et al., 1993)	Z22151
Fnz	Fibronectin binding	*S. equi*	**LPQTS**	(Nilsson, et al., 1998)	Y17116
SeM	M-like	*S. equi*	**LPSTG**	(Timoney, Artiushin, & Boschwitz, 1997)	U73162
SzPSe	M-like	*S. equi*	**LPQTS**	(Timoney, Artiushin, & Boschwitz, 1997)	U73163
Prot G	IgG binding protein	Gr. G strep	**LPTTG**	(Olsson, et al., 1987)	X06173
EmmG1	M protein	Gr. G Strep	**LPSTG**	(Collins, Kimura, & Bisno, 1992)	M95774
DG12	Albumin binding protein	Gr. G strep	**LPSTG**	(Sjöbring, 1992)	M95520
GfbA	Fibronectin binding	Gr. G. strep	**LPATG**	(Kline, Xu, Bisno, & Collins, 1996)	U31115
MRP	Surface protein*	*S. suis*	**LPNTG**	(Smith, Vecht, Gielkens, & Smits, 1992)	X64450
PAc	Surface protein	*S. mutans*	**LPNTG**	(Okahashi, Sasakawa, Yoshikawa, Hamada, & Koga, 1989)	X14490
spaP	Surface protein	*S. mutans*	**LPNTG**	(Kelly, et al., 1989)	X17390
spaA	Surface protein	*S. sobrinus*	**LPATG**	(Tokuda, et al., 1991)	D90354
wapA	Wall-associated protein A	*S. mutans*	**LPSTG**	(Ferretti, Russell, & Dao, 1989)	M19347

Table 1 continued from previous page.

NAME / GENE	FUNCTION / NAME	ORGANISM	LPXTG	REFERENCE	ACCESS#
fruA	Fructosidase	*S. mutans*	**LP**DTG	(Burne & Penders, 1992)	L03358
Sec10	Surface protein	*E. faecalis*	**LP**QTG	(Kao, Olmsted, Viksnins, Gallo, & Dunny, 1991)	M64978
Asc10	Surface protein	*E. faecalis*	**LP**KTG	(Kao, Olmsted, Viksnins, Gallo, & Dunny, 1991)	M64978
asa1	Aggregation substance	*E. faecalis*	**LP**QTG	(Galli, Lottspeich, & Wirth, 1990)	X17214
wg2	Cell wall protease	*S. cremoris*	**LP**KTG	(Kok, Leenhouts, Haandrikman, Ledeboer, & Venema, 1988)	M24767
Hyal1	Hyaluronidase	*S. pneumoniae*	**LP**QTG	(Berry, et al., 1994)	L20670
nanA	Neuraminidase	*S. pneumoniae*	**LP**ETG	(Cámara, Boulnois, Andrew, & Mitchell, 1994)	X72967
glnA	Glutamine synthetase	Gr. B strep	**LP**ATL	(Suvorov, Flores, & Ferrieri, 1997)	U61271
Protein F2	Fibronectin binding	*S. pyogenes*	**LP**ATG	(Jaffe, Natanson-Yaron, Caparon, & Hanski, 1996)	U31980
Fbe	Fibrinogen binding	*S. epidermidis*	**LP**DTG	(Nilsson, et al., 1998)	Y17116
SfBP1	Fibronectin binding	*S. pyogenes*	**LP**XTG	(Rocha & Fischetti, 1999)	AF071083
dex	Dextranase	*S. sobrinus*	**LP**KTG	(Wanda & Curtiss, 1994)	M96978
FAI	Fibrinogen/albumin/IgG	Group C strep	**LP**STG	(Talay, Valentin-Weigand, Jerlström, Timmis, & Chhatwal, 1992b)	NA
SfbII	Fibronectin binding	*S. pyogenes*	**LP**ASG	(Kreikemeyer, Talay, & Chhatwal, 1995)	X83303
spy0130	Minor pilin protein	*S. pyogenes*	**LP**STG	(Scott & Zähner, 2006)	NA
Fba	Fibronectin binding	*S. pyogenes*	**LP**XTG	(Terao, et al., 2001)	AB040536

* Surface proteins: proteins that have been identified to have a C-terminal anchor motif, but the function is unknown.
** NA: Not available

Wall-Associated Region

Immediately N-terminal from the LPXTG motif is the wall-associated region, which spans from about 50 to as many as 125 amino acid residues, and is found in nearly all C-terminal anchored surface proteins analyzed. Although this region does not exhibit a high degree of sequence identity among the known proteins, it is characterized by a high percentage of proline/glycine and threonine/serine residues. For some proteins (like the M protein), the concentration of proline/glycine is significantly higher than threonine/serine, while in others this relationship is either reversed or nearly equal. Because of its proximity to the hydrophobic domain, which is imbedded in the cell membrane, this region would be positioned within the peptidoglycan layer of the cell wall (Wannamaker, 1973), (Figure 4). The reason for the presence of these particular amino acids at this location is unknown. One hypothesis suggests that the prolines and glycines, with their ability to initiate bends and turns within the secondary structure of proteins, allow the peptidoglycan to more easily become cross-linked around these folds, which further stabilizes the molecule within the cell wall (Pancholi & Fischetti, 1988). The function of the threonines and serines within this region is not immediately apparent. While these amino acids are commonly used as O-linked glycosylation sites in eukaryotic proteins, such substitutions have not yet been established in this region of surface molecules.

Surface Exposed Region

As the C-terminal region is characteristically conserved among the various streptococcal surface proteins, the regions exposed on the cell surface are characteristically unique. Despite their differences, these molecules

appear to fall into three groups: i) those with several domains; ii) those with repeat domains located close to and within the cell wall region; and iii) those without any repeat domains (Figure 7). Streptococcal M protein and protein G may be considered representative molecules of those containing several sequence repeat domains. However, the most commonly found structures are those with repeats located close to the cell wall and an extended non-repetitive region N-terminal to this segment.

Conformationally analyzing 12 representative surface molecules through the algorithm of Garnier et al. (Garnier, Osguthorpe, & Robson, 1978) revealed that those proteins that contain repeat sequences were predominantly helical within the region containing repeat segments (Figure 7). On the other hand, regions and molecules without repeat blocks were predominantly composed of amino acids that exhibit a high β-sheet, β-turn, and/or random coil potential. Generally, within most of these molecules, the presence of repeat segments usually predicts the location of a helical domain. One of the possible pressures for the maintenance of repeat blocks is the preservation of the helix potential within specific regions of these molecules, the presence of which may determine an extended protein structure as has been shown for the M protein (Li, et al., 2013). An exception to this is found in the Sec10 protein, which is a predominantly helical molecule with limited repeat segments.

7-residue periodicity

Conformational analysis has revealed that most of the surface molecules that contain repeat sequences were found to be α-helical in those regions composed of repeats, while molecules without repeat blocks exhibited a high degree of β-sheet, β-turn, and random coil. However, except for M6 protein, little information is available to indicate that any of these surface proteins are also in a coiled-coil conformation. To answer this question, an algorithm was developed (Fischetti, Landau, Sellers, & Schmidt, 1993) and used to determine if a 7-residue periodicity also exists in those molecules that contain α-helical regions. When this algorithm was applied to representative sequences, extended regions of 7-residue periodicity were found in Arp4, FcRA, protein H, Pac, and spaP proteins. This strongly suggests that these molecules may attain a coiled-coil conformation within these α-helical segments. It is apparent that only those proteins that contained sequence repeats and exhibited high α-helical potential showed the presence of a 7-residue pattern of hydrophobic amino acids. On the other hand, even though the protein G molecule exhibited an extensive helix potential, the heptad pattern was only found in a limited region. Interestingly, however, the PAc protein from *S. mutans*, only has one small segment between residues 120 to 520 that displays repeat blocks, and this segment exhibits strong helix potential. This is precisely the region that was also found to contain a regular 7-residue heptad pattern. When taken together, these data strongly suggest that the coiled-coil conformation may also be a characteristic of surface proteins other than the M protein.

Size Variation

Size variation is a unique property of the M molecule. Using a broadly cross-reactive monoclonal antibody as a probe, the size of the M protein, which was extracted by solubilizing the cell wall from a number of streptococcal strains with a phage lysin, was examined by Western blot. M protein derived from 20 different serotypes exhibited variation in size ranging from 41 kDa to 80 kDa in molecular weight, depending on the strain (Fischetti, Jones, & Scott, 1985). Similar size variation was observed when streptococcal strains of the same serotype (M6) isolated over a period of 40 years were examined in a similar way. This variation in size may be explained by the observation that the M sequence contains extensive repeats both at the protein and DNA levels. Long, reiterated DNA sequences likely serve as substrates for recombinational events or for replicative slippage, generating deletions and duplications within the M protein gene and leading to the production of M proteins of different sizes.

Sequence analysis of the M6 gene isolates from local streptococcal outbreaks revealed that they are clonally related and are not the result of separate acquisitions of unrelated M6 strains during the course of the infection

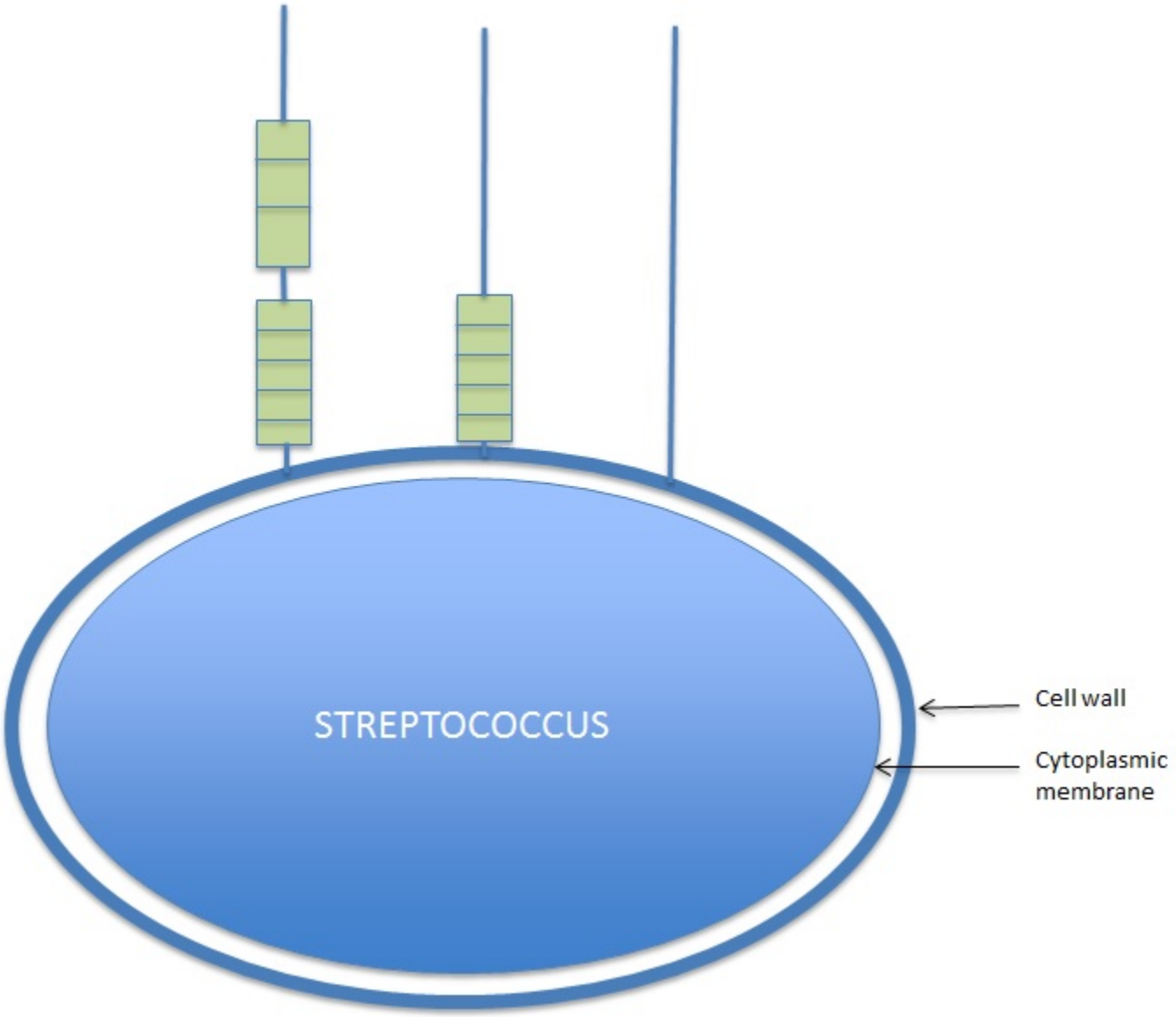

Figure 7. Conformational characteristics of the proteins found on the surface of streptococci. Molecules with multiple repeats (like M protein), molecules with few repeats (usually located close to the wall), and molecules with no repeats are found.

(Fischetti, Jarymowycz, Jones, & Scott, 1986). The observation that size variants occur among these clinical isolates suggests that the size mutant constituted the major organism in the streptococcal population at the time of isolation, and also suggests that a given size mutant has a selective advantage under clinical conditions. Perhaps serological pressure forces the appearance and maintenance of the size variant as a result of a local immune response.

Size variation is not only found in the M protein molecule but is also seen in other surface proteins on Gram-positive bacteria that also exhibit sequence repeats; for example, PspA from *S. pneumoniae* (Wanda & Curtiss, 1994), HagA from *P. gingivalis* (Kozarov, Whitlock, Dong, Carrasco, & Progulske-Fox, 1998), and protein A from *S. aureus* (Cheung & Fischetti, 1988).

Multifunctional Proteins

Of the numerous proteins identified on the surface of streptococci and other Gram-positive organisms, the great majority contain domains that bind molecules found in body secretions (Table 1), which include

immunoglobulins (IgA and IgG), albumin, fibronectin (Yamaguchi, Terao, & Kawabata, 2013), and fibrinogen (Anderson, et al., 2014). Other proteins have also been identified, based on the presence of domains exhibiting certain enzymatic activity. From current data, it is clear that the majority of these surface proteins are multifunctional, where the identified function is limited to only one domain of the multi-domain protein. In several cases, the function of the other region/s has not been identified. For example, as described above, it appears that many of the surface proteins contain a repeat region close to the cell wall and an extended non-repetitive domain N-terminal to this (see Figure 7). In most cases, the binding domain (i.e., fibronectin or immunoglobulin) is localized to the repeat domain, while the N-terminal region exhibits no known function. In the case of streptococcal serum opacity factor, both domains have been defined (Rakonjac, Robbins, & Fischetti, 1995); the repeats bind fibronectin, while the N-terminal domain has the catalytic function that cleaves ApoA1 of high-density lipoprotein and results in serum opacity. In the case of highly repetitive proteins, such as M protein and protein G, each repeat domain has a specific function. In M protein, the A repeats bind albumin, the B repeats fibrinogen (Rýc, Beachey, & Whitnack, 1989; Horstmann, Sievertsen, Leippe, & Fischetti, 1992), and the C repeats factor H of complement (Horstmann, Sievertsen, Knobloch, & Fischetti, 1988) and keratinocytes (Okada, Liszewski, Atkinson, & Caparon, 1995). In protein G, one repeat domain binds IgG, while the second binds albumin. The multifunctional feature of surface proteins is not limited to molecules anchored via their C-terminal region; proteins bound through hydrophobic/charge interactions also exhibit these same characteristics (see below).

Thus, in general, surface proteins on streptococci and other Gram-positive organisms are multifunctional molecules with at least two (and in some cases, three or more) independent functions. Given the fact that several different proteins that contain multifunctional domains may be found on the cell surface of a single streptococcus, the complexity associated with the bacterial surface can become enormous.

Surface Proteins Anchored by Charge and/or Hydrophobic Interactions

While the great majority of proteins identified on the surface of Gram-positive bacteria anchor through their C-termini, a few have been recently identified to bind through less defined charge and/or hydrophobic interactions.

Surface glycolytic enzymes

An in-depth analysis of the individual proteins identified in a cell wall extract of *S. pyogenes* revealed that some proteins were composed of cytosolic enzymes that are normally found in the glycolytic pathway (Pancholi & Fischetti, 1997). A total of five enzymes have been identified (trios-phosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase [GAPDH], phosphoglycerate kinase, phosphoglycerate mutase, and α-enolase), which form a short contiguous segment of the glycolytic pathway (Reiss, Kanety, & Schlessinger, 1986) (Figure 8). Interestingly, these enzymes form a complex involved in the production of ATP. All five of these enzymes have been identified on the surface of nearly all streptococcal groups, and certain ones have been identified on the surface of fungi, such as *Candida albicans*, and parasites, such as trypanosomes and schistosomes (Table 2). Thus, given the right substrate, whole streptococci have the capacity to produce ATP on their cell surface, increasing the complexity and potential of such bacterial surfaces.

Two surface glycolytic enzymes on the streptococcus have been the best characterized, GAPDH and α-enolase (Pancholi & Fischetti, 1993; Pancholi & Fischetti, 1998). Like other surface proteins on Gram-positives, GAPDH is a multifunctional protein with binding affinities for fibronectin and lysozyme, as well as cytoskeletal proteins, like actin and myosin. GAPDH has also been shown to have ADP-ribosylating activity in addition to its GAPDH activity. α-Enolase, is also multifunctional in its enzymatic activity and has the ability to specifically bind plasminogen, a plasma protease precursor (Pancholi & Fischetti, 1998). In some pathogens, only enolase is found on the surface. In these organisms, the surface enolase is used in the invasion of their host cells by binding

plasminogen. Evidence shows that there is only one gene each for encoding GAPDH and enolase, and that both are produced in the cytosol and partially (30–40%) translocated to the cell surface (Derbise, Song, Parikh, Fischetti, & Pancholi, 2004). Since these molecules are not synthesized with an N-terminal signal sequence, it is unclear how they are ultimately translocated outside the bacteria. Also, because they do not contain an apparent anchor motif, the ways in which they are attached to the bacterial surface are also unknown. Since they can be removed with chaotropic agents, it is reasonable to assume that they are bound through charge and/or hydrophobic interactions.

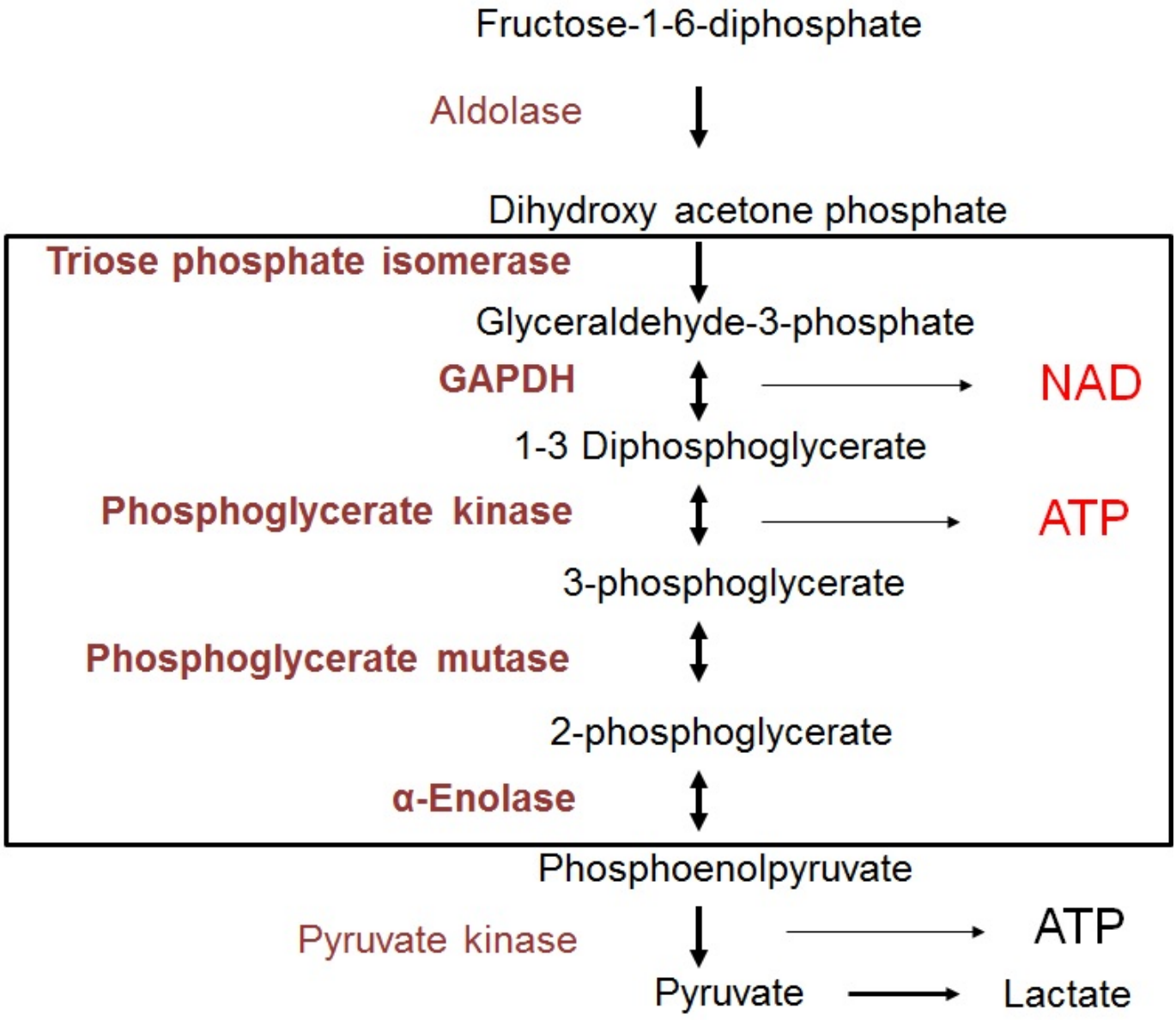

Figure 8. A segment of the glycolytic pathway that depicts the enzymes located on the surface of the streptococcal cell wall (box) that produce NAD and ATP.

Table 2: Enzymes identified on the surface of streptococcal groups, fungi, and parasites

Enzyme	Bacteria	Fungi	Parasites
GAPDH	Streptococci Pneumonococci Staphylococci	Candida M. avium	Schistosoma Trypanosome
PGK	Streptococci	Candida	Trypanosome
TPI	Streptococci		Trypanosome
PGM	Streptococci		
Enolase	Streptococci Pneumonococci	Candida	

Lipoproteins

Bacterial proteins may be lipid modified to facilitate their anchoring to the cytoplasmic membrane. Acylation effectively provides a membrane anchor allowing the now lipoprotein to function the aqueous environment. Bacterial lipoproteins have been shown to perform various roles, including nutrient uptake, signal transduction, adhesion, conjugation, and sporulation, and participate in antibiotic resistance, transport (such as ABC transporter systems) (Sutcliffe & Russell, 1995; Lampen & Nielsen, 1984). While a great deal is known about lipoproteins in Gram-negative bacteria, little is known about those from Gram-positive bacteria. In the case of pathogens, lipoproteins have been shown to play a direct role in virulence-associated functions, such as colonization, invasion, evasion of host defenses, and immunomodulation. Using a sequence-based analysis, Sutcliffe and Harrington examined the sequence of *S. pyogenes* for an N-terminal lipidation pattern G+LPP, and found that at least 25 probable lipoproteins may be present (Sutcliffe & Harrington, 2002). For example, lipoprotein FtsB in *S. pyogenes* is a component of the FtsABCD transporter that is responsible for ferrichrome binding and uptake (Li, et al., 2013). However, lipoproteins isolated from *S. pyogenes* after treatment with penicillin were complex, containing penicillin binding proteins, membrane proteins of the ExPortal (a membrane region responsible for assembly of secreted proteins), and hypothetical proteins (Biagini, et al., 2015).

Conclusion

Based on current information, the surface of the Gram-positive bacterial cell wall is highly complex and could even be considered a distinct organelle, since it is composed of proteins with specific binding functions, enzymatic activity, and the ability to generate energy. Considering the fact that there could be more than 25 different proteins (anchored in different ways) on the cell surface, each with the potential of up to three functional domains, suggests that more than 75 independent functions could potentially be present on the cell surface—much more than had ever been anticipated.

Because surface proteins on streptococci and other Gram-positives do not have cytoplasmic domains, it is unlikely that the binding of these molecules to specific ligands induces a signal in the microbe to activate a gene product. It is more likely that binding initiates a conformational signal on the cell surface to perform a specific function. For example, streptococci usually infect the pharynx (and particularly the tonsils) through contact with contaminated saliva. Upon entering the oral cavity, the organism first encounters the mucus, which coats the mucosal epithelium. Soluble components found in the mucus such as IgA, IgG, albumin, fibronectin, and others, are able to interact with their respective binding proteins on the streptococcal surface. Among other functions, this binding may initiate a set of conformational events on the surface of the bacterial particle to drive the organism through the mucus to the epithelial surface. The energy required for these processes may be derived from surface glycolytic enzymes necessary to generate ATP. This must all occur quickly or the organism will be swept into the gut and eliminated. Thus, the molecules necessary to initiate infection at all body sites are poised and ready on the cell surface.

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Streptococcus pyogenes Pili

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Abstract

Group A Streptococcus pili remained undetected until 2005, when immunogold electron microscopy using sera raised against their protein components revealed long flexible rods that protruded outside the bacteria. Interestingly, the variable protein that constitutes the pilus backbone turned out to be the target of the Lancefield T-typing system used for decades to classify *Streptococcus pyogenes* isolates. In addition to the backbone protein, *S. pyogenes* pili are constituted by two ancillary proteins, which are generally found on the pilus tip and its base. The three pilus subunits are encoded in the highly variable FCT genomic region, together with the sortase enzymes responsible for their polymerization. Recent protein crystallography studies have revealed the mechanism of pilus assembly and some unexpected structural features, including the formation of covalent isopeptide bonds that link different subunits and intramolecular isopeptide bonds that render the pilus proteins highly stable. The transcription of the FCT genes is controlled by the small RNA FasX and by stand-alone regulators, which are in turn integrated in the wider *S. pyogenes* regulatory network. *S. pyogenes* pili play an important role in pathogenesis by mediating bacterial adhesion to host cells and the formation of three-dimensional biofilms. Importantly, the pilus proteins show promise as vaccine targets, as pilus-specific antibodies were shown to mediate bacterial opsonophagocytic killing and provide animal protection against streptococcal infection.

Discovery of *Streptococcus pyogenes* pili and the genomic organization of pilus islands

The long pili that extend outside the surface of Gram-positive bacteria have remained largely unnoticed over many years of electron microscopic studies that apply negative staining. Experimental evidence for the presence of pili in *Streptococcus pyogenes* was first obtained by Mora and colleagues in 2005 (Mora, et al., 2005), soon after the discovery of similar structures in *Actinomyces naeslundii* (Yeung & Ragsdale, 1997), *Corynebacterium diphteriae* (Ton-That & Schneewind, 2003) and *Streptococcus agalactiae* (Lauer, et al., 2005). When labelled with specific sera, SDS-PAGE/immunoblot analysis showed these pili as a ladder of high molecular weight bands, while immunogold electron microscopy portrayed them flexible rods about 2 nm wide and >1 μm long that protrude outside the bacteria.(see panels A and B in Figure 1) (Telford, Barocchi, Margarit, Rappuoli, & Grandi, 2006; Murphy, Janulczyk, Karlsson, Mörgelin, & Frick, 2014)

Like in other Gram-positive bacteria, *S. pyogenes* pilus polymers were shown to be composed of proteins that bear a C-terminal LPTXG-like motif, covalently assembled and attached to the cell wall by a series of sortase mediated transpeptidase reactions. In their pioneering study, Mora and coauthors identified four pilus types, each recognized by a specific serum of the Lancefield T-typing system used for decades to sub-classify *S. pyogenes* isolates (Lancefield & Dole, 1946).

A genomic search revealed that the genes encoding the *S. pyogenes* pilus proteins and their polymerizing machinery were found to cluster in the FCT region, which was previously known to encode the T6 antigen and surface adhesins with binding capacity towards human collagen and fibronectin (Bessen & Kalia, 2002; Kreikemeyer B. , et al., 2005). Epidemiological studies uncovered nine different variants of this region that are

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designated FCT types 1–9, which vary in their gene composition and sequence (Kratovac, Manoharan, Luo, Lizano, & Bessen, 2007; Falugi, et al., 2008).

The 11–16 Kbp highly recombinatorial FCT gene cluster is flanked by the conserved *hsp33* and *spy0136* genes and encodes three main protein families: (1) transcriptional regulators, like RofA, Nra and MsmR; (2) fibronectin-binding proteins, like SfbI/F1 and F2; and (3) pilus-related proteins. This last group includes the three pilus components; namely, the backbone protein (BP/FctA, which is present in multiple copies per pilus unit) plus two ancillary proteins (AP1/Cpa and AP2/FctB, of which there is one copy of each per pilus), and the polymerase machinery, which consists of one or two sortase enzymes (SrtB and SrtC2), plus a putative signal peptidase (SipA). Analysis of 57 *S. pyogenes* strains that belonged to the nine FCT types identified a total of 15, 16, and 5 sequence variants of BP, AP1 and AP2 respectively, and the BP alleles in the different *S. pyogenes* isolates were confirmed to be the main determinants of the T type (Falugi, et al., 2008).

Biochemical and structural analysis of *Streptococcus pyogenes* pilus polymerization

The biochemical characterization of pilus polymers in Gram-positive bacteria followed the discovery of the mechanism by which sortase enzymes anchor surface proteins to the cell wall. Gram-positive bacteria are devoid of an outer membrane and use the cell wall peptidoglycan as a scaffold for displaying a wide variety of proteins on their surfaces. All these cell-wall–anchored proteins contain a signal peptide in their N-terminal region for Sec dependent secretion, and an LPxTG specific motif followed by a hydrophobic membrane that spans the domain, as well as a positively charged tail within their C-terminus. In 1992, Schneewind and colleagues discovered Sortase A of *Staphylococcus aureus* as a prototype enzyme that cleaves the LPXTG motif between the T and the G residues, forming a thioester-linked acyl-enzyme intermediate that is subsequently resolved by the nucleophilic attack of lipid II, the precursor of cell wall peptidoglycan synthesis; consequently, the substrate protein is incorporated into the cell wall by an amide (isopeptide) bond. A conserved cysteine residue in Sortase A acts as a nucleophile, and a conserved histidine is involved in the transpeptidation reaction (Navarre & Schneewind, 1999; Marraffini, Dedent, & Schneewind, 2006)

Pilus assembly in Gram-positive bacteria occurs through a similar mechanism of ordered cross-linking. In this case, the pilin-specific sortase enzymes encoded in the FCT region cleave membrane-attached precursor pilus proteins at their LPxTG-like sorting signals, which forms an acyl-enzyme intermediate. Next, a nucleophilic attack of a lysin side chain amino group present in the neighboring pilus protein is used to generate isopeptide bonds between two pilin subunits. Finally, covalent attachment of the completed pilus structure to the cell wall is accomplished by the previously described "housekeeping" sortase A, which is encoded elsewhere in the genome (Ton-That & Schneewind, 2004; Ton-That & Schneewind, 2003; Hendrickx, Budzik, Oh, & Schneewind, 2011).

The essential role of the FCT encoded SrtB/SrtC2 sortases in the formation of *S. pyogenes* pili was confirmed by knockout mutants, which failed to display the pilus proteins on the bacterial surface as well as to build polymers (Barnett & Scott, 2002; Mora, et al., 2005; Nakata, et al., 2009; Quigley, Zähner, Hatkoff, Thanassi, & Scott, 2009).

Recently, three-dimensional studies undertaken on *S. pyogenes* pilus proteins and their sortase enzymes have elucidated the mechanisms of pilus polymerization and have revealed unexpected structural features. First, Kang and collaborators have demonstrated the involvement of specific lysine residues withinSpy0128, the BP from a M1 T1 (FCT-2) strain in pilus assembly (Kang, Coulibaly, Clow, Proft, & Baker, 2007). Although this protein lacks the pilin motif WxxxVxVYPK that was formerly identified in *C. diphtheria* as essential for nucleophilic activity, an invariant lysine was found in Spy0128 (Lys161) and in other BP pilus variants. The crystal of Spy0128 at 2.2 Ångström of resolution uncovered an extended structure that comprised 2 Ig-like domains with antiparallel β-strands. Different Spy0128 molecules in the crystal were assembled head-to-tail in columns, with the side chain of Lys161 close to the C-terminus of the next molecule. This assembly process suggests that the

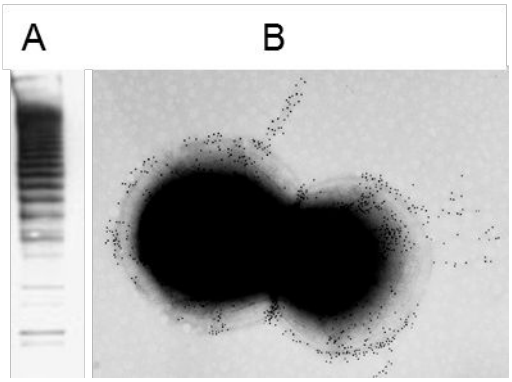

Figure 1. *S. pyogenes* pili as they appear on SDS-PAGE/immunoblot analysis (panel A) and immunogold electron microscopy (panel B), after labeling with antibodies against the pilus backbone protein.

formation of covalent isopeptide bonds in native pili that linked the C and N domains of different subunits occurs by the action of the sortase. Mass spectroscopy (MS) analysis of purified M1 T1 (FCT-2) pilus polymers treated with trypsin did reveal the presence of a non-linear peptide fragment in which the ε-amino group of Lys161 was joined to the carboxyl group of the C-terminal Thr311 from another BP subunit.

Remarkably, 3D and MS analysis also revealed two intramolecular isopeptide bonds in Spy0128 (one in the N domain and one in the C domain of the protein) that were formed by covalent links between lysine and asparagine ε-amino side chains, in a spontaneous reaction that involved a nearby glutamic acid and a surrounding cluster of aromatic residues. This autocatalytic process contrasts with the inter-molecular isopeptide bonds that are exclusively generated by the action of the sortase enzymes. Similar Lys-Asn isopeptide bonds were later detected in other pilus proteins from *S. pyogenes* and various Gram-positive bacteria (Kang, Middleditch, Proft, & Baker, 2009; Kang & Baker, 2012; Walden, Crow, Nelson, & Banfield, 2014), and were shown to confer high stability to the pilus structure. Indeed, mutant protein derivatives devoid of isopeptide bonds showed a significant loss of trypsin-resistance and thermal stability. Further, atomic force microscopy analysis of Spy0128 polymers revealed that the native subunits were unable to extend, even when pulled at forces of up to 800 pN, while mechanical resilience was greatly reduced by the abrogation of the intramolecular isopeptide bonds (Alegre-Cebollada, Badilla, & Fernández, 2010).

The FCT-3 sortase SrtC2 was shown to attach AP1 via the VPPTG motif to the K173 residue of the BP pilin subunit. However, since assembly between two BP subunits also required the BP residue K173, the two processes were expected to be mutually exclusive, and AP1 was hypothesized to be located exclusively at the pilus tip. This hypothesis was confirmed by immunogold labeling and transmission electron microscopy (Quigley, Zähner, Hatkoff, Thanassi, & Scott, 2009). A covalent bond between the AP1 C-terminal threonine residue and a lysine residue from the BP was subsequently confirmed by mass spectrometry analysis of trypsin digested M1 T1 (FCT-2) pili (Smith, et al., 2010).

Concerning the location of the second ancillary protein in the pilus structure, Nobbs et al. showed that AP2 from *S. agalactiae* constitutes the pilus target of the housekeeping Sortase A, during the cell wall anchoring step that terminates pilus growth. Indeed, knockout mutant bacteria lacking either Sortase A or the AP2 presented shedding of pili from the cell surface (Nobbs, et al., 2008). A similar location of AP2 at the pilus base was found in *S. pyogenes* M1 T1 (FCT-2) (Smith, et al., 2010). Further, the crystal structure of AP2 from an FCT-9 strain revealed the determinants for AP2 linkage to the proximal subunit of the pilus shaft and by pilus anchoring (Linke, et al., 2010). In contrast to AP1 and BP, AP2 presents a classical LPXTG-type motif at its C-terminus, which is consistent with its attachment to the peptidoglycan by Sortase A as the final step of pilus formation.

Despite low sequence identity between the three *S. pyogenes* pilins, the striking structural similarity of their domains explains their recognition by the same class B sortase for the formation of inter-molecular isopeptide bonds linking AP1 to BP, the BP subunits, and the basal AP2 to BP (Kang & Baker, 2012).

Finally, the putative signal peptidase SipA was shown to be required for pilus formation in both the *S. pyogenes* serotype M3 and M49 strains, most likely by acting as a potential chaperon-like protein (Zähner & Scott, 2008; Nakata, et al., 2009). Young and colleagues (Young, Proft, Harris, Brimble, & Baker, 2014b) recently showed that the substitution of an FCT-2 SipA by its FCT-3 counterpart allowed for the polymerization of FCT-2 pili, which implies that the SipA function is conserved across *S. pyogenes* strains. The authors obtained the crystal structure of the protein, which contains a peptide-binding groove similar to that of *E. coli* signal peptidase I, but is devoid of the catalytic apparatus typical of signal peptidases. They confirmed the lack of peptidase activity and were not able to detect any interaction with peptide fragments of the pilus proteins. They proposed that SipA could either function in the direct recognition of pilin sorting signals in concert with class-B sortases, or could form a scaffold to modify and orientate the pilin proteins for optimal sortase activity; the chemical basis for the involved interactions remains unknown.

Regulation of pilus expression in *Streptococcus pyogenes*

The transcription of the pilus-encoding *S. pyogenes* FCT region is known to be controlled by stand-alone regulators encoded in the same locus; namely, RofA/Nra and MsmR. Transcription of FCT-2 and FCT-1 proteins is positively regulated by Rof A(Beckert, Kreikemeyer, & Podbielski, 2001). The roles of MsmR and Nra have been investigated in detail in FCT-3 strains, where they were shown to exert their function in opposite directions, acting either as positive or negative regulators depending on the genetic background. In particular, in a M49 strain, expression of pili and the F2 protein were shown to be repressed by Nra and activated by MsmR (Nakata, Podbielski, & Kreikemeyer, 2005; Kreikemeyer B. , et al., 2007). Conversely, in a M53 isolate, Nra acted as an activator of pilus expression without affecting F2, while MsmR repressed pilus transcription and activated the expression of F2 (Luo, Lizano, & Bessen, 2008).

The FCT pilus region and the associated transcriptional regulators are not an isolated regulatory block. Both are integrated and cross-linked with other pathogenicity island-like regions in a growth phase-dependent regulatory network; namely, the Mga locus and the novel ERES-pathogenicity region (Kreikemeyer, et al., 2011). Additionally, transcriptional analysis indicated a partial down regulation of the gene that encodes BP in *in vivo* selected mutations of the two-component regulator *covR/S* in a M1 T1 (FCT-2) strain background (Sumby, Whitney, Graviss, DeLeo, & Musser, 2006).

A newly described post-transcriptional regulatory mechanism of *S. pyogenes* pilus expression involves the small RNA FasX (Liu, Treviño, Ramirez-Peña, & Sumby, 2012). This sRNA was shown to reduce the stability of the pilus operon mRNA in a M1 T1 (FCT-2) strain and to anneal to a region upstream the AP1 ORF, thus blocking the translation of this pilus protein.

Among the environmental signals that affect *S. pyogenes* pilus expression are the growth temperature (Nakata, et al., 2009), oxygen availability (Granok, Parsonage, Ross, & Caparon, 2000), and low pH (Manetti, et al., 2010). The effect of these signals varies between the different FCT types. For instance, despite the fact that FCT-1 and FCT-2 *rofA* genes and their inter-genic regions share 98% of their identity, FCT-1 pili were highly and constitutively transcribed, irrespective of environmental pH, while the transcription of FCT-2 was enhanced only in acidic conditions (Manetti, et al., 2010). Similarly, Granok and collaborators detected high transcription rates of the genes located downstream *rofA* in a M6 T6 (FCT-1) strain both under aerobic and anaerobic conditions, while in M1 T1 (FCT-2) strains high expression occurred only under aerobic conditions, suggesting independent regulation pathways in addition to RofA (Granok, Parsonage, Ross, & Caparon, 2000). The exact mechanism by which the above described regulators are affected by external signals remains unknown.

Finally, the study of pili in a M49 strain revealed some unexpected expression patterns, in that the pili were restricted to 20–50% of the cells within an otherwise homogenous population (Nakata, et al., 2009).

Streptococcus pyogenes pili as virulence factors and vaccine targets

Since their discovery, the highly exposed and tightly regulated pili were expected to play an important role in *S. pyogenes* pathogenesis. Genetic modification of pilus components in different background strains has shed light on the function of these structures during *S. pyogenes* infection. In the *S. pyogenes* M1 T1 (FCT2) strain SF370, pilus-defective mutants showed a decreased capacity to form three-dimensional biofilms on solid surfaces, to attach to pharyngeal cells, and to form micro-colonies on the cell surface (Manetti, et al., 2007). Abbot and colleagues observed that pili also mediated the adhesion of the SF370 strain to human tonsillar epithelium and primary human keratinocytes, and postulated an adhesive role for the AP1 pilin subunit (Abbot, et al., 2007). Notably, the three-dimensional structure of the AP1 Spy125 uncovered an internal Cys-Gln thioester analogous to the reactive thioester bonds used by the human complement proteins for covalent attachment to target molecules. Removal of this internal thioester by allele-replacement mutagenesis severely compromised the adhesion of strain SF370 to a human keratinocyte cell line (Pointon, et al., 2010; Walden, Crow, Nelson, & Banfield, 2014). An additional thioester motif was recently detected in the AP1 Spy125 N-terminal domain. Both Spy125 thioester domains were shown to react with the polyamine spermidine, indicating a preference for amino groups (Linke-Winnebeck, et al., 2014).

All of these results point towards the role of pili, and particularly their AP1 ancillary proteins, in the initial steps of *S. pyogenes* colonization of human tissues (Danne & Dramsi, 2012). However, the receptors to which the pilus proteins attach to mediate host cell adhesion have only partially been identified. Several variants of the protein were found to contain a highly conserved domain that mediated binding to collagen type I(Kreikemeyer B. , et al., 2005). Additionally, pili of different *S. pyogenes* serotypes were found to bind to the salivary glycoprotein gp340, which resulted in bacterial auto-aggregation when exposed to human saliva (Edwards, et al., 2008).

The contribution of pili to *S. pyogenes* pathogenesis has also been evaluated by *in vivo* experiments. Lizano and colleagues found that an in-frame deletion of the gene that encodes AP1 in a FCT-3 strain recovered from an impetiginous skin lesion presented attenuated virulence in a humanized mouse model of superficial skin infection; in contrast, a BP knockout mutant that was incapable of forming pili was as virulent as the wild type (Lizano, Luo, & Bessen, 2007). Another study investigated the role of pili in a hypervirulent M1 T1 (FCT-2) *S. pyogenes* clone. In this case, pilus expression was found to promote neutrophil recruitment, bacterial killing within neutrophil extracellular traps (NETs), and reduced *S. pyogenes* virulence in murine models of necrotizing fasciitis, pneumonia, and sepsis (Crotty Alexander, et al., 2010).

The high divergence in the *S. pyogenes* FCT region suggests that there may be differences in the biological function between the pilus variants. Indeed, it was shown that M6 T6 (FCT-1) isolates formed stronger biofilms than the other *S. pyogenes* FCT types, due to their capacity to highly express pili under diverse growth conditions (Manetti, et al., 2010). In a further study, the FCT-1 AP1 protein was demonstrated as a strong adhesin that could also mediate inter-bacterial contact through homophilic interactions, and promoted the formation of microcolonies on host epithelial cells and enhanced bacterial survival in human blood; in-frame deletion mutant derivatives lacking this protein showed impaired virulence in a mouse model of *S. pyogenes* systemic infection (Becherelli, et al., 2012).

An important feature that has emerged from the study of pili in streptococcal species is that their components could be used as vaccine targets for the prevention of streptococcal infections. In fact, pilus-specific antibodies mediated bacterial opsono-phagocytic killing and protection against lethal challenges in different animal models of infection.

Mora et al. showed that a combination of recombinant pilus proteins conferred protection against *S. pyogenes* challenge in a mouse model of invasive disease (Mora, et al., 2005). Moreover, high levels of pilus expression during human infection were confirmed by analyzing a collection of sera from *S. pyogenes* pharyngitis patients for the presence of anti-pili antibodies; when BPs from *S. pyogenes* strains that belonged to four different FCT and M types (M1, M3, M6, and M12) were screened as part of a *S. pyogenes* protein array, 76% of the sera reacted with at least one BP variant (Manetti, et al., 2007).

One possible drawback of the use of pili as *S. pyogenes* vaccine targets may be the high sequence variability of the pilus proteins, as compared to those of other Gram-positive pathogens. Previous estimates suggested that a vaccine that contains a combination of 12 BP variants would cover about 90% of the most predominant *S. pyogenes* strains in Europe and the US (Falugi, et al., 2008). The modular nature revealed by the crystal structure of the M6 T6 (FCT-1) BP pilus protein (Young, et al., 2014a) may aid the future design of multivalent vaccine chimeras, based on the fusion of protective domains from different pilus variants, an approach that has proved to be highly successful in the case of Group B streptococcal pili (Nuccitelli, et al., 2011).

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Cell Wall and Surface Molecules: Capsule

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Abstract

In the 1920s, it was recognized that a mucoid or matte colony phenotype of *Streptococcus pyogenes* was associated with virulence in mice and with resistance to killing by human blood leukocytes. The gelatinous material produced by the mucoid isolates, currently known as capsule, was later characterized as hyaluronic acid, a linear polymer of N-acetylglucosamine and glucuronic acid with a high molecular mass that is structurally identical to hyaluronic acid found in the extracellular matrix of many higher animals, including human beings. It is now recognized that most (but not all) clinical isolates of group A streptococci produce hyaluronic acid, which is associated with the cell surface during exponential growth and shed during stationary phase. This chapter presents a summary of information on the capsule of *Streptococcus pyogenes*, its biochemistry, genetics, and role in virulence.

For many years, clinical microbiologists and infectious diseases clinicians have noted that group A streptococci freshly isolated from patients with pharyngitis or invasive infection often grow as large, wet-appearing, translucent colonies on blood agar. With prolonged incubation, these "mucoid" colonies collapse and assume an irregular or "matte" appearance. Laboratory passage of such isolates frequently resulted in loss of the mucoid phenotype and conversion to small, compact, or "glossy" colonies (Figure 1). In the 1920s, Lancefield and Todd recognized that the mucoid or matte colony phenotype was associated with virulence in mice and with resistance to killing by human blood leukocytes (Lancefield & Todd, 1928; Todd & Lancefield, 1928). Kendall and co-workers characterized the gelatinous material produced by mucoid isolates as hyaluronic acid, a linear polymer of N-acetylglucosamine and glucuronic acid with a high molecular mass that is structurally identical to hyaluronic acid found in the extracellular matrix of many higher animals, including human beings (Figure 2) (Kendall, Heidelberger, & Dawson, 1937). Wilson demonstrated that the growth of such isolates on media containing hyaluronidase prevented the development of mucoidy, which confirmed that the production of hyaluronic acid was responsible for the mucoid colony phenotype (Wilson, 1959). It is now recognized that most (but not all) clinical isolates of group A streptococci produce hyaluronic acid, which is associated with the cell surface during exponential growth and shed during stationary phase. Presumably because it is recognized by the host immune system as a self-antigen, the group A streptococcal hyaluronic acid capsule is poorly immunogenic in animals, including human beings. In contrast to capsular polysaccharides of *Streptococcus pneumoniae* and *S. agalactiae*, hyaluronic acid polymers are not covalently linked to the group A streptococcal cell wall, but rather are associated with the cell surface in a dynamic fashion, as they are synthesized by a cell membrane-associated polymerase. Although its mode of attachment to the bacterial surface is more tenuous than that of covalently bound polysaccharide capsules in other species, there is abundant evidence that the hyaluronic acid capsule is an important virulence determinant as a modulator of multiple interactions between group A streptococci and their human hosts.

Genetics and biochemistry of hyaluronic acid biosynthesis

Studies in the 1990s used transposon mutagenesis to identify a chromosomal locus required for hyaluronic acid production in group A streptococci (DeAngelis, Papaconstantinou, & Weigel, 1993a; Dougherty & van de Rijn,

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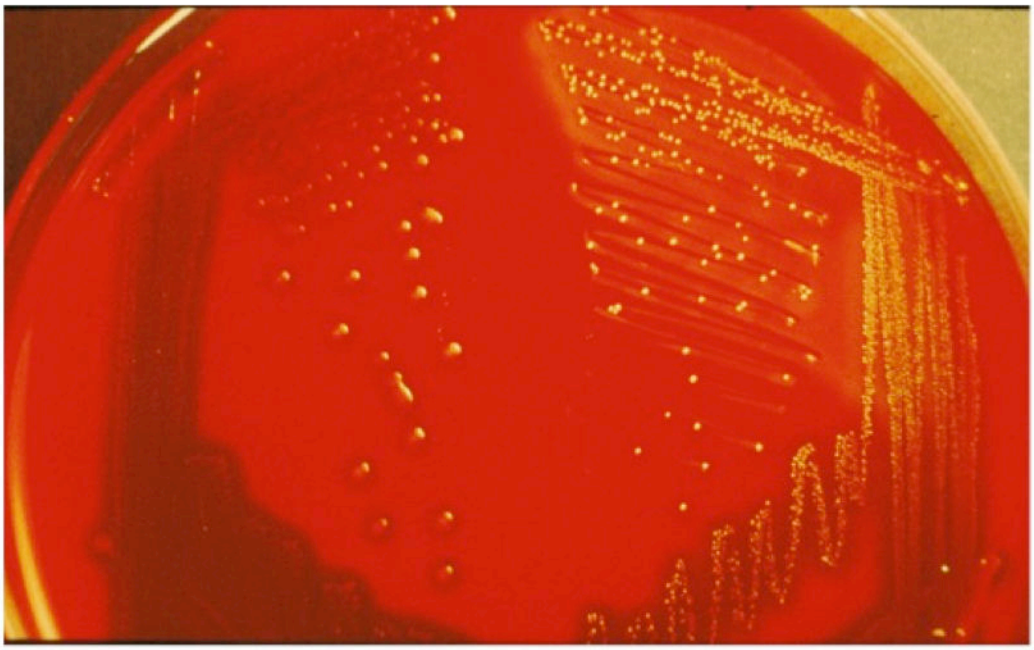

Figure 1. Blood agar plate with typical colonies of a mucoid strain of group A streptococcus (left) and non-mucoid (glossy) colonies of an acapsular mutant (right).

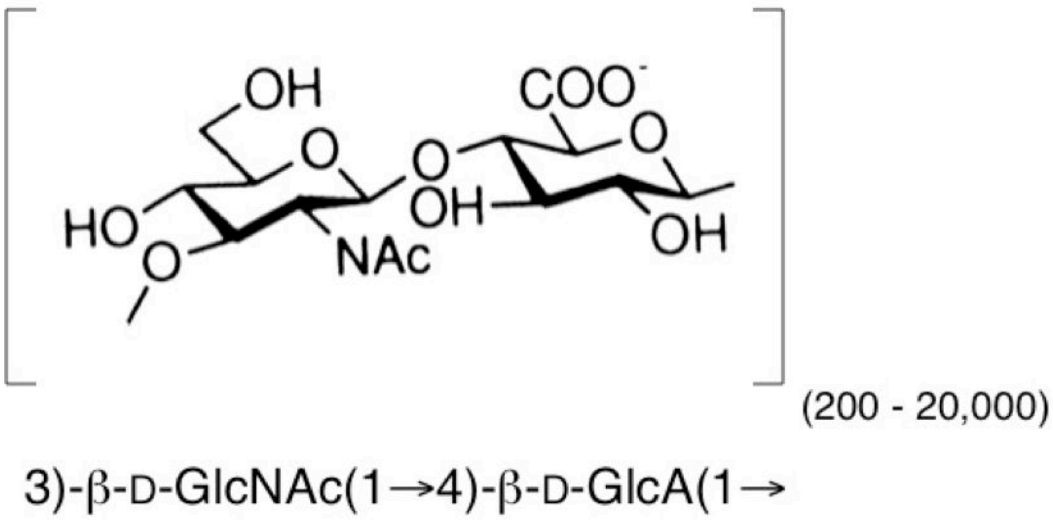

Figure 2. Repeating unit structure of hyaluronic acid.

1992; Wessels, Moses, Goldberg, & DiCesare, 1991). Further characterization of the locus revealed an operon of three genes, *hasA*, *hasB*, and *hasC*, each of which encodes an enzyme involved in hyaluronic acid synthesis (DeAngelis, Papaconstantinou, & Weigel, 1993a; Crater, Dougherty, & van de Rijn, 1995; DeAngelis, Papaconstantinou, & Weigel, 1993b; Dougherty & van de Rijn, 1993; Dougherty & van de Rijn, 1994) (Figure 3). The 4.2 kb *has* operon is highly conserved among group A streptococcal strains, but is notably absent in isolates

of M-types 4 and 22, which do not produce capsules (Henningham, et al., 2014; Flores, Jewell, Fittipaldi, Beres, & Musser, 2012). Notably, strains of M-types 4 and 22 produce hyaluronidase, which degrades hyaluronic acid, while the vast majority of other group A streptococcal isolates harbor an inactivating point mutation in the chromosomal hyaluronidase gene, *hylA* (Henningham, et al., 2014; Hynes, Johnson, & Stokes, 2009). This association suggests an evolutionary branch point in which group A streptococcal strains developed alternative strategies for adaptation through expression of either the anti-phagocytic hyaluronic acid capsule or hyaluronidase, which facilitates the spread of secreted toxins by degrading the host's extracellular matrix, but can also digest the group A streptococcus capsule.

Hyaluronic acid is synthesized from the nucleotide sugar precursors UDP-glucuronic acid and UDP-N-acetylglucosamine by a membrane-associated enzyme, hyaluronan synthase, encoded by *hasA*. High-*Mr* hyaluronic acid can be produced by the incubation of cell-free membrane extracts of group A streptococci with the two substrate UDP-sugars in the presence of divalent cations (Markovitz, Cifonelli, & Dorfman, 1959; Stoolmiller & Dorfman, 1969; Sugahara, Schwartz, & Dorfman, 1979). The *hasA* gene product has a predicted *Mr* of 47.9 kD and includes at least four predicted membrane-spanning domains, which is consistent with evidence that the enzyme is localized at the cell membrane where it mediates both polymer formation and export (DeAngelis, Papaconstantinou, & Weigel, 1993a; Dougherty & van de Rijn, 1994). The group A streptococcus hyaluronan synthase shares significant similarity with hyaluronan synthases from other microbial and higher animal species (DeAngelis, Yang, & Weigel, 1994; Weigel, Hascall, & Tammi, 1997). The second gene of the *has* operon, *hasB*, encodes UDP-glucose dehydrogenase, a 45.5 kD protein that catalyzes the oxidation of UDP-glucose to UDP-glucuronic acid (Dougherty & van de Rijn, 1993). The third gene in the cluster, *hasC*, encodes a predicted 33.7 kD protein identified as UDP-glucose pyrophosphorylase (Crater, Dougherty, & van de Rijn, 1995). This enzyme catalyzes the condensation of UTP with glucose-1-phosphate to form UDP-glucose. Thus, the reaction catalyzed by the *hasC* product yields a substrate for UDP-glucose dehydrogenase encoded by *hasB*, whose reaction product is, in turn, a substrate for hyaluronan synthase encoded by *hasA*. While the enzyme protein encoded by *hasC* is enzymatically active, it is not required for hyaluronic acid synthesis by group A streptococci. The inactivation of *hasC* resulted in no reduction in hyaluronic acid synthesis by a highly encapsulated strain of group A streptococci—a finding that implies that another source of UDP-glucose is available within the cell (Ashbaugh, Alberti, & Wessels, 1998a). Furthermore, expression of recombinant *hasA* and *hasB* (without *hasC*) conferred the capacity to synthesize hyaluronic acid in *Escherichia coli* and *Enterococcus faecalis* (DeAngelis, Papaconstantinou, & Weigel, 1993a; DeAngelis, Papaconstantinou, & Weigel, 1993b). A *hasB* paralog, *hasB2*, is widely conserved among group A streptococcus isolates. This gene is located at a site remote from the *has* operon; it encodes a protein with the same enzymatic activity as *hasB* and can support a reduced level of capsule synthesis in the absence of *hasB* (Cole, et al., 2012).

Regulation of capsule expression

While the *has* operon is highly conserved, there is wide variation in production of the hyaluronic acid capsule among group A streptococcus isolates and under different growth conditions in an individual strain. Transcription of the *has* operon and synthesis of hyaluronic acid is maximal during exponential phase in liquid cultures, and declines to very low levels during the stationary phase (Crater & van de Rijn, 1995; Unnikrishnan, Cohen, & Sriskandan, 1999). Cessation of capsule synthesis is associated with shedding of hyaluronic acid from the cell surface into the culture medium. Capsule production is highest in a nutrient-rich environment. Expression of the *has* operon is rapidly induced upon introduction of the bacteria into the peritoneal cavity of mice or into the pharynx of baboons in a non-human primate model of pharyngeal colonization (Gryllos, et al., 2001). Strain-to-strain variation in capsule production may be partially explained by polymorphisms in the promoter region upstream of the *has* operon (Albertí, Ashbaugh, & Wessels, 1998).

The CsrRS (also known as CovRS) two-component system is a critical regulator of *has* operon transcription in response to environmental signals. The CsrRS system was discovered by a transposon mutagenesis screen for

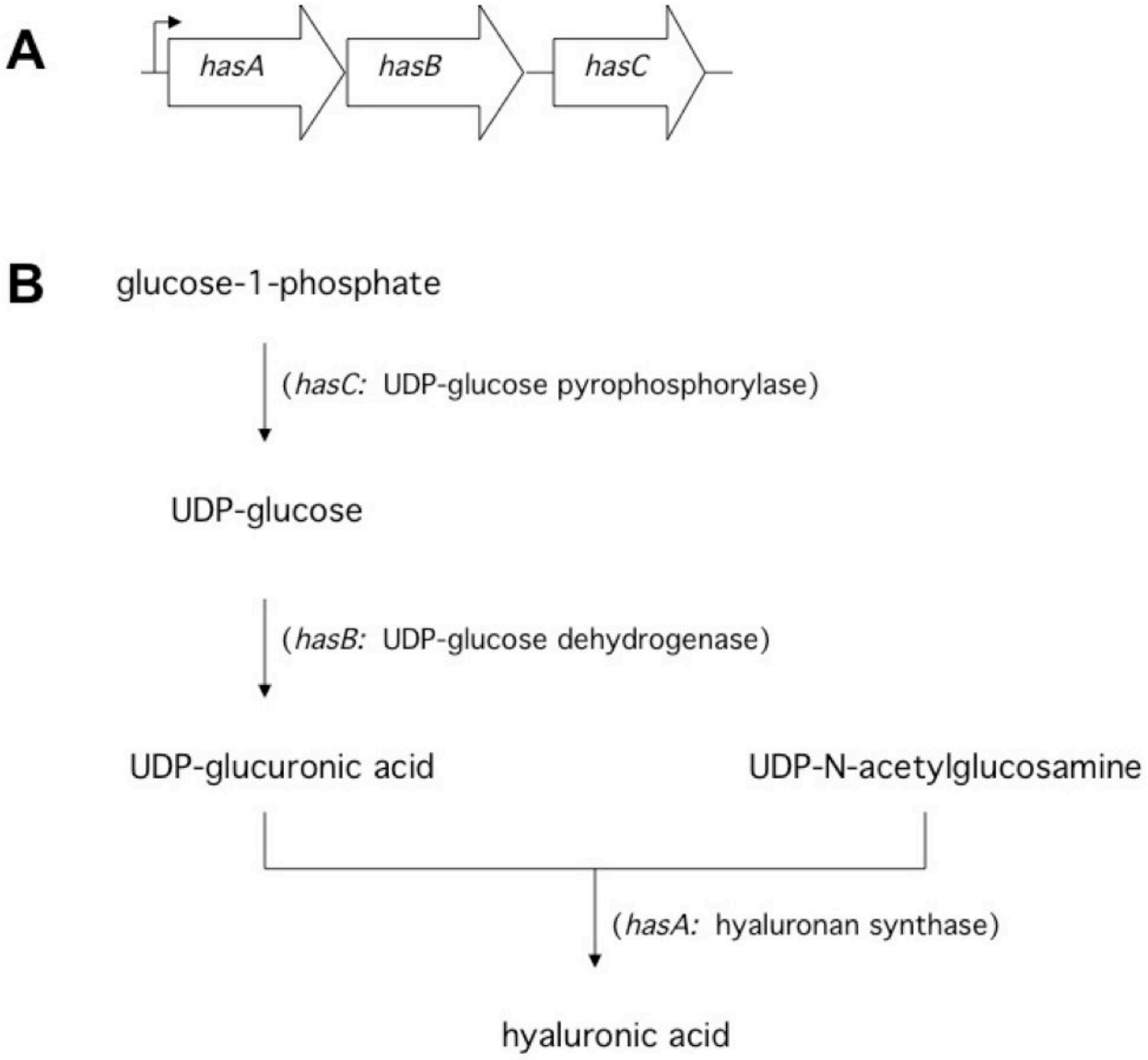

Figure 3. (A) Schematic of the *has* operon encoding enzymes involved in hyaluronic acid biosynthesis in group A streptococci. (B) Diagram illustrating the enzymatic function in hyaluronic acid synthesis of the proteins encoded by *hasA*, *hasB*, and *hasC*.

mutants that formed mucoid colonies; a phenotype that is shown to be the result of inactivation of the *csrRS* locus (Levin & Wessels, 1998). Subsequent work has shown that CsrRS regulates approximately 10% of group A streptococcal genes, including several virulence factors, in addition to the *has* operon (Dalton, Collins, Barnett, & Scott, 2006; Federle, McIver, & Scott, 1999; Graham, et al., 2002; Gryllos, et al., 2007; Heath, DiRita, Barg, & Engleberg, 1999). According to our current model of the CsrRS system, CsrS is a cell membrane-associated histidine kinase that is activated by extracellular magnesium, and perhaps also by other unknown environmental signals (Gryllos, et al., 2007; Gryllos, Levin, & Wessels, 2003). Autophosphorylation of the cytoplasmic domain of CsrS is followed by phosphotransfer to CsrR, which increases the affinity of the latter protein for target promoters—including the *has* operon promoter, where it represses transcription and reduces capsule production. The human antimicrobial peptide LL-37 appears to signal through the CsrRS system in a manner opposite to that of magnesium: exposure of group A streptococci to 100-300 nM LL-37 (concentrations far

below those that inhibit bacterial growth) results in stimulation of expression of the *has* operon (and of other CsrR-repressed genes) (Gryllos, et al., 2008; Tran-Winkler, Love, Gryllos, & Wessels, 2011). LL-37 has been shown to bind to the predicted extracellular domain of CsrS in vitro, and studies of smaller peptides have identified a 10-amino acid internal fragment of LL-37 that is completely devoid of antimicrobial activity against group A streptococci, but retains its CsrS-signaling activity (Velarde, Ashbaugh, & Wessels, 2014). These findings suggest that LL-37 signaling reflects a specific interaction with CsrS rather than a response to a non-specific membrane injury. It has been suggested that the CsrRS system enables group A streptococci to detect the host's innate immune response to group A streptococcal infection by sensing any subinhibitory concentrations of LL-37 secreted by leukocytes and/or epithelial cells. LL-37 signaling through CsrS results in upregulation of capsule production, as well as that of other antiphagocytic factors that enhance group A streptococcal virulence (Gryllos, et al., 2008; Tran-Winkler, Love, Gryllos, & Wessels, 2011).

RocA is another regulatory protein that affects capsule expression. Inactivating mutations in *rocA* have been shown to reduce expression of CsrR and are associated with increased capsule production (Biswas & Scott, 2003). In M-type 18 strains that form mucoid colonies as a result of abundant capsule production, the RocA protein is truncated and non-functional. Replacement of the mutated *rocA* gene with the consensus *rocA* sequence in a mucoid M18 strain suppressed the mucoid phenotype (Lynskey, et al., 2013).

Role of the capsule in pathogenesis

Early studies by Lancefield, Todd, and others found an association between the presence of the mucoid colony type and virulence in mice (Todd & Lancefield, 1928; Ward & Lyons, 1935). However, because mucoid strains tended to be rich in M protein as well as in hyaluronic acid capsule, it was difficult to confidently ascribe an independent role in virulence to the capsule. Investigators in the mid-twentieth century found that hyaluronidase treatment increased the susceptibility of mucoid strains to killing by human blood phagocytes, supporting a role for the capsule in resistance to phagocytosis (Rothbard, 1948; Foley & Wood, Jr., 1959; Stollerman, Rytel, & Ortiz, 1963). Kass and Seastone showed that hyaluronidase treatment of mice reduced the virulence of group A streptococci during experimental infections (Kass & Seastone, 1944). Epidemiological observations have also suggested a link between capsule expression and virulence. Mucoid strains of group A streptococci have been associated with invasive infections and with outbreaks of acute rheumatic fever (Johnson, Stevens, & Kaplan, 1992; Tamayo, Montes, García-Medina, García-Arenzana, & Pérez-Trallero, 2010; Veasy, et al., 2004).

The development of methods for genetic manipulation of streptococci permitted more direct assessment of the role of the capsule in pathogenesis. Acapsular mutants derived by transposon mutagenesis and subsequently by targeted deletion of the *hasA* gene were found to have reduced virulence in systemic infection models in mice and in chicken embryos, in a murine model of invasive soft tissue infection, and in airway challenge models in mice (Wessels, Moses, Goldberg, & DiCesare, 1991; Ashbaugh, Warren, Carey, & Wessels, 1998b; Husmann, Yung, Hollingshead, & Scott, 1997; Schmidt, Günther, & Courtney, 1996; Schrager, Rheinwald, & Wessels, 1996; Wessels & Bronze, 1994). Capsule-deficient mutants have increased susceptibility to complement-mediated opsonophagocytic killing by human blood leukocytes, as compared to their respective encapsulated parent strains, and resistance to phagocytosis is thought to be a major mechanism through which the capsule enhances virulence. The presence of the capsule does not inhibit complement activation or deposition of complement fragments on the bacterial cell wall, but rather interferes with access of leukocyte receptors for opsonic complement proteins on the bacterial surface (Dale, Washburn, Marques, & Wessels, 1996).

In a baboon model of group A streptococcal pharyngeal colonization, an acapsular mutant colonized at a similar efficiency as the parent strain, but was cleared more rapidly (Ashbaugh, et al., 2000). This result suggested that the capsule contributed to persistence in the pharynx. However, genomic analysis of serial isolates from the pharynges of experimentally infected macaques showed the development over time of mutations in the *has*

operon and promoter that reduced transcription of the hyaluronic acid biosynthesis genes (Shea, et al., 2011). Similar mutations were detected in sequential group A streptococcal isolates from human pharyngeal samples (Flores, et al., 2014). When taken together, these observations suggest that the down-regulation of capsule production may favor chronic pharyngeal carriage.

In vitro studies of group A streptococcal adherence to epithelial cells have shown that the capsule reduces bacterial attachment (Hollands, et al., 2010; Schrager, Albertí, Cywes, Dougherty, & Wessels, 1998). The capsule itself can act as an adhesin by mediating attachment to the hyaluronic acid binding protein CD44, which is expressed on multiple cell types including oropharyngeal keratinocytes (Schrager, Albertí, Cywes, Dougherty, & Wessels, 1998). The potential role of CD44 as a group A streptococcus receptor was supported by studies showing reduced pharyngeal colonization in mice after intranasal administration of monoclonal antibody to CD44 together with a group A streptococcal challenge or after pretreatment with exogenous hyaluronic acid (Cywes, Stamenkovic, & Wessels, 2000). Mice expressing a CD44 anti-sense transgene targeted to stratified squamous epithelia also were resistant to group A streptococcal pharyngeal colonization. In addition to the role of CD44 in adherence, the binding of encapsulated group A streptococcus to CD44 on human oropharyngeal keratinocytes induces an intracellular signaling cascade that results in disruption of intercellular junctions and enhancement of group A streptococcal translocation across the epithelial barrier. In this way, CD44-mediated signaling by the hyaluronic acid capsule may facilitate group A streptococcus tissue invasion (Cywes & Wessels, 2001).

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The spatial regulation of protein sorting in *Streptococcus pyogenes*

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Abstract

Surface proteins play a critical role in the pathogenesis and survival of Gram-positive bacteria in an infected host. Anchoring of surface proteins to the cell wall is highly regulated in both space and time, and is coordinated with cell division. Newly synthesized surface proteins contain an N-terminal signal sequence that targets them for secretion across the membrane. Depending on the type of signal sequence, proteins are translocated either at the septum or cell periphery, resulting in distinct cellular distribution patterns. The C-terminus of surface proteins contain a cell wall sorting signal (CWS), composed of an LPXTG motif, a hydrophobic stretch, and a few positive amino acids. Translocation of surface proteins stalls when the CWS reaches the secretion channel, leaving the LPXTG motif exposed outside the membrane. This motif is then cleaved by the enzyme sortase, and covalently attached to lipid II, leading to cell wall anchoring. Sortase is dynamically localized to membranal foci that are primarily associated with the division septum, and is recruited to the septum at an early stage. In this chapter, we review the spatial regulation of surface anchoring in *Streptococcus pyogenes* and how it relates to protein secretion and cell division.

The cell wall of Gram-positive bacteria is composed of cross-linked peptidoglycan, teichoic and lipoteichoic acids, polysaccharides, and proteins (Navarre & Schneewind, 1999; Vollmer, Blanot, & de Pedro, 2008; Weidenmaier & Peschel, 2008). Many of the proteins found on the surface of gram-positive bacteria are covalently anchored to the peptidoglycan by sortase (Marraffini, Dedent, & Schneewind, 2006). This section will discuss the spatial regulation of surface protein anchoring to the *Streptococcus pyogenes* cell wall. In order to provide a context for the different patterns of surface protein anchoring, this chapter will begin with a short overview of cell division. For an in-depth discussion of cell division in streptococci, please refer to two excellent review articles (Philippe, Vernet, & Zapun, 2014; Zapun, Vernet, & Pinho, 2008).

Recently, the term ovococcus was introduced to describe bacteria that have a slightly elongated spherical form, such as streptococci and enterococci, and which are distinct from true cocci (Zapun, Vernet, & Pinho, 2008). True cocci are completely spherical and typically divide in two or three alternating division planes. For example, division of staphylococci occurs in three altering planes and results in the formation of cell clusters (Tzagoloff & Novick, 1977). Ovococci, on the other hand, maintain the same division plane from one division to the next, and in many cases, do not completely separate following division, which results in the formation of chains of organisms. Cell wall synthesis is carried out by specialized machineries, composed of penicillin binding proteins (PBPs), a lipid II translocase, and regulatory proteins. While true cocci have a single, septum associated, peptidoglycan synthesis machinery, ovococci have two such mechanisms. The first mechanism is responsible for septation, while the second mediates peripheral peptidoglycan synthesis, and is analogous to the one used by rod-shaped bacteria, such as *Escherichia coli* and *Bacillus subtilis*, for cell elongation. While the peripheral peptidoglycan synthesis machinery is located in foci along the cylindrical surface of the bacteria in those organisms (Margolin, 2009), in ovococci, both of the peptidoglycan synthesis machineries are localized at mid-cell (Zapun, Vernet, & Pinho, 2008). Nevertheless, the two fill different functions; for example, *S. pneumoniae* Pbp2x (part of the septal machinery) localizes to a ring that closely follows the constriction of the septum, while

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Pbp1a (part of the elongation machinery) forms a ring that trails that of Pbp2x during septum closure (Land, et al., 2013). The presence of a peripheral peptidoglycan synthesis machinery in ovococci is believed to contribute to their slightly elongated form. *S. pyogenes*, which is less elongated than many other ovococci, lacks key components of the peripheral peptidoglycan synthesis machinery, Pbp2b and RodA (Zapun, Vernet, & Pinho, 2008).

Cell division and peptidoglycan synthesis are both controlled by cytoskeletal elements. True cocci, ovococci, and rod-shaped bacteria all have a FtsZ ring, which is composed of polymerized tubulin-like FtsZ molecules (Margolin, 2009). The FtsZ-ring is a dynamic structure that assembles at the site of the future division and that controls the division process through the sequential direct or indirect recruitment of regulatory factors, lipid II translocase, and penicillin binding proteins (Egan & Vollmer, 2013; Errington, Daniel, & Scheffers, 2003; Goehring & Beckwith, 2005; Mohammadi, et al., 2011; Sauvage, Kerff, Terrak, Ayala, & Charlier, 2008). Rod-shaped bacteria also have one or more actin-like cytoskeletal elements (MreB, Mbl, MreBH) that are dedicated to peptidoglycan synthesis regulation along the cylindrical region of the bacteria, but these are absent from both true cocci and ovococci (Margolin, 2009; Philippe, Vernet, & Zapun, 2014).

Division in ovococci begins with the splitting of the septum into two distinct rings termed "equatorial rings" (Higgins & Shockman, 1970; Tomasz, Jamieson, & Ottolenghi, 1964). These two equatorial rings are gradually "pushed" away from each other by the simultaneous synthesis of septal peptidoglycan and the splitting of the septum to create two new hemispheres. Peptidoglycan synthesis occurs in bands parallel to the short axis of the cell, which result in concentric rings with the same orientation as the division septum (Andre, et al., 2010; Wheeler, Mesnage, Boneca, Hobbs, & Foster, 2011). The equatorial rings are prominent features of ovococcal morphology, and are clearly visible in electron micrographs. They represent the cellular location with the largest diameter, and as such, are the location for future assembly of FtsZ rings and division septa in the daughter cells. There is evidence that in some ovococci two consecutive division cycles can overlap; namely, that division can start in the daughter cell septa (mother cell equatorial rings) before the mother septum is entirely closed. Classical electron microscopy studies by Higgins and Shockman demonstrated that this occurs in certain enterococci (Gibson, Daneo-Moore, & Higgins, 1983; Higgins, Carson, & Daneo-Moore, 1980; Higgins & Shockman, 1970; Higgins & Shockman, 1976). Additionally, fluorescent vancomycin, which labels the D-Ala–D-Ala moieties found in lipid II and newly synthesized peptidoglycan (Daniel & Errington, 2003), labels both the division septum and the equatorial rings of *Streptococcus pneumoniae* (Daniel & Errington, 2003; Ng, Kazmierczak, & Winkler, 2004; Wheeler, Mesnage, Boneca, Hobbs, & Foster, 2011), and *S. pyogenes* (Raz & Fischetti, 2008). However, the overlap of division cycles was not observed in *Lactococcus lactis* and *Enterococcus faecalis*, where fluorescent vancomycin labels daughter septa only after the mother septum is completely closed (Wheeler, Mesnage, Boneca, Hobbs, & Foster, 2011).

Surface protein anchoring to the wall of *S. pyogenes*

Wall-anchored proteins share similar features. These include an N-terminal signal sequence that directs the protein for secretion through the Sec apparatus, and a C-terminal cell wall sorting signal (CWS), which is comprised of an LPXTG motif followed by a hydrophobic membrane spanning stretch, along with a few positively charged amino acids at the C-terminus (Fischetti, Pancholi, & Schneewind, 1990; Schneewind, Model, & Fischetti, 1992). Translocation of surface proteins through the Sec apparatus is halted when the C-terminal sorting signal is reached (Schneewind, Mihaylova-Petkov, & Model, 1993; Schneewind, Model, & Fischetti, 1992). The LPXTG motif, which at the stalled state is exposed outside the membrane, is cleaved between the threonine and glycine residues by the membranal transpeptidase sortase, and the freed threonine is connected to lipid II (Marraffini, Dedent, & Schneewind, 2006; Mazmanian, Liu, Ton-That, & Schneewind, 1999; Perry, Ton-That, Mazmanian, & Schneewind, 2002). The resulting complex is then incorporated into the cell wall through the action of PBPs.

Like peptidoglycan synthesis, anchoring of surface proteins is spatially regulated. Early studies by Cole and Hahn used fluorescence microscopy to examine the manner in which M protein is attached to the wall of *S. pyogenes* (Cole & Hahn, 1962; Hahn & Cole, 1963). Labeling the *S. pyogenes* M protein with fluorescent antibodies results in a roughly uniform labeling of the bacterial surface. When such labeled cells divide, newly synthesized peptidoglycan is marked by patches of unlabeled cell wall. Similarly, pre-existing M protein can be blocked with unlabeled antibodies or removed using proteases, which enables the use of fluorescent antibodies to detect newly deposited M protein. The combined observations from these experiments conclusively demonstrated that M protein is exclusively anchored at the division septum, which is also the sole site for cell wall growth in this organism (Cole & Hahn, 1962; Hahn & Cole, 1963). Similar results were also obtained from studying the location of M protein anchoring using electron microscopy. M protein, which appears as hair-like structures in electron micrographs, is regenerated at the septum following proteolytic removal of pre-existing M protein (Swanson, Hsu, & Gotschlich, 1969). However, the septum is not the only location for protein anchoring in *S. pyogenes*. Protein F (PrtF1 or SfbI), a factor important in the binding of host fibronectin (Hanski & Caparon, 1992; Talay, Valentin-Weigand, Jerlström, Timmis, & Chhatwal, 1992; Yamaguchi, Terao, & Kawabata, 2013), is predominantly found at the cell poles (Ozeri, et al., 2001). For continuity with other sections of this work, we will refer to this protein as PrtF1.

A major breakthrough in understanding surface protein targeting different cellular regions came from a study by Carlsson et al. (Carlsson, et al., 2006). This study showed that the signal sequence is the factor that determines whether proteins are anchored at the septum or the poles. Replacing the signal sequence of M protein with that of PrtF1 results in a molecule secreted and anchored, like wild-type PrtF1, at the poles. Conversely, replacing the signal sequence of PrtF1 with that of M protein results in a molecule that is secreted and anchored, like wild-type M protein, at the septum. A similar phenomenon was later described in *Staphylococcus aureus*, where the replacement of the signal sequence of ClfA (normally secreted at the septum) with that of SasF (normally secreted at the poles) results in a molecule that is anchored at the poles, and vice versa.

The majority of *S. pyogenes* surface proteins are anchored by the housekeeping sortase, SrtA. This has been experimentally validated for M protein, PrtF1, C5a peptidase (ScpA), and protein-G-related-α2-macroglobulin-binding protein (GRAB) (Barnett & Scott, 2002). Depending on the strain, *S. pyogenes* also has one or more sortases that are responsible for pilus polymerization, termed SrtB and SrtC (*srtC* has two alleles, *srtC1* and *srtC2*) (Barnett, Patel, & Scott, 2004; Barnett & Scott, 2002; Mora, et al., 2005). The role of sortases in the streptococcal pilus polymerization is addressed elsewhere in this work, though little is known about the spatial regulation of pilus assembly in *S. pyogenes*. Sortases are membrane-bound enzymes that are found beneath the cell wall, and that are therefore not accessible to antibodies. Using the phage lytic enzyme, PlyC, to gently permeabilize the wall of fixed *S. pyogenes* cells, we were able to use immunofluorescence microscopy to determine the localization pattern of SrtA (Raz & Fischetti, 2008). We found that sortase localizes to foci, which are predominantly associated with the division septum, but are also found in other cellular regions to some extent. The distribution of sortase foci in the cell depends on the stage of the cell cycle. Sortase begins to assemble at a new division site before constriction of the septum is apparent, shows distinct septal localization at mid-division, and remains associated with the septum to a certain extent even after division is complete. In many cases, sortase foci are found simultaneously at the division septum and the equatorial rings, particularly at later stages of the cell cycle.

Septal versus peripheral protein anchoring

Super-resolution 3D structured illumination microscopy (3D-SIM) was used to obtain a better understanding of the pathways that lead to M protein and PrtF1 anchoring. Analysis of a large population of cells following proteolytic removal of pre-existing surface proteins and two minutes regeneration demonstrated that M protein and PrtF1 are simultaneously anchored at their respective cellular locations throughout the cell cycle (Raz, Talay, & Fischetti, 2012). M protein is anchored at a narrow septal band, and is often seen at mother and daughter

septa within the same cell. Simultaneous anchoring to mother and daughter septa was observed in up to 40% of the cells in certain stages of the division cycle, even when M protein was regenerated for only 30 seconds (Raz, Talay, & Fischetti, 2012). This is in agreement with the observed simultaneous localization of sortase foci to both mother and daughter septa, and with the simultaneous labeling of these location by fluorescent vancomycin (Raz & Fischetti, 2008).

While PrtF1 shows a clear polar distribution on *S. pyogenes* cells, anchoring of PrtF1 does not occur exclusively at the poles, but also in the area between the septum and the equatorial rings. Its anchoring pattern, therefore, is better described as peripheral (or non-septal), rather than polar. There is a direct correlation between the age of the pole and the amount of PrtF1 anchored to it—which suggests that PrtF1 accumulates at a steady pace on peripheral peptidoglycan, and that the accumulation of more PrtF1 on mature poles leads to the observed polar distribution (Raz, Talay, & Fischetti, 2012). A schematic representation of protein anchoring in *S. pyogenes* is presented in Figure 1.

Anchoring of M protein to the wall is dependent on the presence of a functional septum. Disruption of the septum does not result in a re-direction of M protein anchoring to other cellular locations, but instead causes a complete shutdown of M protein anchoring. PrtF1 anchoring on the other hand, is not inhibited by the disruption of the septum (Raz, Talay, & Fischetti, 2012). At a low concentration, methicillin preferentially inhibits PBP2x, which results in unbalanced peptidoglycan synthesis and can prevent septum closure in some ovococci (Lleo, Canepari, & Satta, 1990; Pérez-Núñez, et al., 2011). Application of a sub-inhibitory methicillin concentration to *S. pyogenes* results in the formation of rod-like cells with multiple septa (Raz, Talay, & Fischetti, 2012). M protein is anchored to all the septa within such cells, while PrtF1 is anchored both at the poles and the extensive inter-septal regions of these rod-like cells, which further demonstrates that PrtF1 is not targeted to the poles *per se*, but rather is targeted to any non-septal region. A higher concentration of methicillin completely inhibits septum formation and M protein anchoring, but not PrtF1 anchoring. Similar inhibition of M protein anchoring occurs in cells that over-express DivIVA (Raz, Talay, & Fischetti, 2012), an important cell division regulator (Fadda, et al., 2003; Fadda, et al., 2007; Ramirez-Arcos, Liao, Marthaler, Rigden, & Dillon, 2005). These cells display abnormal septa and bloated morphology, and while M protein anchoring is drastically reduced, PrtF1 anchoring increases (Raz, Talay, & Fischetti, 2012).

The different outcomes of septal and peripheral anchoring

Anchoring of M protein concomitantly with peptidoglycan synthesis at the septum assures the coating of the entire cell wall from the moment it is formed. However, if M protein is lost, it can no longer be attached to existing peptidoglycan. Extensive coating of the cell wall is likely required for the proper function of M protein, since wall patches lacking this protein could become targets for complement deposition, leading to opsonization and clearance of the bacteria from an infected host (Perez-Casal, Caparon, & Scott, 1992). The currently available data suggest that septal anchoring may be the dominant anchoring pathway in terms of the number of molecules anchored (although it is possible that a greater variety of molecules is anchored at the periphery; see below). First, sortase foci are highly enriched at the septum throughout the division cycle, and there is a strong correlation between sortase and newly anchored M protein in co-localization studies (Raz & Fischetti, 2008). Secondly, lipid II, the molecule to which surface proteins are anchored, is highly enriched at the septum, as indicated from the strong fluorescent vancomycin labeling at this location (Raz & Fischetti, 2008). Thirdly, PBPs, which are required for the incorporation of the lipid II – surface protein complex into the cell wall, are predominantly associated with the septum (Zapun, Vernet, & Pinho, 2008). And finally, in *S. pyogenes* cells harboring a sub-optimal amount of sortase, which results in delayed processing of surface proteins, sorting intermediates with uncleaved LPXTG motifs are detected primarily at the septum (Raz, et al., 2015).

On the other hand, peripheral anchoring leaves a substantial portion of the wall devoid of anchored proteins, but creates polar distribution. Localization of virulence factors to the poles is a common phenomenon in other

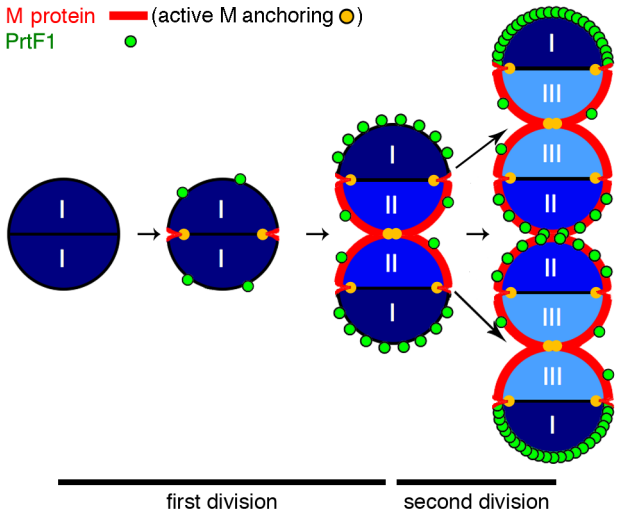

Figure 1. A model representation of M protein and PrtF1 anchoring. The regeneration of surface proteins during two division cycles is presented. M protein (red) is anchored exclusively to newly synthesized peptidoglycan at the septum (sites of active anchoring labeled yellow). Anchoring of M protein at daughter septa begins before the mother septum is completely closed, which results in simultaneous anchoring at both locations. Following two generations, M protein is anchored to all newly synthesized, but not pre-existing, peptidoglycan. PrtF1 (green) is anchored over time in patches to peripheral peptidoglycan, with some preference to the poles. Following two generations, the oldest poles (I) display the most intense PrtF1 labeling, while one-generation-old poles show less labeling (II), and newly formed poles (III) show little PrtF1 labeling. The figure was adopted with permission from Raz et al., 2012 (Raz, Talay, & Fischetti, 2012).

bacterial pathogens (Laloux & Jacobs-Wagner, 2014; Rudner & Losick, 2010; Shapiro, McAdams, & Losick, 2002; Shapiro, McAdams, & Losick, 2009; Treuner-Lange & Søgaard-Andersen, 2014). Older poles at the end of streptococcal chains may be more likely to come in contact with host tissue, which makes polar accumulation of various factors (such as adhesins) advantageous. It was also observed that the polar distribution of PrtF1 results in reciprocal clustering of integrins, which form focal complexes on host cells (Ozeri, et al., 2001).

The role of YSIRK G/S motif in the signal sequence of surface proteins

The signal sequence of *S. pyogenes* M protein contains a YSLRKx_3Gx_2S motif that is conserved among various *S. pyogenes* serotypes, while PrtF1 does not contain this motif (Carlsson, et al., 2006). This motif is similar to the YSIRKx_3Gx_2S (shorthand YSIRK G/S) found in staphylococcal lipases (Götz, Verheij, & Rosenstein, 1998; Nikoleit, Rosenstein, Verheij, & Götz, 1995; Rosenstein & Götz, 2000), the majority of *S. aureus* surface proteins (Bae & Schneewind, 2003; DeDent, Bae, Missiakas, & Schneewind, 2008), 4 of the *S. aureus* secreted proteins (Bae & Schneewind, 2003), and in many *S. pneumoniae* surface proteins (Tettelin, et al., 2001; Tsui, Keen, Sham, Wayne, & Winkler, 2011). A YSIRK G/S motif was identified in only a single *Enterococcus faecalis* surface protein (Kline, et al., 2009), and is absent from *Listeria monocytogenes* (Bruck, Personnic, Prevost, Cossart, & Bierne, 2011), bacilli, clostridia, corynebacteria, *Streptomyces* spp., and *Actinomyces* spp. (Bae & Schneewind, 2003).

The YSIRK G/S motif is important for efficient processing of the signal sequence of *S. aureus* protein A by signal peptidase; however, it is not necessary for anchoring protein A to the cell wall (Bae & Schneewind, 2003). There is a close correlation between the presence of a YSIRK G/S motif in the signal sequence of *S. aureus* surface proteins and secretion at the septum. Five proteins containing a YSIRK G/S motif in their signal sequence (protein A, ClfA, SdrC, SdrD, FnbpB) are secreted and anchored at the septum, while four proteins that do not contain this motif in their signal sequence (SasA, SasD, SasF, SasK) are anchored at the poles (DeDent, Bae, Missiakas, & Schneewind, 2008; DeDent, McAdow, & Schneewind, 2007). Fusion of the signal sequence of the

staphylococcal lipase (contains YSIRK G/S) to mCherry and a CWS motif results in a molecule that is anchored at the septum, while a similar fusion protein, which contains the signal sequence of SasF (no YSIRK G/S), is anchored at the poles. Notably, growth of *S. aureus* in the presence of sub-inhibitory concentrations of penicillin or moenomycin leads to the anchoring of both fusion proteins at the septum (Yu & Götz, 2012). Despite this close correlation between the presence of a YSIRK G/S in the signal sequence and secretion at the septum, the YSIRK sequence itself does not appear to be the septum targeting motif, since mutation of the *S. pyogenes* M protein YSLRK motif to YAARK or YSAAK did not alter the site of protein secretion (Carlsson, et al., 2006), and neither did the use of a scrambled YSIRK motif in *S. aureus* ClfA (DeDent, Bae, Missiakas, & Schneewind, 2008).

Of 20 *S. aureus* surface proteins, 13 have a highly conserved YSIRK G/S motif in their signal sequence. In particular, the G/S residues are absolutely conserved, and the distance between the YSIRK G/S motif and the predicted signal peptidase cleavage site varies by no more than two amino acids (DeDent, Bae, Missiakas, & Schneewind, 2008). In *S. pyogenes* on the other hand, the YSIRK G/S motif is both less common and less conserved (Figure 2A). Of the 16 surface proteins found in serotype M6 *S. pyogenes* MGAS10394 (Banks, et al., 2004), only two have a conserved YSIRK G/S motif (M protein and ScpC), while three additional proteins have a partial YSIRK G/S motif with changes not only in the YSIRK sequence itself but also in the final G/S amino acids (GRAB, PulA, and a highly altered motif in 5'-nucleotidase) (Raz, et al., 2015). Furthermore, the distance between the YSIRK G/S motif and the predicted signal peptidase cleavage site varies by up to eight amino acids, as opposed to two in *S. aureus*. The absence of an intact YSIRK G/S motif from the signal sequence of most *S. pyogenes* surface proteins may indicate that those proteins are secreted at the cell periphery. However, given the high variability observed in the YSIRK G/S motif in *S. pyogenes*, a systematic analysis of the anchoring location of additional surface proteins is required.

In addition to M protein, certain streptococcal strains have one or more M-like proteins, which are clustered together with M protein on the genome, and have likely arisen through gene duplication (Hollingshead, Arnold, Readdy, & Bessen, 1994). The signal sequence of M-like proteins is very similar to that of M protein, and has an intact YSIRK G/S motif (Figure 2B). In *S. aureus*, the presence of a YSIRK G/S motif in the signal sequence contributes to its efficient processing by signal peptidase (Bae & Schneewind, 2003; Yu & Götz, 2012). It might also be important in *S. pyogenes*, given that M protein is among the most abundant of *S. pyogenes* surface proteins, and that it is rapidly anchored at a narrow septal band.

Localized protein secretion and anchoring

To reach their final destination on the cell wall, surface proteins are translocated across the cytoplasmic membrane through the general secretory pathway (Buist, Ridder, Kok, & Kuipers, 2006; de Keyzer, van der Does, & Driessen, 2003; Marraffini, Dedent, & Schneewind, 2006; Schneewind & Missiakas, 2014). *S. pyogenes* surface proteins are not likely to be translocated through the tween arginine (tat) secretion system, since components of the tat system are absent from the genome of this organism (Ferretti, et al., 2001). The location of the secretion apparatus in *S. pyogenes* is a highly contested issue. Rosch et al. used immunoelectron microscopy to study the distribution of the ATPase subunit of the translocon, SecA, and found that it localizes to a single microdomain on the membrane of *S. pyogenes* cells, termed ExPortal. Secretion of the protease SpeB is localized to this microdomain, as well as the chaperone HtrA (Rosch & Caparon, 2004; Rosch & Caparon, 2005). This microdomain is enriched in anionic lipids, and can be visualized using nonyl acridine orange (NAO) (Rosch, Hsu, & Caparon, 2007). On the other hand, Carlsson et al. have studied the location of SecA on the membrane of *S. pyogenes* using similar techniques, and found that SecA is distributed throughout the membrane of this organism (Carlsson, et al., 2006). The reason for this difference in SecA distribution is still unresolved. There is evidence that not all proteins are translocated through the same secretory pathway in *S. pyogenes*. An *ffh*$^{-}$ mutant, which lacks a functional signal recognition particle (SRP), does not secrete NAD^{+} glycohydrolase (SPN) and streptolysin O (SLO), but can secrete SpeB and M protein (Rosch, Vega, Beyer, Lin, & Caparon, 2008).

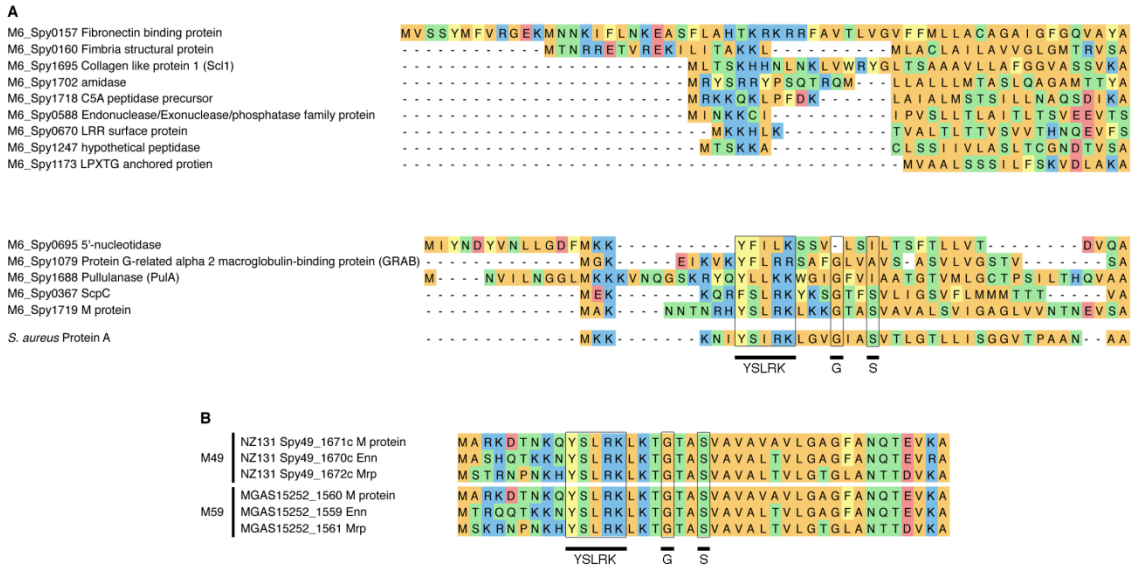

Figure 2. The YSIRK G/S motif in the signal sequence of *S. pyogenes* surface proteins is only partially conserved.
A. Screening the genome of *S. pyogenes* MGAS10394 for proteins with a conserved cell wall sorting signal (CWS) identified 16 proteins. SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP) was used to determine the presence of a signal sequence and to predict the signal peptidase cleavage site. Surface proteins were divided into two groups, based on the presence of a YSIRK G/S motif (including partial YSIRK G/S motif) in their signal sequence. The predicted signal sequences were aligned using MegAlign pro (DNASTAR, Lasergene 12).
B. The signal sequence of M protein and M-like proteins, Enn and Mrp, from strains NZ131 (M49) and MGAS 15252 (M59) were aligned in a similar fashion.

Additionally, the cationic antimicrobial peptide polymyxin B disrupts the ExPortal at a sub-lethal concentration and inhibits the secretion of SpeB and SLO, but not the secretion of streptococcal inhibitor of complement-mediated lysis (SIC) (Vega & Caparon, 2012). This suggests that only a subset of streptococcal proteins is translocated through the ExPortal.

The localization patterns of SecA and sortase A have also been described in several other ovococci. Immuno-EM studies of *Streptococcus mutans* showed that SecA and sortase A co-localize in a single ExPortal on the bacterial membrane, suggesting that the ExPortal controls the location of protein anchoring in this organism (Hu, Bian, Fan, Huang, & Zhang, 2008). Similarly, Immuno-EM studies of *E. faecalis* showed that SecA, sortase A, and sortase C, localize to a single microdomain on the bacterial membrane, which is found at septal or peripheral regions of the cell (Kline, et al., 2009). Later, however, fluorescence microscopy studies have shown that SecA and sortase A often localize to a number of foci per cell, and that these are distinctly associated with the septum (Kandaswamy, et al., 2013). Fluorescence microscopy studies of *S. pneumoniae* showed that SecA and SecY are dynamically distributed in the cells, with preferential localization to the septum, and assembly at equatorial rings early in the division cycle (Tsui, Keen, Sham, Wayne, & Winkler, 2011). *S. pneumoniae* sortase A localizes to membranal foci; however, no specific localization pattern was observed (Tsui, Keen, Sham, Wayne, & Winkler, 2011). Finally, a fluorescence microscopy study of *Streptococcus agalactiae* showed that SecA and Sortase A localize to the septum (Brega, Caliot, Trieu-Cuot, & Dramsi, 2013).

The extensive research regarding translocation of secreted factors thorough the *S. pyogenes* ExPortal (Port, Vega, Nylander, & Caparon, 2014; Rosch & Caparon, 2004; Rosch & Caparon, 2005; Rosch, Hsu, & Caparon, 2007; Vega & Caparon, 2012; Vega, Port, & Caparon, 2013), as well as reports showing that in some ovococci sortase localizes to the ExPortal (Hu, Bian, Fan, Huang, & Zhang, 2008; Kline, et al., 2009), warrant discussion of a possible role for the ExPortal in the translocation of surface proteins in *S. pyogenes*. Targeting of M protein and

PrtF1 for translocation at different cellular locations, guided by information within their signal sequence (Carlsson, et al., 2006), is hard to reconcile with secretion through a single ExPortal. Furthermore, simultaneous anchoring of M protein and PrtF1 was observed at their respective cellular locations throughout the cell cycle (Raz, Talay, & Fischetti, 2012), ruling out the possibility that a single ExPortal could facilitate the anchoring of both proteins by segregating their anchoring to specific stages of the cell cycle. Moreover, following a mere 30 seconds of protein regeneration, M protein was often simultaneously detected at both mother and daughter septa (Raz, Talay, & Fischetti, 2012), which requires secretion at more than one cellular location at once. Additionally, surface protein intermediates with an intact LPXTG motif accumulate primarily at the septum of cells with a sub-optimal amount of sortase, and in larger regions of the membrane of cells lacking sortase altogether; no accumulation in an ExPortal-like pattern was observed (Raz, et al., 2015). While the ExPortal localizes to the septum in some cells, in many cells it localizes to other cellular regions (Rosch & Caparon, 2004; Rosch & Caparon, 2005; Rosch, Hsu, & Caparon, 2007), which is not consistent with the anchoring of M protein at the septum throughout the cell cycle (Raz, Talay, & Fischetti, 2012). Accordingly, when NAO is used to localize the membranal anionic lipid microdomain in which the ExPortal resides, only occasional co-localization with sites of M protein anchoring is seen (Figure 3). Given these observations, the ExPortal is not likely to control localized surface protein anchoring in *S. pyogenes*.

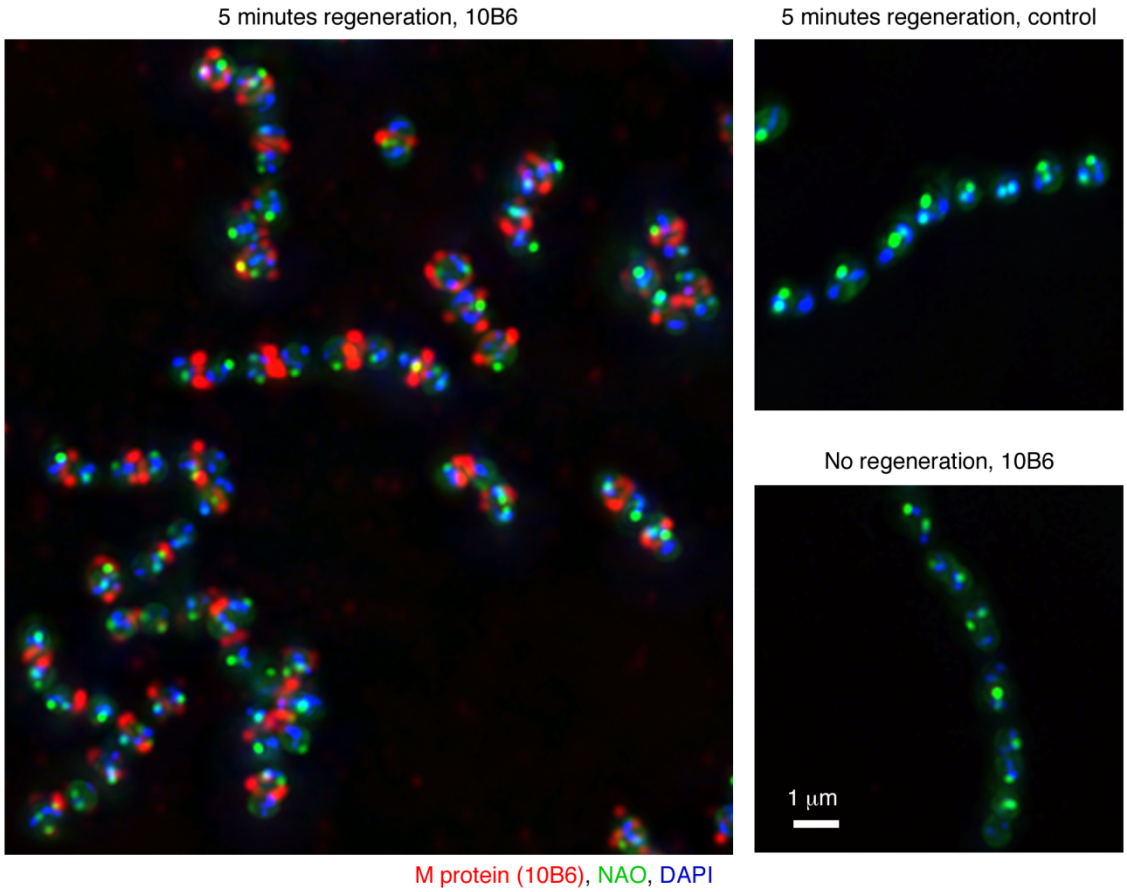

Figure 3. Poor co-localization between the ExPortal and sites of M protein anchoring. *S. pyogenes* D471 was grown to the log phase in the presence of trypsin. Cells were either fixed immediately ("No regeneration" control), or washed and allowed to regenerate M protein for five minutes in medium without proteases, and then fixed. M protein (red) was labeled with the 10B6 monoclonal antibody and Alexa Fluor 647 conjugate; anionic lipids (green) were labeled with nonyl acridine orange (NAO); and DNA (blue) was labeled with DAPI. Images were obtained using deconvolution immunofluorescence microscopy and are presented as maximum intensity projections, composed of all the Z-sections.

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Molecular Basis of Serotyping and the Underlying Genetic Organization of *Streptococcus pyogenes*

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Abstract

Historically, three serological typing schemes were used to classify *Streptococcus pyogenes* isolates. All are based on LPXTG-linked (or similar) surface proteins that exhibit high levels of antigenic heterogeneity. The serological targets include M proteins, T-antigens (which form pili), and serum opacity factor (SOF). More recently, the genetic basis for serological typing has been elucidated. Three *emm* pattern groupings, defined by 3' ends of *emm* and *emm*-like genes (encoding distinct peptidoglycan-spanning domains), display a strong correspondence with streptococci that tend to cause infection at the epithelium of the throat versus at the skin. Significant correlations extend to *emm* cluster groups (defined by surface-exposed, functional portions of M protein genes), and to T-antigen (FCT-region) and SOF genes as well. A deeper understanding of the genetic organization and population biology of this species was revealed through analysis of the genes that encode the serological targets. Horizontal transfer of serotype-related genes and the emergence of new strains/clones may be a result of selective pressures conferred by the host immune response. Furthermore, against a background of extensive lateral gene flow, the strong linkage observed among serotype-related genes may signify that M-proteins, T-antigens and/or SOF (or products of other linked genes) are direct determinants of host tissue site preferences of infection.

Throughout most of the 20th century, the serological typing of *Streptococcus pyogenes* led to key insights on the association of specific serotypes of *S. pyogenes* with specific diseases. Historically, three serological typing schemes were used to classify *S. pyogenes* isolates; the targets are M protein, T-antigens, and serum opacity factor (SOF). All three schemes are based on LPXTG-linked (or similar) surface proteins that exhibit high levels of antigenic heterogeneity. More recently, the genetic basis for serological typing has been elucidated, and has led to a far deeper understanding of the genetic organization and population biology of this species.

M-type-specific antibodies provide protective immunity against organisms sharing the same serotype and therefore, M-typing has important biological implications beyond simply identifying strains of *S. pyogenes*. The molecular basis for the *emm* sequence-based typing scheme lies within the 5' end region of the *emm* gene that encodes the N-terminus of the mature M protein molecule, located at the fibril tips. By setting the threshold for defining a unique *emm* type at <92% sequence identity over 30 codons, there is a strong correspondence between the traditional M serological- and *emm* sequence type-based assignments (Beall, Facklam, & Thompson, 1996; Facklam, et al., 1999). At present, 234 distinct *emm* types have been defined. *emm* subtypes—of which there are ~1,200—provide finer granularity and are based on SNPs, as described (US Department of Health and Human Services, 2012).

Numerous epidemiological studies show that there are *S. pyogenes* strains with certain M-types that have a strong tendency to cause pharyngitis ("strep" throat), but not impetigo (a superficial skin infection), and similarly, there is a set of M-types that are often recovered from impetigo lesions, but that are rarely recovered from cases of pharyngitis (Anthony, Kaplan, Wannamaker, & Chapman, 1976; Bisno & Stevens, 1990; Wannamaker, 1970). This finding gave rise to the important concept of distinct throat and skin type strains, and

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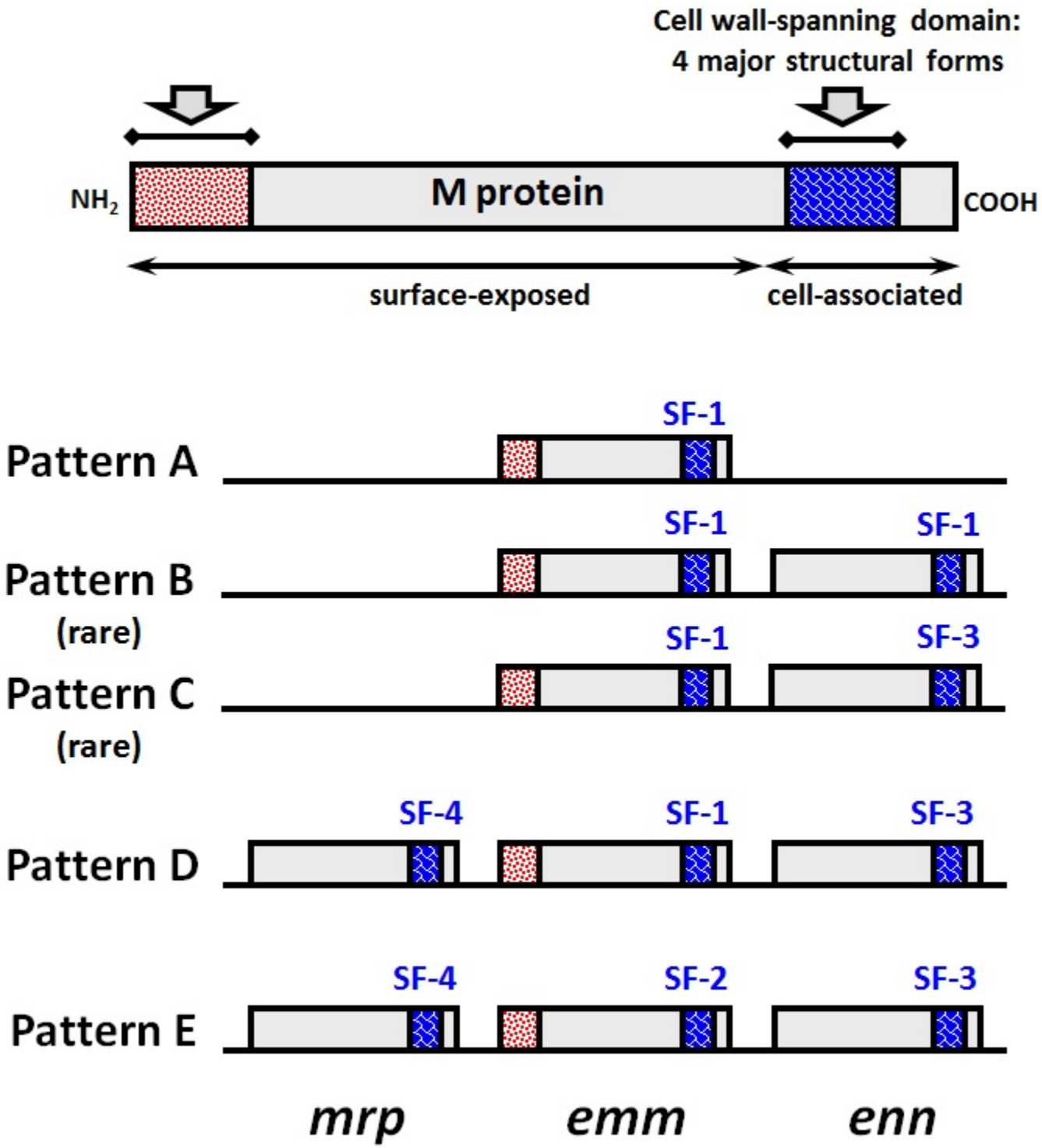

Figure 1: *emm* pattern groups and the structure of the *emm* chromosomal region. The gene content and organization of the *emm* region is depicted, based on the four major sub-family (SF) forms of the peptidoglycan-spanning domain. Relative positions of *emm* (which contains the *emm* type-specific determinants) and *emm*-like (*mrp, enn*) genes are indicated. *S. pyogenes* strains with *emm* patterns B or C are rare, and are usually combined with pattern A to form the *emm* pattern A–C grouping.

supports the idea that some degree of specialization exists among *S. pyogenes* strains. This makes sense because *S. pyogenes* strains that cause pharyngitis and those that cause impetigo are widely separated in both time and space. Not only do the disease-associated *S. pyogenes* strains tend to infect different tissue sites, but *S. pyogenes*

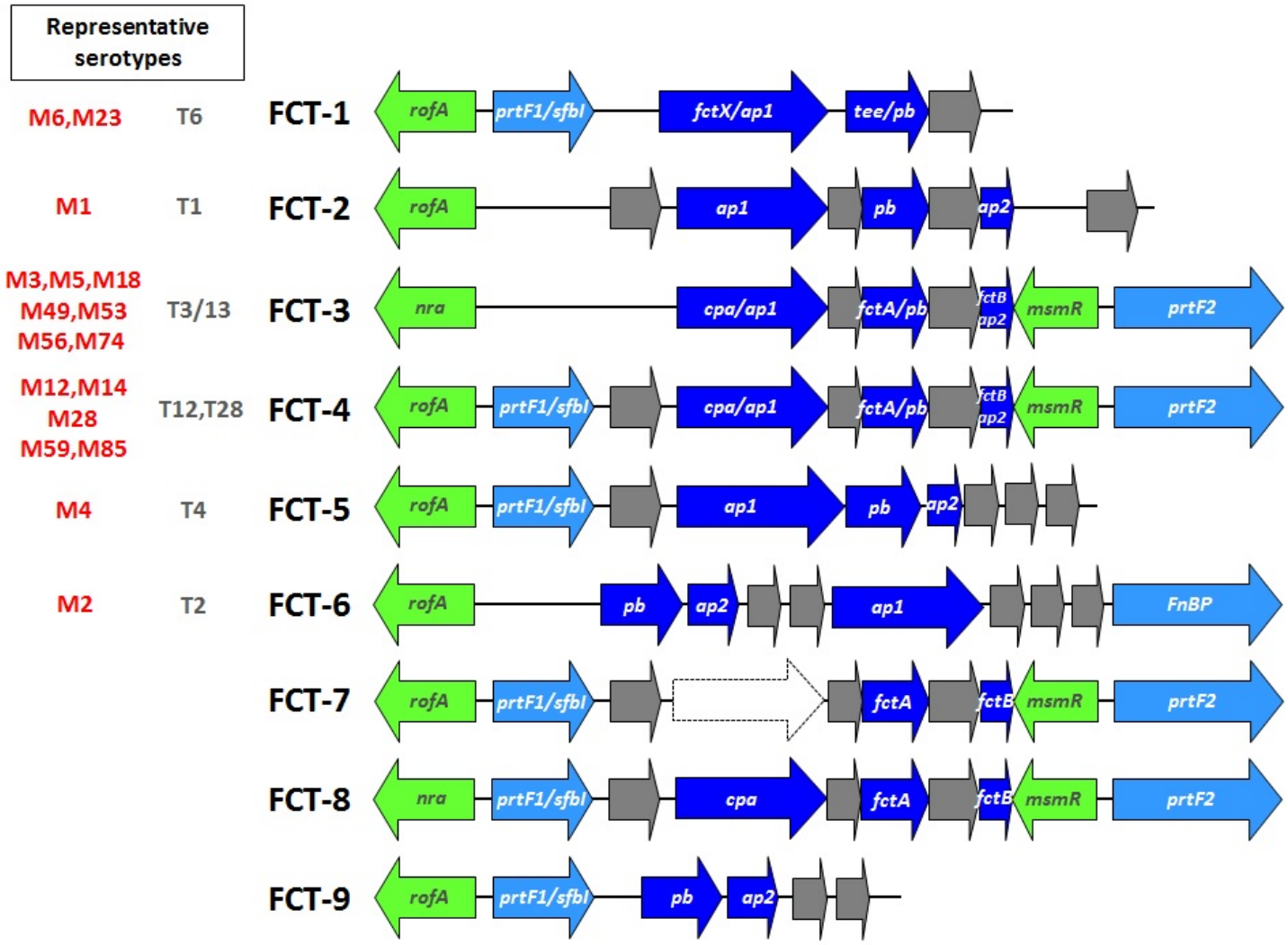

Figure 2: Structure of the FCT region encoding pili. The nine characterized FCT region forms are shown. Genes encode transcriptional regulators (RofA/Nra, MsmR; green), pilus structural proteins (PB/FctA/Tee, AP1/Cpa/FctX, AP2/FctB; dark blue), other (putative) fibronectin-binding proteins (PrtF1/SfbI, PrtF2/Pfbp/FbaB, other FnBP; light blue), and processing enzymes (signal peptidase, sortases; gray); transposases are not shown. The gene content and organization of the FCT regions is based on either nucleotide sequence determination or PCR-mapping. Representative M-serotypes are assigned, based on the sequenced genomes of numerous *S. pyogenes* strains; our current understanding of the relationship between the T type and the FCT form is incomplete. Data is based on findings reported in (Beres & Musser, 2007; Bessen, et al., 2015; Falugi, et al., 2008; Kratovac, Manoharan, Luo, Lizano, & Bessen, 2007).

pharyngitis predominates in temperate regions, whereas *S. pyogenes* impetigo is mostly found in tropical and sub-tropical locales. Also, the seasonal peaks for pharyngitis (winter) and impetigo (summer) disease differ.

A detailed understanding of the structure of the non-type-specific portions of *emm* genes and paralogous *emm*-like genes has provided key insights on the genetic organization of this species (Haanes, Heath, & Cleary, 1992; Hollingshead, Readdy, Yung, & Bessen, 1993; Podbielski, 1993), by virtue of the strong correlation between *emm* genotype and strains that have a predilection to cause either pharyngitis or impetigo (Bessen, 2009; Bessen, et al., 2000; Bessen & Lizano, 2010; Bessen, McShan, Nguyen, Shetty, Agrawal, & Tettelin, 2015; Bessen, Sotir, Readdy, & Hollingshead, 1996). The extreme 3' ends of *emm* and *emm*-like genes encode the peptidoglycan cell wall-spanning domain, whereby a different sub-family (SF) gene forms provide the basis for the *emm* pattern scheme (Figure 1). The *emm* gene, which contains the *emm*-type-specific determinant, has two major forms of the peptidoglycan-spanning coding region, and are known as sub-families 1 and 2 (SF-1 and SF-2) (Hollingshead, Arnold, Readdy, & Bessen, 1994). In addition, many *S. pyogenes* strains harbor an *emm*-like gene

(known as *enn*) that lies immediately downstream of *emm*, which has either an SF-1 or a distinct SF-3 form. A subset of *S. pyogenes* strains that have a downstream SF-3 *enn* gene also have, in addition, an upstream SF-4 *emm*-like gene (known as *mrp*). In all, there are five basic chromosomal arrangements of *emm* and *emm*-like genes and their SF forms, which are designated as *emm* patterns A through E (Figure 1). *emm* patterns B and C are rare and are grouped together with pattern A strains (referred to as *emm* pattern A-C), whereby all have an SF-1 *emm* gene and lack *mrp*. *emm* pattern D and E strains have *mrp* and the SF-3 form of *enn*, but are distinct in that their *emm* genes are of the SF-1 and SF-2 forms, respectively. The functional significance of the distinct cell wall-spanning domains is not known. However, the domains are predicted to be 58 and 39 amino acids in length for SF-1 and SF-2 *emm* genes, respectively, and the size differential conceivably reflects adaptations to distinct cell wall structures.

The large central portion of *emm* and *emm*-like genes encode functional domains that bind host proteins, such as IgG, IgA, and plasminogen. The correlation between *emm* pattern and functional domains varies. For example, the plasminogen-binding domain is exclusive to SF-1 *emm* genes of pattern D strains (Berge & Sjöbring, 1993), whereas the IgA-binding domain can be found in association with either SF-2 *emm* genes or SF-3 *emm*-like genes of pattern E strains (Bessen, 1994; Johnsson, Andersson, Lindahl, & Hedén, 1994). The C repeat regions of SF-1 and SF-2 *emm* genes also appear to be antigenically distinct (Bessen & Fischetti, 1990).

For the vast majority of *emm* types examined, multiple isolates that share an *emm* type belong to the same *emm* pattern group (McGregor, et al., 2004). Thus, *emm* type is highly predictive of *emm* pattern group, and reasonable inferences can be made for *emm* pattern group, based on knowledge of the *emm* type (McGregor, et al., 2004; McMillan, et al., 2013). Organisms (~5,400) of known *emm* type, derived from 29 population-based collections of *S. pyogenes* recovered from cases of either pharyngitis or impetigo throughout the world (Steer, Law, Matatolu, Beall, & Carapetis, 2009), were evaluated to determine their inferred *emm* pattern group. Data show that *emm* pattern A–C strains account for 47% of pharyngitis isolates but only 8% of impetigo isolates, whereas *emm* pattern D strains account for 50% of impetigo isolates, but only <2% of pharyngitis isolates (Bessen, et al., 2011; Bessen, et al., 2015). Thus, *emm* pattern A–C strains are considered to be "throat specialists," whereas the pattern D strains are "skin specialists." *emm* pattern E isolates account for almost equal fractions of throat and skin infections (52 and 42%, respectively) and as a group, they are designated as “generalists.”

In a more recent phylogenetic analysis of the portion of 175 *emm* genes that encode the entire surface-exposed regions of M proteins, two major clades (X and Y) were identified (Sanderson-Smith, et al., 2014). Of the pattern E *emm* types (i.e., generalists), 98% belong to clade X, whereas 92% of pattern A–C *emm* types (namely, throat specialists) fall into clade Y. Thus, for these two *emm* pattern groups, the excluded cell wall-spanning domain (SF-1 for pattern A–C, SF-2 for pattern E) is tightly linked to the phylogeny based on the surface-exposed portion. In contrast, the pattern D *emm* types (SF-1) form three major groupings (or clusters) distributed among both clades. In all, 16 major clusters of *emm* genes were identified; 90% of pattern D and E *emm* types belong to one of the 16 *emm*-clusters, whereas ~half of pattern A–C *emm* types stand alone and could not be assigned to a distinct cluster (Sanderson-Smith, et al., 2014). This latter finding highlights a distinct dynamic for the evolution of many pattern A–C *emm* types, which is further underscored by data showing that the *emm* type-specific region of pattern A–C *emm* genes display substantially higher levels (~3- to 5-fold) of positive (that is, diversifying) selection as compared to pattern D or E *emm* genes (Bessen, McGregor, & Whatmore, 2008).

The geographic partitioning observed for many throat and skin specialist *S. pyogenes* strains can potentially create ecological barriers that may eventually lead to allopatric speciation, due to reduced opportunities for horizontal gene transfer (HGT). However, when one considers the seven core housekeeping genes that are used for the multilocus sequence typing (MLST) of *S. pyogenes*, there is no evidence for genetic divergence between *emm* pattern A–C (throat specialist) and *emm* pattern D (skin specialist) strains (Kalia, Spratt, Enright, & Bessen, 2002). In fact, numerous studies based on core housekeeping genes point to very high rates of homologous recombination (following HGT) among *S. pyogenes* (Feil, et al., 2001; Hanage, Fraser, & Spratt,

2006; Vos & Didelot, 2009). Thus, despite wide spatiotemporal distances, *S. pyogenes* of different *emm* pattern groups contain the signatures of an extensive past history of HGT events. Furthermore, against a background of highly random associations among core housekeeping genes, as observed for *S. pyogenes*, the strong associations between *emm* pattern group and preferred tissue sites for infection are indicative of a direct role for *emm* and/or *emm*-linked genes in establishing tissue site tropisms. An *emm*-linked gene that contributes to establishing the tissue site specificity for *S. pyogenes* infection could potentially have a map position close to *emm* on the chromosome; or alternately, it is physically distant, and linkage disequilibrium is maintained by strong co-selection pressures that act to preserve the adaptive phenotype.

Clones of *S. pyogenes* can be defined by their sequence type (ST) based on MLST (Enright, Spratt, Kalia, Cross, & Bessen, 2001) (http://pubmlst.org/spyogenes/). The correspondence between *emm* type and clone (or clonal complex) varies in accordance with *emm* pattern group, where isolates of pattern D and E *emm* types are ~4 times more likely to be associated with multiple, distant genetic backgrounds (a result of HGT), as compared to *emm* types of pattern A–C strains (Bessen, McGregor, & Whatmore, 2008). Thus, for pattern A–C strains, most *emm* types closely correspond to a single clone (ST) or clonal complex. Furthermore, distinct *emm* types assigned to pattern A–C are occasionally observed on the same clonal background (i.e., ST). The recovery of both types of strain pairs—same *emm* type/distant ST (patterns D and E), and different *emm* type/same ST or clonal complex (pattern A-C)—may reflect serotype replacements that emerge due to positive selection pressures driven by immune escape. Thus, the relationships between *emm* type and clone that are uncovered in nature may have important implications for a differential role of host immune selection in shaping the population genetic structure of clinically important subsets of strains.

A second major serotyping scheme for *S. pyogenes* is based on trypsin-resistant surface antigens. The T-antigens are contained within extended surface pili that consist of covalently-linked polymers of two or three distinct gene products, following their digestion by trypsin (Mora, et al., 2005). There are ~20 recognized T-serotypes, and many *S. pyogenes* strains have multiple T-types (e.g., T3/13/B, T8/25/Imp19). Although the precise relationship between T-serotypes and antigenic epitopes within the (trypsin-treated) pilus structural proteins has not yet been fully elucidated, the genetic analysis of pilus genes shows strong correspondence to most T-serotypes (Falugi, et al., 2008).

The first *tee* gene whose nucleotide sequence was determined is *tee6* (Schneewind, Jones, & Fischetti, 1990), which was later mapped (Bessen & Kalia, 2002) to a genomic region encoding the fibronectin-binding protein PrtF1 and transcriptional regulator RofA (Fogg, Gibson, & Caparon, 1994). This genomic region has structural similarities to another virulence regulon that encodes the RofA-like protein (RALP) known as Nra and a collagen-binding protein (Cpa) (Podbielski, Woischnik, Leonard, & Schmidt, 1999). This genomic region became known as the FCT region, in which FCT stands for Fibronectin- and Collagen-binding proteins and T-antigen (Bessen & Kalia, 2002).

A genetic assessment of >150 *S. pyogenes* isolates led to the characterization of nine basic forms of the FCT region (Beres & Musser, 2007; Falugi, et al., 2008; Kratovac, Manoharan, Luo, Lizano, & Bessen, 2007), many of which have been confirmed by whole genome sequencing (reviewed in (Bessen, et al., 2015) and shown in Figure 2). Each FCT region contains five to 10 ORFs, and all are bounded at one end by either *rofA* or *nra*. In addition, all FCT regions contain genes that encode the major pilin backbone protein (BP, which is often FctA), one or two accessory pilin proteins (for example, AP2 or FctB), and at least one specialized sortase (Barnett & Scott, 2002; Falugi, et al., 2008; Kratovac, Manoharan, Luo, Lizano, & Bessen, 2007; Kreikemeyer, et al., 2011; Mora, et al., 2005; Scott, 2014). The gene that encodes the collagen-binding protein (AP1, or Cpa) is present in many, but not all FCT region forms. The nine FCT regions also vary in their content of fibronectin-binding protein genes (for example, *prtF1/sfbI* and *prtF2/pfbpI/fbaB*) whose products do not appear to be part of the pilus structure, and a second transcriptional regulator gene (*msmR*). Extensive diversity in nucleotide sequencing is observed among *cpa*, *fctA*, and *prtF1*alleles (Falugi, et al., 2008; Kratovac, Manoharan, Luo, Lizano, & Bessen, 2007). The RALPs

RofA and Nra regulate the expression of pilin genes, and depending on the *S. pyogenes* strain, the regulatory effect can be positive or negative (Kreikemeyer, Beckert, Braun-Kiewnick, & Podbielski, 2002; Luo, Lizano, & Bessen, 2008; Podbielski, Woischnik, Leonard, & Schmidt, 1999). Thus, not all *S. pyogenes* strains express pili, and those with a negative regulator may be T-nontypable organisms.

As previously recognized for M- and T-serotypes (Johnson, Kaplan, VanGheem, Facklam, & Beall, 2006), there is also a reasonably high concordance between *emm* type and FCT region, in that multiple *S. pyogenes* isolates with the same *emm* type also have a strong tendency to harbor the same FCT region form (Falugi, et al., 2008; Kratovac, Manoharan, Luo, Lizano, & Bessen, 2007). However, there is also evidence for a history of horizontal transfer of FCT region genes between *S. pyogenes* strains of different *emm* types, and between *S. pyogenes* and related streptococcal species (such as group B streptococci); the *emm* and FCT regions lie ~250 kb apart on the ~1.8-1.9 Mb *S. pyogenes* genome. The FCT region forms found in association with the highest number of distinct *emm* types are FCT-3 and FCT-4 (Falugi, et al., 2008; Köller, et al., 2010; Kratovac, Manoharan, Luo, Lizano, & Bessen, 2007). Strikingly, of 113 *S. pyogenes* strains that represent 112 *emm* types, 83% of strains that harbor FCT-3 are *emm* pattern D (30/36), whereas 84% of strains with FCT-4 are *emm* pattern E (26/31) (Kratovac, Manoharan, Luo, Lizano, & Bessen, 2007). Thus, despite their physical distance on the genome (combined with extensive recombination among core housekeeping genes), there is a strong linkage between the FCT region form and *emm* pattern, which supports the notion that the FCT region gene products may play an adaptive role in establishing tissue tropisms.

Serum opacity factor (SOF) is an LPXTG-anchored, multifunctional surface protein that also appears in a secreted form. SOF binds fibronectin and enzymatically disrupts the structure of high-density lipoproteins that are present in blood (Courtney & Pownall, 2010). The original SOF typing scheme was serologically based, wherein SOF type-specific serum neutralized enzymatic activity. More recently, *sof* sequence types have been defined by a 450-bp region at the 5' end of the *sof* gene (Beall, et al., 2000; Rakonjac, Robbins, & Fischetti, 1995); the genome map position of *sof* lies ~16.5 kb from *emm*. All (or nearly all) *emm* pattern E strains have a *sof* gene and an enzymatically active SOF protein (Beall, et al., 2000; Johnson, Kaplan, VanGheem, Facklam, & Beall, 2006; Kratovac, Manoharan, Luo, Lizano, & Bessen, 2007). Several *emm* pattern D strains also have a *sof* gene; these represent a subset of pattern D strains that are assigned to clade X of the *emm* cluster scheme (Sanderson-Smith, et al., 2014). Despite the close physical distance between *sof* and *emm* on the chromosome, several *emm* types are found in association with >1 *sof* type (and vice versa), which is indicative of HGT of *emm* or *sof* to new genetic backgrounds (Beall, et al., 2000). Attempts to generate a phylogeny for the *sof*-specific determinants have been largely unsatisfactory, due to extensive intergenic recombination (Wertz, McGregor, & Bessen, 2007).

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Streptococcus pyogenes Metabolism

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Introduction

Streptococcus pyogenes (Group A *Streptococcus or S. pyogenes*) is endowed with a formidable arsenal of virulence factors that allow it to evade a host's innate immune responses (Cole, Barnett, Nizet, & Walker, 2011; Olsen, Shelburne, & Musser, 2009; Olsen & Musser, 2010). Additionally, *S. pyogenes* responds to a variety of host environmental conditions to maintain its metabolic fitness (Kreikemeyer, McIver, & Podbielski, 2003). Together, these properties enable *S. pyogenes* to cause a variety of localized and invasive diseases, such as pharyngitis, impetigo, cellulitis, necrotizing fasciitis, and toxic shock syndromes (Aziz & Kotb, 2008; Chapnick, et al., 1992; Fox, Born, & Cohen, 2002; Ralph & Carapetis, 2013; Schlievert, Assimacopoulos, & Cleary, 1996; Sharkawy, et al., 2002). Thus, despite important advances in hygiene and modern methods of prevention, *S. pyogenes* remains a very successful pathogen, and is responsible for more than half a million deaths per year worldwide (Carapetis, Steer, Mulholland, & Weber, 2005; Sanderson-Smith, et al., 2014).

Historical perspective

The desire of researchers to understand and define the metabolism of *S. pyogenes* is not new, although the purpose and perspective of such studies have changed over time. Up until the serological differentiation of human and other groups of hemolytic streptococci (Brown, 1919; Schottmüller, 1903) was established in 1933, by Rebecca Lancefield (Lancefield, 1933), β-hemolytic *S. pyogenes*, like other living organisms, was originally thought to possess cytochromes for respiration. However, the early attempts of Farrell (Farrell, 1935; Fujita & Kodama, 1935) to find cytochromes in *S. pyogenes* were unsuccessful, as the treatment of *Streptococcus* with potassium cyanide, which is a potent inhibitor of cytochromes and the respiratory chain, was unable to inhibit the growth of this bacterium. Instead, *S. pyogenes* was found to contain peroxidase (but not catalase) as a hydrogen acceptor and utilized glucose as the sole carbon source for growth (Farrell, 1935). Hewitt investigated the manner in which *S. pyogenes* oxidized simple sugars, such as glucose, to identify the end product (Hewitt, 1932); his study found that nearly 75% of the glucose that was initially present was converted to lactic acid. The early attempts of Chu and Hastings, as well as Farrell, to measure oxygen consumption in the presence of glucose failed (Chu & Hastings, 1938; Farrell, 1935).

In an attempt to detect respiration, Sevag and Shelburne added yeast extract or normal horse serum to the glucose medium, which enabled the detection of a significant increase in *S. pyogenes* respiration (Sevag & Shelburne, 1942a; Sevag & Shelburne, 1942b). Waring and Weinstein's systematic experimental approaches that used Warburg's measurement of oxygen consumption confirmed that the active constituents of heated or non-heated Sevag's enriched medium that supported *S. pyogenes* growth were present in the dialyzable material (i.e., amino acids) (Waring & Weinstein, 1946). However, these authors were perplexed by their observations that the growth of *S. pyogenes* was inhibited after the addition of glucose in the absence of simple nitrogen. Although the significance of the simultaneous requirement of carbohydrates and simple nitrogen for the growth of *S. pyogenes* was not clear, it supported the earlier findings of Berman and Rettger (Berman & Rettger, 1918), which indicated that unlike Kendall's "protein-sparing effect" (Kendall, 1922), *S. pyogenes* prefers not to utilize carbohydrates until the nitrogen-containing material is present. Subsequent studies also revealed the importance of glutamine,

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serum, and other bodily fluids for the growth of *S. pyogenes* (McIlwain, Fildes, Gladstone, & Knight, 1939). Additionally, the new growth factor streptogenin, which was required for the growth of *S. pyogenes,* was initially identified in a casein hydrolysate basal medium (Sprince & Woolley, 1945; Woolley, 1941; Woolley, 1948). Subsequently, streptogenin was also detected in the pancreatic digest of casein (Slade, Knox, & Slamp, 1951), in heated ovalbumin (Slade & Slamp, 1955), and even in Todd-Hewitt broth, the most commonly used growth medium (Todd & Hewitt, 1932). In 1946, Funk demonstrated that streptogenin is likely to consist of vitamins adsorbed onto protein fragments (McIlwain, Fildes, Gladstone, & Knight, 1939). Based on individual amino acid requirements for the normal and steady-state growth of *S. pyogenes*, it became clear that *S. pyogenes* is auxotrophic for (unable to synthesize) nearly 15 amino acids (Davies, Karush, & Rudd, 1965; Slade H. , 1954).

These early attempts to grow *S. pyogenes* in a chemically-defined medium (CDM) required either the supplementation of Todd-Hewitt broth to acclimatize *S. pyogenes* to a new environment or a large inoculum size (Mickelson, 1964). However, such attempts yielded growth with either reduced or absent expression of the M protein, which is a major known virulence factor (Davies, Karush, & Rudd, 1968; Lancefield, 1962), thus defeating the purpose of growing *S. pyogenes* in CDM. Finally, the efforts of Van de Rijn and Kessler resulted in a CDM that had a well-defined formulation of salt, vitamins, amino acids, nucleotides, and metal ions (van de Rijn & Kessler, 1980). This widely used CDM supports the growth of *S. pyogenes* as effectively as enriched media. While these early attempts did not aim to investigate *S. pyogenes* metabolism *per se* but instead sought to determine the basic requirements for obtaining optimal *S. pyogenes* growth, they set the stage for many subsequent studies related to the new phase of interest in *S. pyogenes* metabolism.

Contemporary interest in S. pyogenes metabolism

Information on whole-genome sequence analysis of several *S. pyogenes* strains belonging to different M types in the last few decades has had a tremendous impact, not only in understanding the global molecular epidemiology of *S. pyogenes* and its mode and spread of infection, but also its mechanisms of pathogenesis (Musser, 1996; Musser & DeLeo, 2005; Olsen & Musser, 2010; Sanderson-Smith, et al., 2014). A wide spectrum of *S. pyogenes* diseases, which range from asymptomatic carriage and self-limiting symptomatic pharyngitis or purulent pyoderma to invasive and often fatal diseases, such as cellulitis, necrotizing fasciitis, and toxic shock syndrome, establishes that *S. pyogenes* can invade, survive, and proliferate within the diverse micro environments of human cells and tissues (Carapetis, Steer, Mulholland, & Weber, 2005; Ralph & Carapetis, 2013). Thus, in a broad sense, *S. pyogenes* infections range from severe invasive (sepsis) and superficial symptomatic diseases (pyoderma, pharyngitis) to asymptomatic commensal colonization. Each type of infection may occur in isolation, or as integrated into a sequence progressing in severity. During any of these infections, *S. pyogenes* primarily replicates as an extracellular pathogen. However, a subpopulation may be found in an intracellular location, where it may or may not replicate but can promote various forms of programmed death of the infected cells (Bricker, Cywes, Ashbaugh, & Wessels, 2002; Medina, Goldmann, Toppel, & Chhatwal, 2003a; Medina, Rohde, & Chhatwal, 2003b; Osterlund, Popa, Nikkilä, Scheynius, & Engstrand, 1997). Given such various infection niches, invasive *S. pyogenes* diseases likely represent a combination of nutritionally distinct microenvironments that include the one that allows *S. pyogenes* to grow extracellularly, and the one that is created intracellularly subsequent to the *S. pyogenes* invasion of non-phagocytic cells and/or its phagocytosis by neutrophils and macrophages. All of these events ultimately lead to host cell death (Agarwal, Agarwal, Jin, Pancholi, & Pancholi, 2012; Barnett, et al., 2013; Cywes Bentley, Hakansson, Christianson, & Wessels, 2005; Ito, et al., 2013; Marouni & Sela, 2004; Molinari, Rohde, Guzmán, & Chhatwal, 2000). Each of these microenvironments challenges *S. pyogenes* with a unique nutritional landscape.

Key differences between these diverse microenvironments include variations in the number and abundance of different substrates, including sugar/glucose, proteins/amino acids, oxygen/CO_2, pH values, and osmolarity. Since *S. pyogenes* is a multiple amino acid auxotroph that preferentially uses glucose as a primary source of energy, progression through each of these diverse environments requires *S. pyogenes* to adapt its metabolism to

continuously adjust to a changing host nutritional environment. For example, while blood and plasma are extremely rich in proteins and amino acids, other body structures and fluids (including skin/sweat) contain a nearly ten-fold or lower concentration of free amino acids (Calderón-Santiago, et al., 2015; Greiling & Gressner, 1995). Similarly, compared to the glucose content of human blood, the amount of glucose in the saliva (Gough, Luke, Beeley, & Geddes, 1996), nasal secretions (Wood, Brennan, Philips, & Baker, 2004), lower airway secretions (de Prost & Saumon, 2007), and skin/sweat (Calderón-Santiago, et al., 2015) have been reported to be equally poor in content. Further, as *S. pyogenes* is a lactic acid bacterium, it relies exclusively on acid-generating fermentation pathways to generate energy. Thus, during active infection, *S. pyogenes* must adapt to nutritional stress caused by the depletion of glucose, as well as to pH stress caused by the accumulation of organic acids that are its fermentative end products.

Not only does *S. pyogenes* have to adapt nutritionally to a dynamic host environment, but it must also coordinate the expression levels of its virulence factors with these changes. Transcriptome analyses of several fully sequenced genomes of *S. pyogenes* grown in various *in vitr*o and *in vivo* models, including primate and non-primate animal models (Banks, et al., 2004; Beres, et al., 2002; Graham, et al., 2005; Graham, et al., 2006; Green, et al., 2005; Musser & DeLeo, 2005), have unequivocally established that *S. pyogenes* metabolism and virulence are closely linked, which reveals the bidirectional "cause and effect" host-parasite relationship. An important component of this relationship is the linkage between substrate growth selection and transcriptional regulation of virulence genes, including those that are not required to metabolize specific substrates, but are required to resist host defenses. This linkage suggests that *S. pyogenes* senses a variety of environmental adversities, including changes in the availability of preferred growth substrates for the global regulation of its virulence network in both temporal and host compartment-specific patterns, and maintains its reproductive capacity and transmissibility from one human host to other.

Typically, the life cycle of *S. pyogenes* starts *with* asymptomatic carriage on a mucosal or epithelial surface, where it does not cause acute disease, but simply remains in a quiescent state. Based on the research by Ferrieri et al. (Ferrieri, Dajani, Wannamaker, & Chapman, 1972), *S. pyogenes* can maintain a quiescent state on normal skin for an average period of 8 days before skin lesions develop, and remain in this state for about 2–3 weeks prior to the establishment of a new niche in the oropharynx. Nutrients in this latter microenvironment are primarily derived from saliva, and competition with commensal oral bacteria for the limited availability of essential nutrients forces *S. pyogenes* to transform its metabolism from a growth mode to a survival mode. As a consequence, the survival mode is characterized by a temporal induction of several *S. pyogenes* virulence factors in response to nutrient depletion, including toxins (Kreikemeyer, McIver, & Podbielski, 2003), and these promote sustained asymptomatic colonization. Subsequently, *S. pyogenes* can invade and proliferate in the equally nutritionally challenged environment of the host's intracellular and deeper tissues. In certain hosts, *S. pyogenes* can reemerge after an extended period of quiescence (Osterlund, Popa, Nikkilä, Scheynius, & Engstrand, 1997) and overcome a host's innate immune responses, leading to life-threatening necrotic diseases (Agarwal, Agarwal, Jin, Pancholi, & Pancholi, 2012; Barnett, et al., 2013; Cywes Bentley, Hakansson, Christianson, & Wessels, 2005; Ito, et al., 2013; Marouni & Sela, 2004; Molinari, Rohde, Guzmán, & Chhatwal, 2000). As part of this lifecycle, the survival mode requires *S. pyogenes* to confront the challenge of host neutrophils/host immune cells by adapting its cellular redox status, by detoxifying intracellular oxygen radicals, and by adapting to low pH and osmotic insults to prevent its elimination from the infection site.

Therefore, contemporary interest in understanding *S. pyogenes* metabolism has transitioned from the analysis of coupled biochemical reactions to studies that focus on revealing the molecular and genetic basis of how *S. pyogenes* adapts its metabolic status in the context of a dynamic host environment, as well as the consequences of adaptation on virulence factor expression and pathophysiology. Against the backdrop of these historical and contemporary perspectives of *S. pyogenes* metabolism, the purpose of the present chapter is: (i) to describe various metabolic pathways present in *S. pyogenes*; (ii) to elucidate the manner in which important nutritional factors are transported and utilized; and (iii) to set the stage for virulence regulation, which is described in detail

in one of the subsequent chapters. A unifying theme of this chapter will be to highlight reports that have addressed important questions about the bidirectional "cause and effect" host-pathogen relationship, such as: How does *S. pyogenes* survive in a nutritionally limited environment? What is the metabolic status of the *S. pyogenes* carrier state? How does *S. pyogenes* use its metabolic potential to grow efficiently in different types of tissues? What is the metabolic status of *S. pyogenes* during infection of different microenvironments? How does *S. pyogenes* adapt its metabolism over time in response to changes in substrate availability, including changes that are caused by its consumption of nutrients and/or the host's response to infection?

Any discussion of *S. pyogenes* metabolism would be incomplete without a consideration of its diverse population structure. Presently, more than 240 *emm* serotypes of *S. pyogenes* have been reported (Carapetis, Steer, Mulholland, & Weber, 2005; Ralph & Carapetis, 2013; Sanderson-Smith, et al., 2014; Steer, Law, Matatolu, Beall, & Carapetis, 2009). Type M1 *S. pyogenes* was the very first serotype. Although the detection of this type through the early 20th century was fairly common, a new hypervirulent clone (M1T1) bearing a specific prophage that contained a gene encoding SpeA toxin emerged in the population in early 1980. This newly evolved highly pathogenic *emm* type 1 strain became the most commonly isolated *emm* type in many parts of the world (Nasser, et al., 2014). Thus, in the present chapter, the gene annotations that correspond to all of the discussed enzymes/proteins have been adopted from the genome sequence of the type M1 *S. pyogenes* strain MGAS5005 (Sumby, et al., 2005). Where necessary, they are indicated as the annotated gene name or ORF # Spy_XXXX (lower-case *italics* for genes,i.e. "*gene*" or "*spy_XXXX*", or Capitalized non-italics for translated proteins, i.e. "Gene" or "Spy_XXXX"), where XXXX refers to a specific number of a genetic locus within the open reading frame of the genome (NCBI, 2015). Annotations of some of the genes or proteins of type M1 *S. pyogenes* MGAS5005 are based on genetic, biochemical, and functional analyses carried out by various investigators, or based on the homology with the annotated genes from other *emm* types or homologous genes reported in other Gram-positive pathogens, but that are not annotated in the publicly available type M1 MGAS5005 genome.

The present chapter describes the contributions of numerous investigators who have enhanced our understanding of (i) sugar and amino acid transport and their utilization during depleted vs. enriched environments; (ii) factors that neutralize the reactive oxygen-mediated assault during phagocytosis and high acidic pH in purulent conditions when various metabolic end products, including lactic acid are accumulated; and (iii) environmental cues that trigger adaptive responses in *S. pyogenes* that complement the environment-associated nutrient deficiencies. While the extensive literature on *S. pyogenes* carbohydrate and amino acid metabolisms has been published, other well-established metabolic pathways have not yet been investigated in *S. pyogenes*. In the forthcoming description in this chapter, those pathways are described in context with published *S. pyogenes* genome sequences and transcriptome analyses, or corresponding pathways described in other Gram-positive pathogens, to emphasize their pathophysiologically relevant contribution to *S. pyogenes* virulence and the mechanism of *S. pyogenes* pathogenesis.

Carbohydrate metabolism

Glycolysis

As glucose is the primary carbon source for obtaining energy, *S. pyogenes* has an intact glycolytic pathway (namely, the Embden-Meyerhof-Parnas (EMP) pathway). This pathway is constituted of 12 coupling enzymes and is responsible for the net gain of 2 ATP molecules and 2 $NADH_2$ molecules. These enzymes are: phosphoglucomutase (Spy_0938 and Spy_1235); glucose-specific transporter IIABC (Spy_1692 and Spy_1693); glucokinase (Spy_1257); glucose-6-phosphate isomerase (Spy_0185); 6-phosphofructokinase (Spy_0989); fructose-bisphosphate aldolase (Spy_1707); triosephosphate isomerase (Spy_0509); glyceraldehyde-3-phosphate dehydrogenase (Spy_0233); NADP-dependent glyceraldehyde-3-phosphate dehydrogenase (Spy_1119); phosphoglycerate kinase (Spy_1599); phosphoglycerate mutase (Spy_0497, Spy_1164, and Spy_1503); enolase (Spy_0556); and pyruvate kinase (Spy_0988) (Figure 1). The activation of EMP enzymes is dependent on

divalent cations (Mg^{2+} or Mn^{2+}) that allow enzymes to bind to negatively charged, energy-rich phosphorylated substrates. While most individual glycolytic reaction steps are reversible, two steps each in the first and the second half of the EMP pathway (the conversion of glucose to glucose-6-P and fructose-6-P to fructose 1,6–bis-P, and the conversion of 1,3-diphosphoglycerate to 3-phosphoglycerate and phosphoenolpyruvate to pyruvate, respectively) are irreversible. The significance of the presence of multiple copies of phosphoglucomutase and phosphoglycerate mutase in *S. pyogenes* is presently unknown, and their presence is likely due to gene duplication. It is also unknown whether other copies are functionally active and possess a function other than the glycolytic function. The presence of multiple copies of this enzyme could be another example that may justify the concept of neofunctionalism (as discussed later in this chapter) for tagatose-6-phosphate metabolism (Loughman & Caparon, 2007).

All of the enzymes in this pathway typically reside in the cytoplasm in *S. pyogenes*. However, many of these enzymes, such as GAPDH and enolase, are also exported to the cell surface through an unknown mechanism. These proteins are, therefore, known as anchorless surface proteins (Fischetti, 2006; Pancholi & Chhatwal, 2003; Pancholi & Fischetti, 1992; Pancholi & Fischetti, 1993; Pancholi & Fischetti, 1998). The biochemical properties of these anchorless surface-exported glycolytic enzymes indicate that they possess multiple virulence-related functions; some of these enzymes, such as GAPDH, also called streptococcal surface dehydrogenase (SDH), serve as major virulence determinants (Jin, Song, Boel, Kochar, & Pancholi, 2005; Pancholi & Fischetti, 1992; Pancholi & Fischetti, 1993; Pancholi & Fischetti, 1997). In fact, the surface exportation of SDH is essential for the maintenance of *S. pyogenes* virulence (Boël, Jin, & Pancholi, 2005; Jin, Agarwal, Agarwal, & Pancholi, 2011).

Genome analyses of several strains of *S. pyogenes* have also revealed that, unlike many other Gram-positive pathogens, *S. pyogenes* does not possess the genes that encode enzymes associated with the aerobic tricarboxylic acid cycle (TCA), also known as the Krebs cycle. However, *S. pyogenes* contains several genes that encode enzymes of the specific pathway that leads to the TCA cycle identified in other prokaryotes, such as *spy_0751/acoA* (pyruvate dehydrogenase E1 α), *spy_0752/acoB* (pyruvate dehydrogenase E1 β), *spy_0753/acoC* (α-ketoacid dehydrogenase E2), and *spy_0755/acoL* (dihydrolipoamide dehydrogenase). *S. pyogenes* represents these enzymes in the form of a truncated TCA pathway. Although the functional significance of the acoA-C-encoded enzymes is currently unknown, these enzymes appear to be involved in the conversion of pyruvate to acetyl-CoA (Figure 1).

Similarly, *S. pyogenes* also contains an incomplete Entner-Doudoroff (ED) pathway, which is an alternative glycolytic pathway (Conway, 1992); this incomplete pathway is characterized by the presence of a few key enzymes. The ED pathway is responsible for the catabolism of glucose to pyruvate via non-phosphorylated intermediates. *S. pyogenes* possesses a key gene that encodes keto-hydroxyglutarate aldolase/keto-deoxy phosphogluconate aldolase (KDPGA/EDA/KgdA) (Spy_0527). The ED pathway differs from the classical glycolytic EMP pathway, based on the nature of the 6-carbon metabolic intermediate. In the EMP pathway, fructose 1,6-bisphosphate aldolase catalyzes the conversion of fructose 1,6-bisphosphate to glyceraldehyde-3-phosphate. In contrast, in the ED pathway, KDPG aldolase catalyzes the conversion of 2-keto 3-deoxy-6-phosphogluconate to glyceraldehyde-3-phosphate and pyruvate. The pathophysiological significance of this orphan enzyme is currently unknown, as *S. pyogenes* cannot convert glucose to gluconolactone and 6-phosphogluconate to enter the ED pathway (Figure 1). The absence of this pathway makes GAPDH/Plr or SDH (Spy_0233) the key metabolic enzyme in *S. pyogenes*. Therefore, the second half of the EMP pathway is crucial to the formation of pyruvate, which is required for *S. pyogenes* growth and survival.

Phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS)

S. pyogenes is a human pathogen and uses glucose as the primary carbon source for its growth. During systemic or deep-wound infection surrounded by blood products and innate immune cells, plasma and serum can provide a glucose-rich environment for *S. pyogenes* growth. However, during the colonization of the oral cavity or skin tissues, where the glucose content is low (Calderón-Santiago, et al., 2015; de Prost & Saumon, 2007;

Fig.1

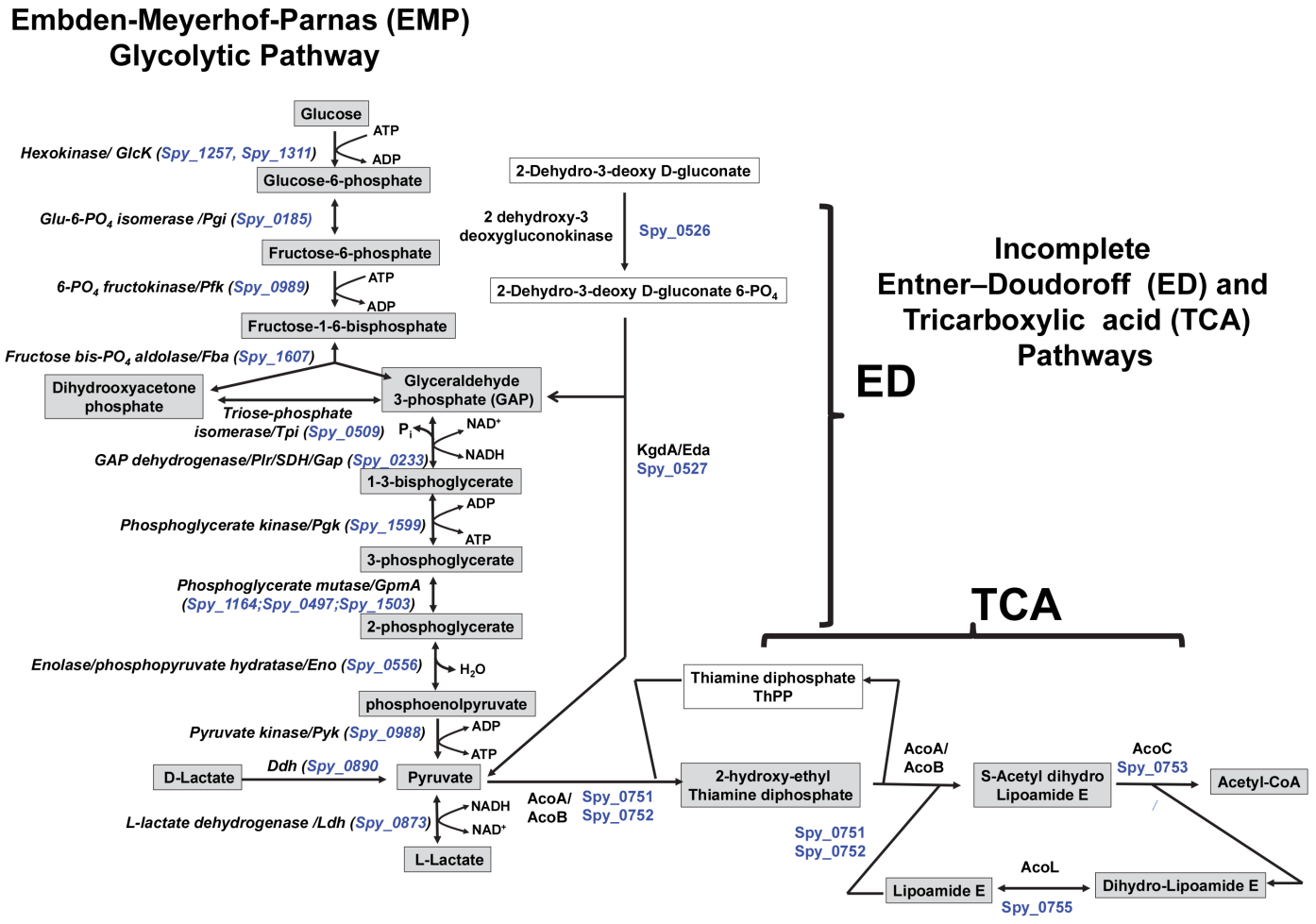


Figure 1: The Embden-Meyerhof-Parnas (EMP) pathway and its association with the incomplete Entner-Doudoroff (ED) and tricarboxylic acid (TCA) pathways present in *Streptococcus pyogenes*. *S. pyogenes* uses glucose as its sole carbon source for energy. The significance of the presence of incomplete ED and TCA pathways is currently unknown. The EMP pathway is responsible for the net gain of two ATP molecules and two $NADH_2$ molecules. The incomplete nature of alternate energy sources makes many glycolytic enzymes essential for survival, especially those that belong to the second half of the EMP pathway. The genes that encode the enzymes involved in the glycolysis process are shown as the ORF numbers (shown in blue fonts) annotated in the genome of the type M1 *S. pyogenes* MGAS5005 strain. The choice to use this genome over other published *S. pyogenes* genomes is based entirely on the worldwide prevalence of MGAS5005-like type M1 strains.

Gough, Luke, Beeley, & Geddes, 1996; Wood, Brennan, Philips, & Baker, 2004), *S. pyogenes* is forced to use non-glucose complex carbohydrates for its growth and survival. Like most bacteria, *S. pyogenes* also utilizes a salvage pathway as an alternative energy source; this pathway is activated only when glucose is present in scarce amounts. When glucose is present in excess, the genes that are responsible for catabolizing complex carbohydrates to glucose are repressed: a phenomenon known as carbon catabolite repression (CCR). CCR allows *S. pyogenes* to adapt quickly to a preferred or rapidly metabolizable carbon and energy source. As early as the 1960s and 1970s, the relationship between the nature and amount of carbohydrate and the production of major virulence factors, such as the M protein and streptolysin O, was already established (Davies, Karush, & Rudd, 1968; Pine & Reeves, 1978; Todd & Hewitt, 1932). However, the detailed mechanism that underlies CCR in *S. pyogenes* was not known until recently (Almengor, Kinkel, Day, & McIver, 2007).

In virtually all low-GC Gram-positive organisms, including *S. pyogenes*, CCR is regulated by the phosphoenolpyruvate (PEP)-dependent phosphotransferase (PTS) system (Deutscher, et al., 2014; Deutscher, Francke, & Postma, 2006). This system catalyzes the uptake, transport, and phosphorylation cascades of several carbohydrates. Reizer and Saier's group first discovered the PTS system in *S. pyogenes* during their studies of the transport of β-galactosides in resting cells when they observed the expulsion of sugars (Reizer, Novotny, Hengstenberg, & Saier, 1984). In particular, those authors observed that resting or starved cultures use the PTS and the cytoplasmic storage of PEP for the uptake and phosphorylation of PTS sugars. Further, the addition of a metabolizable PTS sugar elicited the rapid dephosphorylation of intracellular sugar-phosphate and the energy-independent efflux of the sugar. This energy-independent vectorial system generally consists of sugar/carbohydrate-specific PTS permeases (also known as enzyme II or EII), enzyme EI (PtsI or EI, Spy_1120), and a histidine-containing phosphocarrier protein (PtsH/HPr, Spy_1121). Sugar-specific permeases, in general, are composed of three/four protein domains that are referred to as EIIA, B, and C/D. Ten sugar-specific permease systems are present in *S. pyogenes*. These systems are dedicated to the transport of ascorbate (KEGG spz M00283), lactose (KEGG spz M00281), galactitol (KEGG spz M00279), mannose (KEGG spz M00276), cellobiose (KEGG spz M00275), fructose (KEGG spz M00273), trehalose (KEGG spz M00270), sucrose (KEGG spz M00269), glucose (KEGG spz M00265), and N-acetylglucosamine.

As Figure 2 illustrates, the phosphotransfer cascade starts with EI and PEP (and is thus PEP-dependent) and proceeds via HPr to the sugar-specific EIIA and EIIB domains of the PTS permeases. PEP-mediated phosphorylation of the EI enzyme targets the His15 residue of HPr. HPr can be found in different phosphorylated forms (Deutscher, Francke, & Postma, 2006). Deutscher, Francke, and Postma (Deutscher, Francke, & Postma, 2006) demonstrated that HPr is also phosphorylated by HPr kinase/phosphorylase (HPrK/P) (Spy_0484). This phosphorylation requires ATP or pyrophosphate as a phosphoryl donor, instead of PEP. HPrK/P–mediated phosphorylation of HPr occurs at the Ser46 residue. The kinase activity of HPrK/P is primarily activated by fructose 1,6-bisphosphate (FBP). In general, the high FBP stimulates the kinase activity, and low FBP allows the enzyme to function as a phosphatase (32). The resultant HPrSer46~P serves as a co-repressor for catabolite control protein A (CcpA, Spy_0424). The CcpA-HPrSer46~P dimer binds more efficiently to its specific catabolite control element (*cre*) sites (Almengor, Kinkel, Day, & McIver, 2007; Deutscher, Francke, & Postma, 2006; Shelburne, et al., 2008a). Based on 126 *B. subtilis* consensus "*cre*" (TGW[A/T]AAR[A/G]CGY[C/T]TW[A/T]N[A/T/G/C]CW[A/T]) sites, 98 potential CcpA-binding sites (RegPrecise, 2015) have been detected in the type M1 SF370 *S. pyogenes* strain (Almengor, Kinkel, Day, & McIver, 2007). Several studies have characterized CcpA networks both biochemically and functionally by using both DNA-binding assays and comparison of transcriptional profiles of wild-type versus CcpA-deficient mutants (Almengor, Kinkel, Day, & McIver, 2007; Kietzman & Caparon, 2010; Kietzman & Caparon, 2011; Kinkel & McIver, 2008; Shelburne, et al., 2010). While the kinase activity of HPrK/P plays a key role in the modulation of the DNA-binding affinity of CcpA, its phosphatase activity is modulated and stimulated in the presence of increased amounts of inorganic phosphate (P_i) which results from the increased catalysis of intracellular ATP and fructose 1,6-bisphosphate (Deutscher, et al., 2014; Deutscher, Francke, & Postma, 2006). Depending on the metabolic demand, the ATP- and P_i-dependent dual functions of *S. pyogenes* HPrK/P coordinate and balance the relative concentrations of the HPrSer46~P and His15-phosphorylated HPr (HPrHis15~P).. While the former serves as an active CcpA cofactor but an inactive sugar transport mediator, the latter form plays an active role in the PTS sugar transport but remains as an inert CcpA-cofactor. Thus, CcpA-mediated CCR activity requires the sustained maintenance of HPrSer46~P, which also inhibits the E1-mediated HPrHis15~P to exclude uptake of PTS sugars (Deutscher, et al., 2014; Deutscher, Francke, & Postma, 2006). The detailed mechanism of transcriptional regulation is described in another chapter in this book.

Complex carbohydrate metabolism and transport

The predilection of *S. pyogenes* to colonize the oropharynx and cause pharyngitis reflects the dual nature of the regulation of carbohydrate utilization. During colonization or biofilm formation (Tylewska, Fischetti, &

Fig. 2

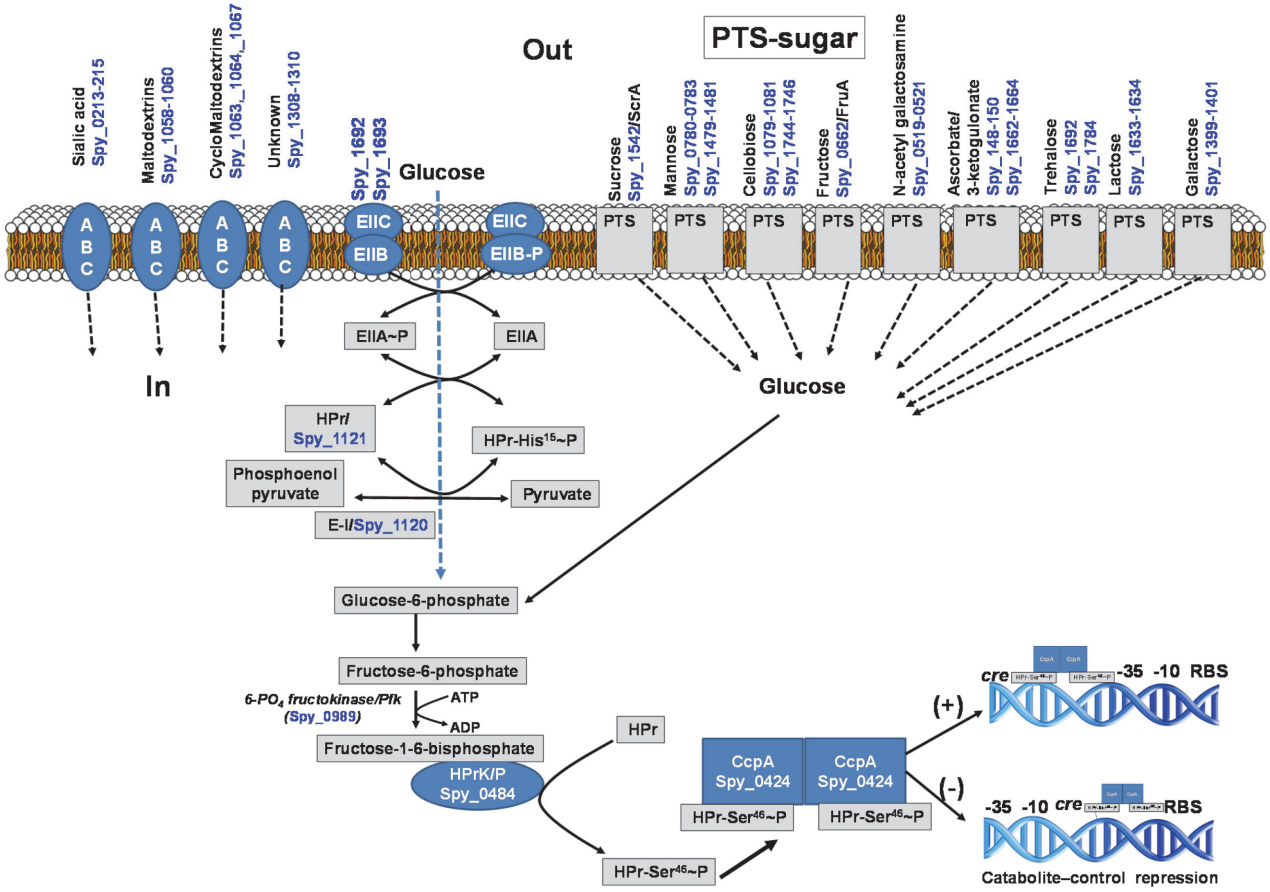


Figure 2: Phosphoenol pyruvate (PEP)-dependent phosphotransferase (PTS) pathway and catabolite repression. The PEP-PTS pathway in *S. pyogenes* couples the phosphorylation and import of glucose and non-glucose sugars. *S. pyogenes* possesses 14 PTS and four ABS transport systems to transport glucose and non-glucose carbohydrates. Some complex carbohydrates are transported via two different PTS systems. As shown, the cytoplasmic enzymes EI, sugar-specific EII, and phosphocarrier protein (HPr)-kinase/phosphorylase (HPrK/P), in conjunction with HPr, form the phosphorelay system that transfers phosphate from the energy-rich PEP (ΔG^{o}= -62.2 kJ/mol) produced by glycolysis to the incoming sugar. The phosphorylation of HPr by EI at histidine residue 15 forms HPrHis15~P, and couples sugar uptake to sugar phosphorylation (e.g., glucose-6-phosphate) through the carbohydrate-specific EII enzyme. Phosphorylated sugars are then channeled into the glycolytic pathways. The bifunctional HPrK/P regulates HPr activity through phosphorylation at serine residue 46 to form HPrSer46~P in the presence of ATP; this form of the protein is unable to phosphorylate EIIs. HPrSer46~P also activates catabolite repression through the catabolite control protein (CcpA), which dimerizes more efficiently with HPrSer46~P. The HPr-CcpA dimer then binds to the "*cre*" locus to execute transcriptional regulatory activity. (+) and (-) denote transcription stimulation and repression, respectively, depending on whether the targeted "*cre*" region is located upstream of the -35 and -10 promoter elements and the ribosomal binding site (RBS), or between the -35 and -10 elements and the RBS. When the glucose content is depleted, the uptake of non-glucose is activated through PTS. The ABC transport system mediates the uptake of certain carbohydrates. The Spy_xxxx assignments are based on the strain type M1 *S. pyogenes* MGAS5005 genome.

Gibbons, 1988; Virtaneva, et al., 2005), the close interaction with saliva and salivary contents with a significantly lesser amount of free glucose requires *S. pyogenes* to adjust its metabolism to use complex carbohydrates as key nutrients for its survival. Maltodextrin, which is an α-1,4-linked glucose polymer (with the dextrose equivalent value of 3-20), is a major constituent of the saliva and is derived from starch (a common component of the human diet) as a salivary α-amylase-digested product. Several studies performed by Shelburne et al.

demonstrated that MalE (Spy_1058) and MalT (Spy_1692) play key roles in the transport of maltodextrin and maltose/maltotriose, respectively (Shelburne, et al., 2007a; Shelburne, et al., 2008a; Shelburne S. A., Keith, Davenport, Horstmann, Brennan, & Musser, 2008b; Shelburne, et al., 2007b; Shelburne, et al., 2010; Shelburne, et al., 2011) (Figure 3). However, as described above, during pharyngitis or deep wound infection, the presence of glucose or other readily metabolized mono- and di-saccharides in the purulent exudate may direct CcpA to repress the expression of genes involved in complex carbohydrate utilization (Almengor, Kinkel, Day, & McIver, 2007; Kinkel & McIver, 2008; Shelburne, et al., 2010). Similar to CcpA, another LacI/GalR family regulator, the maltose repressor (Spy_1057/MalR), affects the catabolism of maltodextrin. While CcpA and MalR of *S. pyogenes* bind to similar promoter regions (NNGCAARCGNTTGCYR, see above), sequence recognition by MalR is much more stringent than CcpA, which can recognize a much wider variation from consensus sequence. As a result, MalR-regulated repression of certain carbohydrate genes is more stringent than that of CcpA and is restricted primarily to maltodextrin metabolism and maltose transport-encoding genes (Shelburne, et al., 2011). CcpA extends its influence over maltodextrin metabolism primarily through the regulation of *malR* expression by binding to the "*cre*" site that is located upstream of *malR*. Subsequently, MalR influences the conversion of maltodextrin to dextrose by the regulation of several genes responsible for maltose transport and metabolism, including those that encode a maltodextrin phosphorylase and 2-α-glucanotransferase activity (*spy_1055*/*malP*/*glgP* and *spy_1056*/*malQ*/*malM*); ABC maltodextrin transporter/permease-mediated transport (*spy_1058*/*malE*, *spy_1059*/*malF*, and *spy_1060*/*malG*); starch degradation/pullulanase activity (*spy_1680*/*pulA*); maltodextrin degradation (*spy_1681*/*dexB*); ATP-dependent *multiple sugar transport (spy_1682); maltodextrin catabolism (spy_1691); and maltose transport (spy_1692*/*malT*) (Figure 3). Dual regulation by CcpA and MalR ensures that the latter contributes to *S. pyogenes* virulence in a site-specific manner at the oropharynx. An additional example is MalR regulation of pullulanase (PulA), an α-glucan binding protein (Shelburne, et al., 2011) that contributes to *S. pyogenes* adherence to the oropharyngeal region, but is dispensable for invasive disease (Shelburne, et al., 2011) (Figure 3). Although PulA is known to degrade and catabolize host cell surface α-glucan, *S. pyogenes* does not use this enzyme for carbon source catabolism.

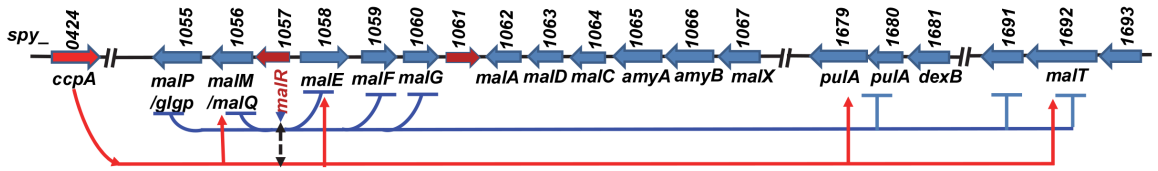
Another regulator important for niche-specific colonization of the oropharynx is the salivary persistence two-component regulator (SptR) (M5005_Spy_0680 in MGAS5005 or SPy_0874 in M1 GAS SF370 strain) (Shelburne, et al., 2005). SptR is activated in the presence of human saliva, as it positively regulates carbohydrate metabolism, and SptR-deficient mutants do not grow in saliva (Shelburne, et al., 2005). Interestingly, a mutant that lacks SptR is hypervirulent in a murine skin infection model, but not in a peritonitis model (Sitkiewicz & Musser, 2006). The mechanism that underlies SptR-regulated carbohydrate metabolism and virulence is currently unknown.

Lactose metabolism and transport

Multifunctional (often termed as "Moonlighting") enzymes like GAPDH (SDH) and the other metabolic enzymes described above (Pancholi, 2001; Pancholi & Chhatwal, 2003; Pancholi & Fischetti, 1992; Pancholi & Fischetti, 1998) defy the one gene-one enzyme theory (Beadle & Tatum, 1941) by performing a variety of functions required for the maintenance of *S. pyogenes* virulence. In contrast, *S. pyogenes* also harbors multiple paralogous genes that encode proteins that apparently perform the same function. A perfect example is the multiple operons that contain genes that are responsible for lactose metabolism and transport. The knowledge of these operons have proven valuable for understanding the functional significance and evolution of paralogous proteins (Loughman & Caparon, 2007). All *S. pyogenes* genomes sequenced to date possess two operons (*lac.1* and *lac.2*) that encode components of the tagatose-6 pathway for lactose and galactose metabolism (Figure 4). *S. pyogenes* lacks the Laloir pathway that is dedicated to galactose transport and metabolism. Each operon consists of eight genes, including *lacA (lacA*.1/*spy_1398*, *lacA.2*/*spy_1638,* 68% identity); *lacB* (*lacB.1*/*spy_1397*, *lacB.2*/*spy_1637,* 76% identity); *lacC* (*lacC.1*/*spy_1396*, *lacC.2*/*spy_1636,* 42% identity); *lacD* (*lacD.1*/*spy_1395*, *lacD.2*/*spy_1635*, 72% identity); *lacE* (*spy_1399*/*ptsIIC*, *lacE*/*spy_1633,* 13% identity); *lacF* (*spy_1400*/*ptsIIB-spy_1401*/, ptsIIA *lacF*/*spy_1634,* 14% identity*); lac.2*-specific *lacG (lacG/spy_1632,); lac.1*-specific *spy_1401/ptsIIA,* and the

Fig. 3

A



B

ORF in MGAS5005	Protein annotation	Function
Spy_0424	**CcpA**	**Catabolite control protein A**
Spy_1055	MalP/GlgP	Maltodextrin phosphorylase
Spy_1056	MalM/MalQ	4-α-glucanotransferase
Spy_1057	**MalR**	**LacI-family repressor**
Spy_1058	MalE	Maltose/maltodextrin transporter
Spy_1059	MalF	Maltodextrin-ABC transporter permease protein
Spy_1060	MalG	Maltodextrin-ABC transporter permease protein
Spy_1061	Spy_1061	LacI-family regulator
Spy_1062	MalA	Maltodextrin utilization protein
Spy_1063	MalD	Maltodextrin ABC-transporter permease
Spy_1064	MalC	Maltose ABC transporter permease
Spy_1065	AmyA	Alpha amylase
Spy_1066	AmyB	Amylopullulanase
Spy_1067	MalX	Maltose-Maltodextrin binding protein
Spy_1679-80	PulA	Pullulanase
Spy_1681	DexB	Glucan 1,6-α glucosidase
Spy_1691	Spy_1691	Intracellular processing of maltotriose and longer maltodextrin
Spy_1692-93	MalT	Maltose/maltotriose

Figure 3: Genes and corresponding proteins that are involved in maltose/maltodextrin transport metabolism and metabolic regulation. (A) Genomic organization of genes involved in maltose/maltodextrin transport metabolism and metabolic regulation. Red arrows depict positive regulation. Blue blunt-ended arrows depict repression. A black arrow with a dotted line between MalR and CcpA regulatory pathway at the *malR* depicts competition between MalR and CcpA at the MalR-binding DNA site. CcpA competes with MalR for the same binding site, and thus the CcpA-mediated activation of certain genes may occur via MarR derepression. In the presence of maltose, MalR is released from its DNA binding site, which results in derepression of key genes involved in the maltose/maltodextrin metabolism. (B) Functional annotation of proteins involved in maltose-maltodextrin transport, metabolism, and regulation. The Spy_xxxx assignments are based on the strain type M1 *S. pyogenes* MGAS5005 genome.

regulator/repressor *lacR (lacR.1/spy_1402, lacR.2/spy_1639,* 54% identity) (Loughman & Caparon, 2007). While these two operons likely arose as an outcome of a gene duplication, they have evolved to perform distinctly separate functions (Loughman & Caparon, 2006b; Loughman & Caparon, 2007).

The *lac.2* operon serves as a canonical *lac* operon with the full set of genes required for lactose metabolism via the tagatose pathway (Figure 4). Functionally, a PTS transporter (ptsIIABC or LacEF) allows exogenous lactose or galactose to be transported to the cytoplasm in the form of lactose-6-phosphate, which is then converted to galactose-6-phosphate by 6-P-β-galactosidase (LacG), or to glucose by β-galactosidase (LacZ/Spy-1304) (Figure 4). Glucose can enter the EMP pathway, while galactose-6-phosphate is metabolized to tagatose-1,6 bisphosphate by the sequential actions of the Lac isomerase and epimerase (LacA/B), and tagatose-6-phospho kinase (LacC). Finally, tagatose bisphosphate aldolase (LacD) converts tagatose-1,6 bisphosphate to

glyceraldehyde 3-phosphate and dihydroxyacetone phosphate, both of which can then enter into the EMP pathway (Figure 2).

In contrast to the functionally active *lac.*2 operon, the highly conserved *lac.1* operon encoded enzymes cannot catabolize lactose in all *S. pyogenes* strains,, because it lacks a gene to encode LacG, and the gene *spy_1396* that encodes LacC.1 is truncated by a number of frame-shift mutations. The alternative function of Lac.1 resides in its LacD.1 aldolase, which possesses a unique function as a transcriptional regulator of several virulence genes, including the SpeB cysteine protease, in a pH- and salt-concentration–dependent manner (Cusumano & Caparon, 2013; Loughman & Caparon, 2006b). Although the enzymatic activity of LacD.1 is not required for its regulatory activity (Cusumano & Caparon, 2013; Loughman & Caparon, 2006b), it retains aldolase enzymatic activity and can complement a LacD.2 mutant for lactose metabolism (Cusumano & Caparon, 2013; Loughman & Caparon, 2007). However, the converse is not the case, as LacD.2 cannot complement a LacD.1 mutant for its regulatory activity (Cusumano & Caparon, 2013; Loughman & Caparon, 2006b). The two LacD proteins (LacD.1 and LacD.2) possess a structural similarity of nearly 82% and an identical catalytic site; however, LacD.1 has a significantly lower enzymatic efficiency that results from divergence in residues outside of its catalytic site. While this reduction in enzymatic activity is necessary for its function in transcriptional regulation, it is not sufficient, as additional divergent residues are also required (Cusumano & Caparon, 2013). This differential enzymatic activity suggests that a decrease in enzymatic activity may have alleviated an adaptive conflict between the two enzymes after their duplication, which has allowed LacD.1 to adapt to its regulatory role. The maintenance of this enzymatic activity suggests that its regulatory function is associated with an ability to sense the concentration of a specific metabolite. A more refined mutational analysis of its catalytic groove suggests that the metabolite may be one of the products of its cleavage reaction, dihydroxyacetone phosphate (Loughman & Caparon, 2006b). The fact that this latter metabolite is a key intermediate in the EMP pathway of carbon and energy metabolism provides an additional example of the bidirectional link between *S. pyogenes* metabolism and regulation of pathogenesis. In the same context, LacR.1 (Spy_1402), but not LacR.2 (Spy_1639) represses the regulatory function and thus the regulation of LacD.1 expression. In turn, the LacR activity is negatively controlled by the concentration of tagatose-6-phosphate (Figure 4) (Loughman & Caparon, 2007).

Malate transport and utilization pathway

In lactic acid bacteria, energy-producing fermentation leads to glucose starvation and the accumulation of acid during *in vitro* or *in vivo* growth. As previously discussed, *S. pyogenes* uses its PTS system for the uptake and metabolism of non-glucose carbohydrate sources to adapt to a glucose-depleted environment. *S. pyogenes* also uses malate, a dicarboxylic organic acid that is abundant in both tissue and in the environment, as a non-glucose, non-complex carbohydrate source for energy. Among lactic acid bacteria (Neijssel, Snoep, & Teixeira de Mattos, 1997), two distinct pathways for malate utilization have been identified. The most common is the malolactic fermentation (MF) pathway, in which the malolactic enzyme converts malate into lactate, which maintains the ATP pool and provides protection against acid killing (Neijssel, Snoep, & Teixeira de Mattos, 1997; Zaunmüller, Eichert, Richter, & Unden, 2006) (Figure 5). While this pathway is commonly found in *Lactobacillus* (Landete, Ferrer, Monedero, & Zúñiga, 2013; Landete, et al., 2010) and *Streptococcus mutans* (Sheng, Baldeck, Nguyen, Quivey, & Marquis, 2010; Sheng & Marquis, 2007), it does not exist in *S. pyogenes*. Instead, *S. pyogenes* has the genes for the malic enzyme (ME) pathway, in which malate is converted to pyruvate and CO_2 in an NAD-dependent manner by malic enzyme (Figure 4). The functional significance of the ME pathway has been evaluated in *Enterococcus faecalis* (London & Meyer, 1970; Mortera, et al., 2012), *Streptococcus* sp. (Kawai, Suzuki, Yamamoto, & Kumagai, 1997), and *Lactococcus* (Landete, Ferrer, Monedero, & Zúñiga, 2013; Landete, et al., 2010), where it primarily contributes to growth yield, with little to no protection against acid killing. However, the global gene expression profiles of *S. pyogenes in vitro* and *in vivo* murine model of soft tissue infection have revealed that the ME genes, *spy_0832/maeP/malP* encoding the malate transporter, and *spy_0833/maeE* encoding the malic enzyme are highly up-regulated in an acidic environment (Loughman & Caparon, 2006a; Paluscio & Caparon, 2015; Port, Paluscio, & Caparon, 2013). Their expression is under the

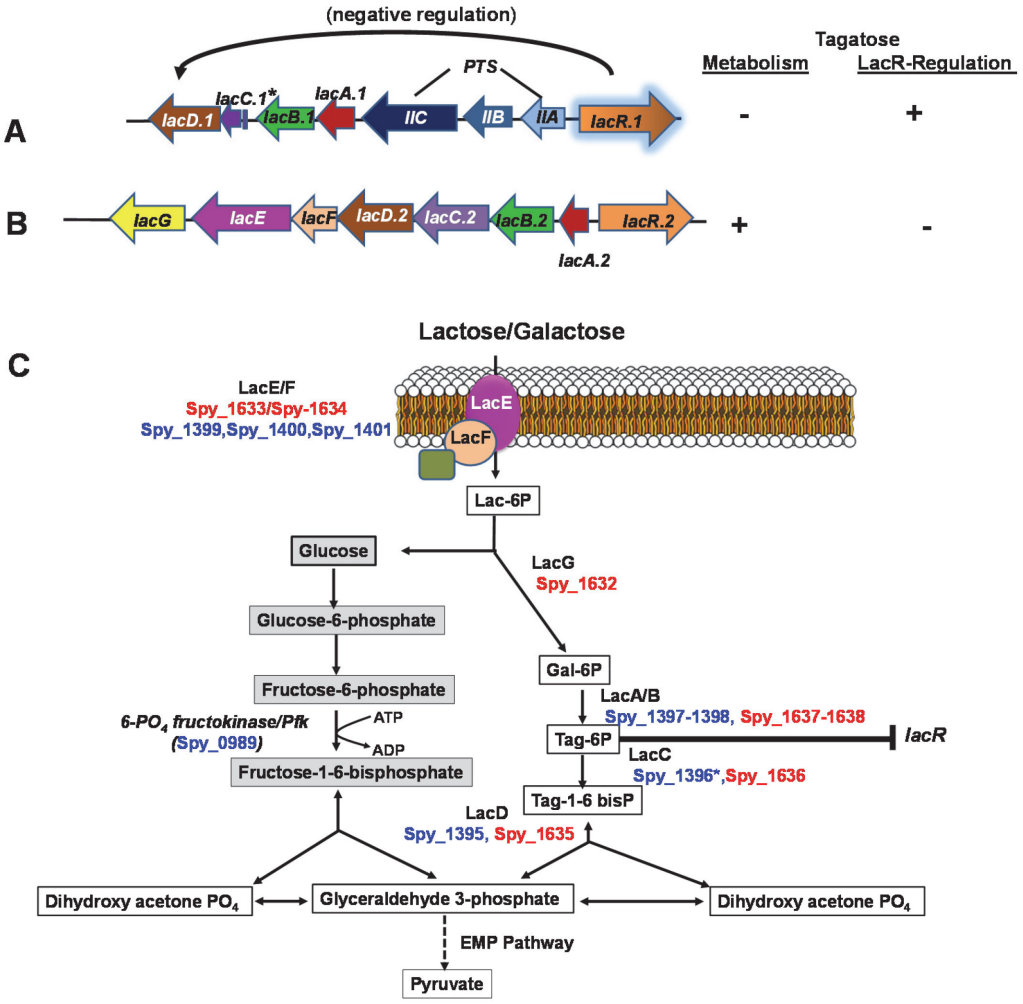


Fig. 4

Figure 4: Organization of *lac.1* and *lac.2* loci and their color-coded genes in the genome of type M1 *S. pyogenes* MGAS5005 strain, highlighting the gene duplication. (A) and (B) show the arrangement of genes that constitute the *lac.1* operon and *lac.2* operon, respectively, that encode the enzymes of the tagatose-6-phosphate pathway. (A) The asterisk on *lac.1** (*spy_1396*) denotes that it is a pseudogene that encodes a truncated product with only the intact N-terminus. Three components of the phosphotransferase system (PTS), IIC, IIB, and IIA, are encoded by *spy_1399-1401* and are homologous to *lacF* and *lacE* of the *lac.2* shown in B. The *lacG* gene is absent in the *lac.1* locus. Tabulated functions shown adjacent to A and B depict the differential roles played by these two loci. Unlike the *lac.2* locus encoding enzymes, those encoded by the *lac.1* locus do not participate in the actual tagatos-6-phosphate metabolism, but do possess regulatory activity. (C) Pathways depicting gene products involved in the transport and metabolism of lactose and/galactose. The Spy_xxxx assignments are based on the strain type M1 *S. pyogenes* MGAS5005 genome and those shown in blue and red fonts belong to Lac.2 and Lac.1, respectively. **LacE**-PTS-specific IIBC, **LacF**- PTS-specific IIA, **LacG**- 6-phosphobetagalactosidase, **LacA**- galactose-6-isomerase, **LacB**-Galactose-6-isomerase subunit, **LacR**-lactose-phosphotransferase system repressor. **LacC**-tagatose-6-phosphate kinase, **LacD**-tagatose-1,6 diphosphate aldolase.

direct control of a two-component system that consists of a histidine kinase (Spy_0831/MaeK/DpiA) and its cognate response regulator (Spy_0830/MaeR/DpiB/) (Figure 5) (Paluscio & Caparon, 2015). Malate stimulates expression of both the ME genes and the genes that encode the regulators; however, a low pH environment (pH 6.0) stimulates only the ME genes (Paluscio & Caparon, 2015) and the regulation in response to both cues requires the two-component regulator (Paluscio & Caparon, 2015; Sitkiewicz & Musser, 2006). Like many other non-glucose complex carbohydrate utilization systems, malate utilization is also subject to CCR regulation (Deutscher, et al., 2014; Deutscher, Francke, & Postma, 2006) and is repressed by glucose (Paluscio & Caparon, 2015), albeit by a CcpA-independent mechanism by utilizing the general PTS proteins PtsI (EI) and HPr

(Paluscio & Caparon, 2015). The latter is similar to the regulation of the *bglPH* operon in *B. subtilis* (Krüger, Gertz, & Hecker, 1996), which prevents the phosphorylation of regulatory transcription factors (Görke & Stülke, 2008). In the case of *S. pyogenes,* this transcription factor has not yet been identified. The loss of either PtsI or HPrHis15~P blocks the transcription of the two operons that encode the four ME genes (Paluscio & Caparon, 2015). Further, the *S. pyogenes* mutant lacking MaeE displays enhanced virulence in a murine soft tissue infection model, indicating that malate metabolism can influence virulence (Paluscio & Caparon, 2015).

Amino acid metabolism

Because *S. pyogenes* is auxotrophic for at least 15 amino acids (Davies, Karush, & Rudd, 1965; Slade, 1954) required for growth and persistence, it must overcome the substantially decreased quantity of protein in bodily fluids vs. the blood (7-8 mg/ml vs. ~70-90 mg/ml). During infection, the progressive exhaustion of protein and amino acid sources is likely to cause localized nutritional deficiencies for the continued growth of *S. pyogenes*. The latter can also occur when *S. pyogenes* persists in a high density at a given infection or colonization site. Therefore, it is not surprising that the *S. pyogenes* genome encodes a plethora of transport, catabolic, and regulatory genes to facilitate its adaptation to a low-protein environment. For example, like several Gram-negative and Gram-positive bacteria (Monnet, 2003; Tam & Saier, 1993), *S. pyogenes* also possesses two independent operons that encode membrane-associated oligopeptide permease complexes. The latter belong to the ABC transporter family that contains five genes (Podbielski & Leonard, 1998; Podbielski, et al., 1996). These operons encode oligopeptide permease (Opp) (Podbielski, et al., 1996) and dipeptide permease (Dpp) (Podbielski & Leonard, 1998). OppA (Spy_0249, 656 aa) and DppA (Spy_1704, 542 aa) are membrane-associated lipoproteins that bind to hexapeptide and dipeptide substrates, respectively. OppB-OppC (Spy_0250, 500 aa; Spy_0251, 308 aa) and DppB-DppC (Spy_1705, 325 aa; Spy_1706, 274 aa) are transmembrane permeases, which form a channel for the passage of the substrate across the membrane. OppD-OppF (Spy_0252, 356 aa; Spy_0253, 307 aa) and DppD-DppE (Spy_1707, 267 aa; Spy_1708, 208 aa) are membrane-associated ATPases, which energize the transport process. The deletion of either transporter gene does not alter the growth in complex media or a chemically-defined medium (CDM), suggesting that the transporter genes are independently regulated and have an overlapping function under these growth conditions (Podbielski & Leonard, 1998; Podbielski, et al., 1996). However, their functions do not completely overlap as supplementation with dipeptides or hexapeptides does not restore the growth of Dpp mutants and Opp mutants respectively in the CDM depleted of two essential amino acids (isoleucine and valine) (Podbielski & Leonard, 1998). *S. pyogenes* mutants lacking either Opp (Podbielski, et al., 1996) or Dpp (Podbielski & Leonard, 1998) exhibit significantly reduced production of the secreted SpeB cysteine protease, indicating that peptide transport directly or indirectly regulates the expression of protease activity and other secreted virulence factors (Podbielski & Leonard, 1998; Podbielski, et al., 1996) (Figure 6).

The uptake of amino acids and their metabolism in *S. pyogenes* are highly regulated. During starvation conditions, the synthesis of ribosomal proteins and transfer RNAs is inhibited. As in most bacteria, the starvation triggers the stringent response in *S. pyogenes*, as a result of the release of RelA from ribosomes, which then synthesize the "alarmone" (p)ppGpp [guanosine pentaphosphate (pppGpp) or guanosine tetraphosphate (ppGpp)] from GTP/GDP using ATP as a phosphate donor (Chatterji & Ojha, 2001; Srivatsan & Wang, 2008). The accumulation of alarmone/(p)ppGpp has a profound effect on cellular processes, as it alters the rates of transcription, bacterial replication, and protein translation, which leads to the altered expression of virulence, differentiation, and persistence (Hogg, Mechold, Malke, Cashel, & Hilgenfeld, 2004; Malke, Steiner, McShan, & Ferretti, 2006; Steiner & Malke, 2000; Wendrich, Blaha, Wilson, Marahiel, & Nierhaus, 2002). As shown in Figure 6, amino acid deprivation also activates a RelA-independent global transcriptional response (Steiner & Malke, 2001). The latter is characterized by up-regulated expression of *opp* and *dpp*, and several other genes, resulting in an increased amino acid pool which, in turn, counterbalances the (p)ppGpp-dependent stringent response. Consequently, the expression of a specific set of virulence factors is repressed. The fact that many other

Fig. 5

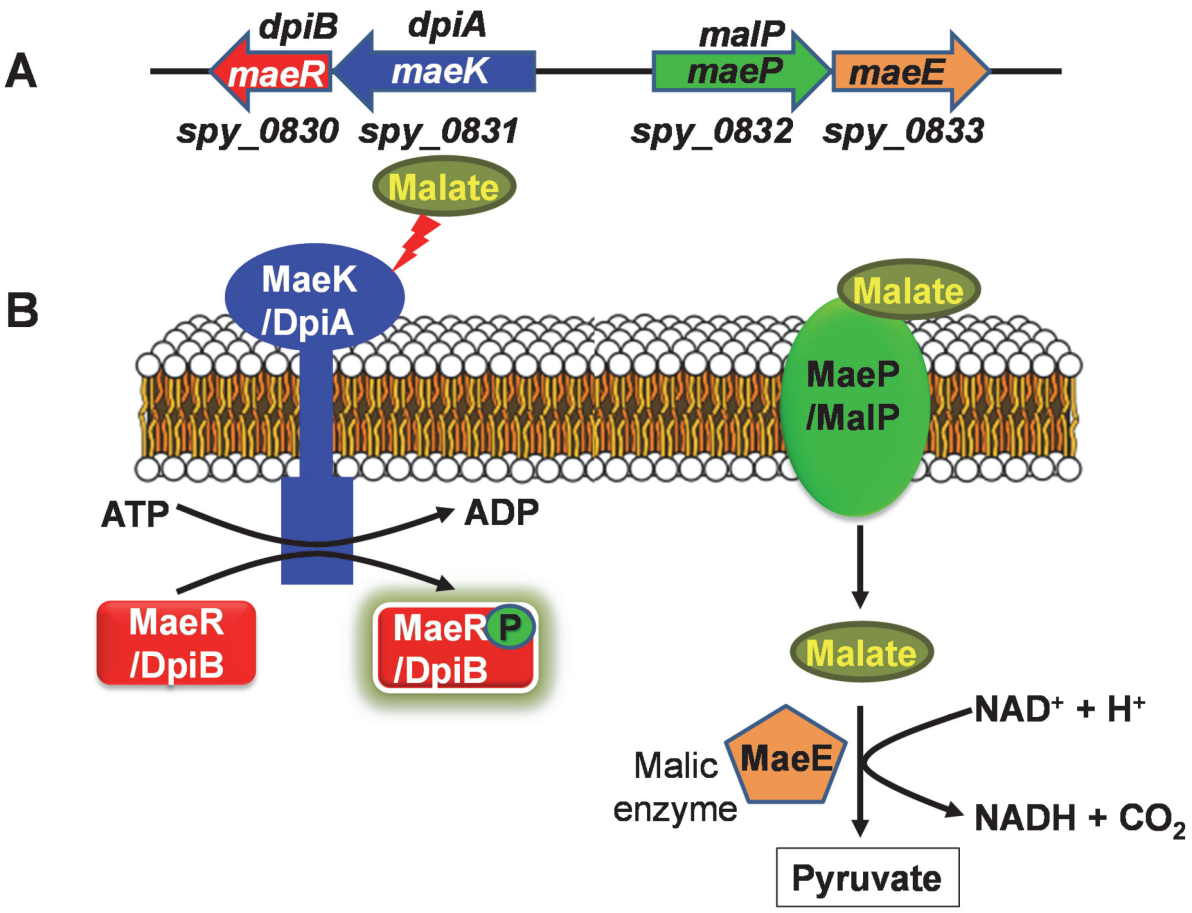


Figure 5: The malic enzyme (ME) pathway in *S. pyogenes*. (A) The arrangement of the open-reading frame that constitutes the ME locus of *S. pyogenes*. (B) A schematic presentation of the ME pathway depicts malate utilization via malate permease (MaeP/MalP). Once transported to the cytoplasm, the intracellular malate is converted to pyruvate in an NAD-dependent manner by malic enzyme (MaeE). Accumulation of malate during infection activates the two-component system constituted by MaeK (histidine kinase) and MaeR (response regulator). The activation of MaeKR regulates the expression of genes, maeP and maeE that encode malate permease and malic enzyme respectively. The Spy_xxxx assignments are based on the strain type M1 *S. pyogenes* MGAS5005 genome.

regulators, including virulence-related two-component regulators (such as FasABC and CovR) and standalone regulators (such as Rgg, MgaA, and CodY) also regulate the expression of Opp and Dpp indicates that amino acid metabolism and virulence in *S. pyogenes* are closely linked (Chaussee, et al., 2002; Dmitriev, McDowell, Kappeler, Chaussee, Rieck, & Chaussee, 2006; Hogg, Mechold, Malke, Cashel, & Hilgenfeld, 2004; Kreth, Chen, Ferretti, & Malke, 2011; Lyon, Gibson, & Caparon, 1998; Malke & Ferretti, 2007).

The metabolism of both glucose and amino acids also remain interdependent during active *S. pyogenes* infection, irrespective of the site of infection. As the glucose concentration is depleted, the catabolism of amino acids acts to increase the content of nitrogen, which in turn, induces the expression of several amino acid catabolic pathways as a positive feedback amplification loop. The induction of these pathways also includes the arginine deiminase pathway (Cusumano, Watson, & Caparon, 2014; Shelburne, et al., 2011). Encoded by the *arc* operon, this pathway results in the fermentation of arginine producing citrulline through the action of arginine deiminase (Spy_1275/ArcA), which is converted to ornithine and carbamoyl phosphate by ornithine carbamoyl

transferase (Spy_1273/ArcB). Finally, in this pathway, carbamoyl phosphate is converted to ammonia and CO_2 by carbamate kinase (Spy_1270/ArcC), producing one molecule of ATP (Figure 7). Ornithine is then exported out by the ArcD antiporter (Spy_1272) in an exchange with concomitant import of an additional arginine molecule in a supplemental energy-independent manner. In murine infection models, *S. pyogenes arcB* knockout mutants, in comparison to *arcA* knockout mutants, show severe attenuation, which emphasizes the unexpected and unusual role of citrulline catabolism in *S. pyogenes* virulence (Cusumano, Watson, & Caparon, 2014). In particular, the Arc pathway inhibits the proliferation of peripheral blood mononuclear cells (Degnan, et al., 1998) and modulates host $NO^{\bullet}$/iNOS-mediated innate immunity (Cusumano, Watson, & Caparon, 2014). Contributions of the Arc pathway include the production of energy in the form of ATP and the release of ammonia, which plays a beneficial role in reducing acid stress (Cotter & Hill, 2003). Thus, arginine catabolism helps neutralize the acidic extracellular environment, and the metabolism of citrulline generates ATP to maintain intracellular pH through the F_1F_0-ATPase-mediated extrusion of protons from the cell, at the expense of ATP hydrolysis (Cusumano & Caparon, 2015). The F_1F_0-ATPase will be discussed in more detail later in the chapter. The retention of GAPDH (also known as SDH or Plr) or prevention of its surface export from the cytoplasm of *S. pyogenes* adversely affects the expression of the Arc operon and the V-type Na^+-ATPase (Jin, Agarwal, Agarwal, & Pancholi, 2011). The biochemical link between GAPDH and arginine metabolism, as well as the possible role of GAPDH in the regulation of pH balance, are currently unknown.

Acetate metabolism

As elucidated above, *S. pyogenes* is equipped with a variety of mechanisms that allow it to switch its metabolism from a program that permits rapid growth when a surplus of nutrients are available to a program that facilitates its survival in the absence of those nutrients. Another potential survival mechanism is the "acetate switch" (Wolfe, 2005), which involves the importation and utilization of acetate as an energy source that had been excreted during growth on glucose. This process of acetate dissimilation (production and excretion) and assimilation (import and utilization) has previously been studied in detail in *E. coli* and several Gram-negative bacteria (Wolfe, 2005; Wolfe, 2008; Wolfe, et al., 2003). Like other streptococcal species, *S. pyogenes* contains an intact metabolic pathway for the dissimilation and assimilation of acetate (Figure 8). Although the implication of this pathway has been analyzed more in detail in pathogenic streptococcal species other than *S. pyogenes* (Ramos-Montañez, Kazmierczak, Hentchel, & Winkler, 2010), indirect evidence has implied that this pathway plays an important role in *S. pyogenes* (Seki, Iida, Saito, Nakayama, & Yoshida, 2004). Acetate dissimilation first involves the conversion of pyruvate to acetyl-CoA by different pathways, depending on whether conditions are oxidative (aerobic) or non-oxidative (anaerobic). The pyruvate dehydrogenase (PDHC) complex (Spy_0751/AcoA and Spy_0752/AcoB) carries out the oxidative decarboxylation, and generates two additional NADH molecules per glucose molecule. PDHC can be repressed by high concentrations of NADH and anaerobic conditions (Wolfe, 2005), and in these cases, pyruvate is converted to acetyl-CoA via the oxygen sensitive pyruvate formate-lyase (Pfl) enzyme complex (Spy_1569/Pfl and Spy_1743/PflD) (Figure 8). The resultant acetyl-CoA may then follow two alternate paths ultimately leading to the production of either acetate or ethanol. The conversion to acetate occurs through the energy-rich intermediate acetyl-phosphate and is catalyzed by phospho-transacetylase (Spy_0851/PTA) and acetate kinase (Spy_0094/Ack), which results in the production of two ATP molecules per glucose without consuming any reducing equivalents (Figure 8). The reduction of acetyl-CoA to ethanol occurs via an acetaldehyde intermediate and is likely to be catalyzed by alcohol dehydrogenase (Spy_0039/Adh1 and Spy_0040/Adh2). While Pta and Ack in pneumococci and other Gram-positive pathogens are associated with various cellular processes, including cell division and cell growth/survival (Ramos-Montañez, Kazmierczak, Hentchel, & Winkler, 2010), the physiological roles of these proteins in *S. pyogenes* have not been investigated to date. The energy-rich acetyl~P may globally affect transcriptional regulation by direct phosphorylation of the response regulators of various two-component systems in the absence of a cognate histidine kinase (Churchward, 2007). In addition, excretion of acetate creates an acidic environment, which can induce acid stress and may accumulate to toxic levels.

Fig. 6

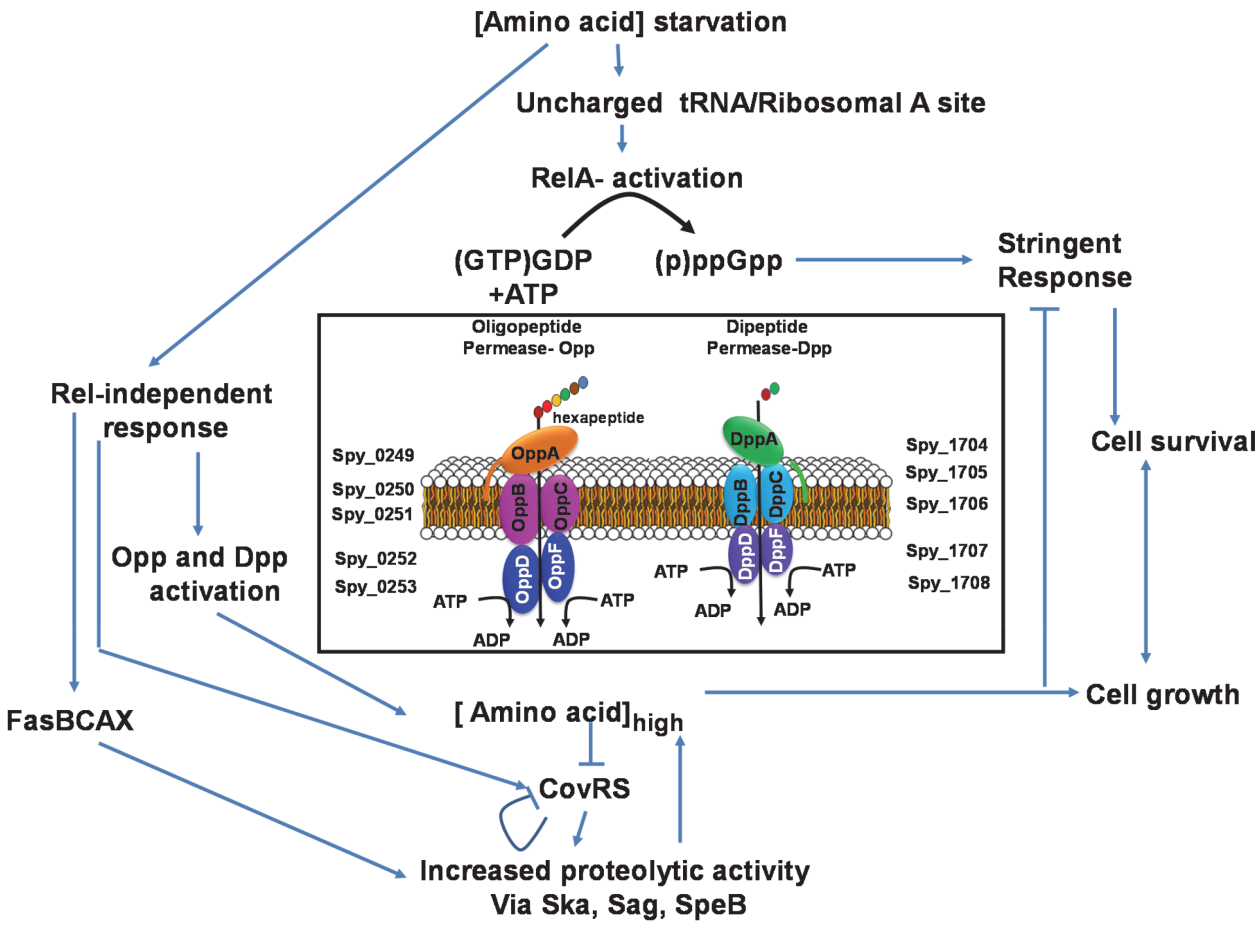


Figure 6: RelA-dependent and -independent amino acid starvation response networks of S. pyogenes. Arrows indicate positive regulation, while blunt-ended arrows indicate negative regulation or repression. The schematically presented network highlights the up-regulation of several genes including those that encode Opp and Dpp and other virulence regulators that are responsible for increasing the amino acid pool to counterbalance the (p)ppGpp-dependent stringent response. A schematic diagram in the center shows the organization of protein complexes formed by Opp and Dpp proteins in the membrane. OppA and DppA are lipoproteins, transmembrane proteins OppBC and DppBC are permeases, and OppDF and DppDF are ATPases. Together these protein complexes facilitate the transport of exogenous hexapeptides or dipeptides to the cell cytoplasm. The Spy_xxxx assignments are based on the strain type M1 S. pyogenes MGAS5005 genome.

Assimilation of acetate is possible because the activities of Ack and PTA are reversible and can convert acetate and ATP to acetyl~P and ADP, and then to acetyl-CoA and inorganic phosphate. However, this requires the importation of acetate from the environment. Based on genome sequence analysis, it is not clear whether *S. pyogenes* contains an acetate-specific permease (ActP) that is commonly found in Gram-negative bacteria (Wolfe, 2005). The sequence of the recently published type M14 genome (strain HSC5) contains an open-reading frame annotated as *actP* (*L897_07030*), which is annotated as a gene encoding copper exporting ATPase in other *S. pyogenes* strains (such as *spy_1405*/*copA*). Thus, it is not known whether this gene encodes a physiologically relevant acetate permease. Alternately, because acetate can freely permeate across the cell membrane (Llarrull, Fisher, & Mobashery, 2009), it is possible that a dedicated acetate transport system is not required.

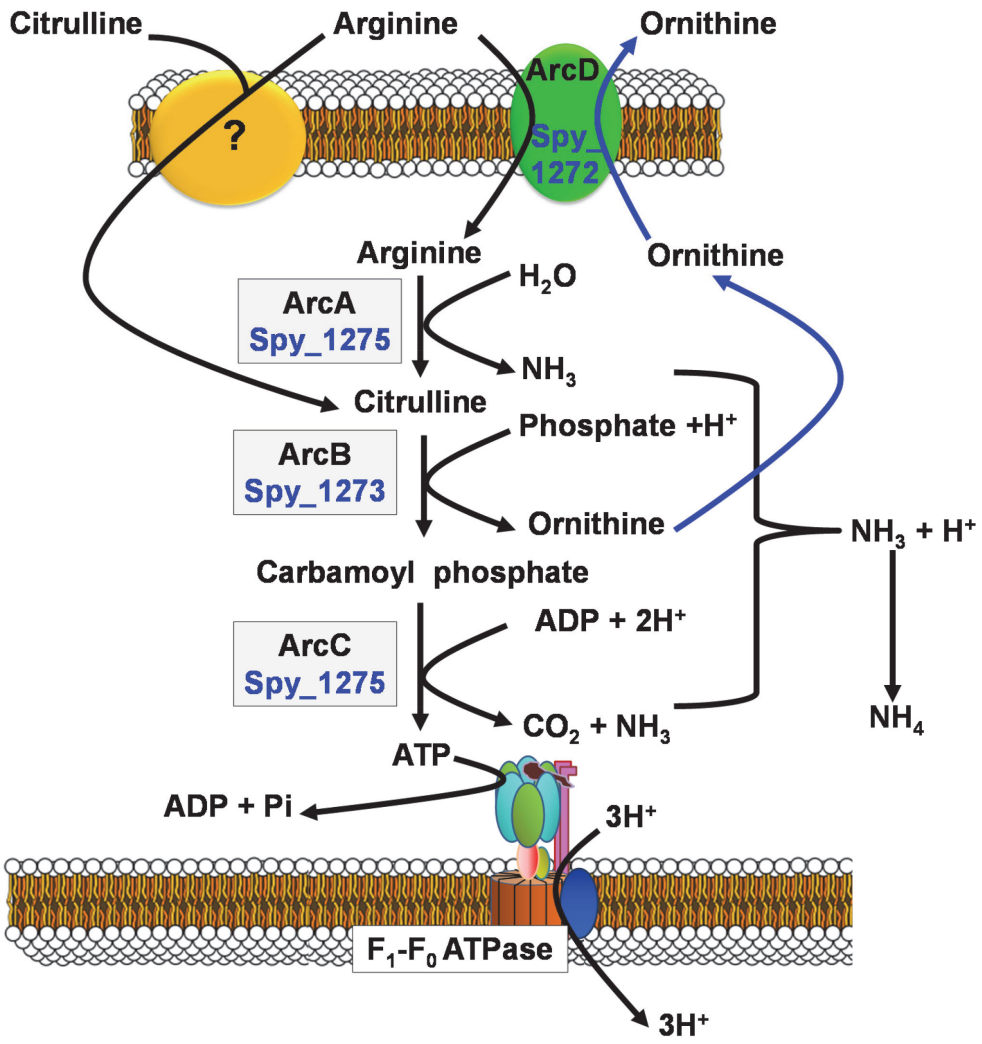


Fig. 7

Figure 7: Schematic diagram showing the arginine and citrulline catabolic pathway in *S. pyogenes* and its coordination with F_1F_0-ATPases. Transport and catabolism of arginine and citrulline in *S. pyogenes* involve a multienzyme arginine deiminase pathway, wherein ArcD serves as an antiporter and/or an unknown transporter. The transported arginine is then catabolized via the enzymes ArcA, ArcB, and ArcC, which results in two molecules of ammonia and one molecule of ATP. Catabolism of citrulline, on the other hand, produces one molecule each of ammonia and ATP. F_1F_0-ATPase then exports three protons outside the cell with simultaneous hydrolysis of ATP to ADP. The Spy_xxxx assignments are based on the strain type M1 *S. pyogenes* MGAS5005 genome.

Lipid metabolism and fatty acid biosynthesis

In bacteria, phospholipids comprise approximately 10% of the dry weight of the cell, and each mole of lipid requires approximately 32 moles of ATP for biosynthesis. Most of the enzymes involved in fatty acid biosynthesis are cytosolic in nature, but the enzymes required for membrane lipid synthesis are primarily found in membranes. Like in other bacteria, the fatty acid biosynthesis machinery in *S. pyogenes* is highly conserved and involves the type II fatty acid synthase (FASII)-containing multi-enzyme complex. The latter is encoded, along with other lipid biosynthesis genes, by a 12-gene operon that includes *Spy_1484/accD*, *Spy_1485/accA*, *Spy_1486/accC*, *Spy_1487/fabZ*, *Spy_1488/accB*, *Spy_1489/fabF*, *Spy_1490/fabG*, *Spy_1491/fabD*, *Spy_1492/fabK*, *Spy_1493/acpP*, *Spy_1494/fabH* and *Spy_1495/marR* (Figure 9). MarR may serve as a putative transcriptional regulator. Further, four additional genes are likely involved in lipid biosynthesis, including three genes (*Spy_0433*, *Spy_0766*, and *Spy_1496*) that encode long chain fatty acid CoA, acyl-ACP-thioesterase, and PhaB/FabM, respectively, and a truncated version of FabG (Spy_0359) that is likely to be the product of a gene duplication.

Fig. 8

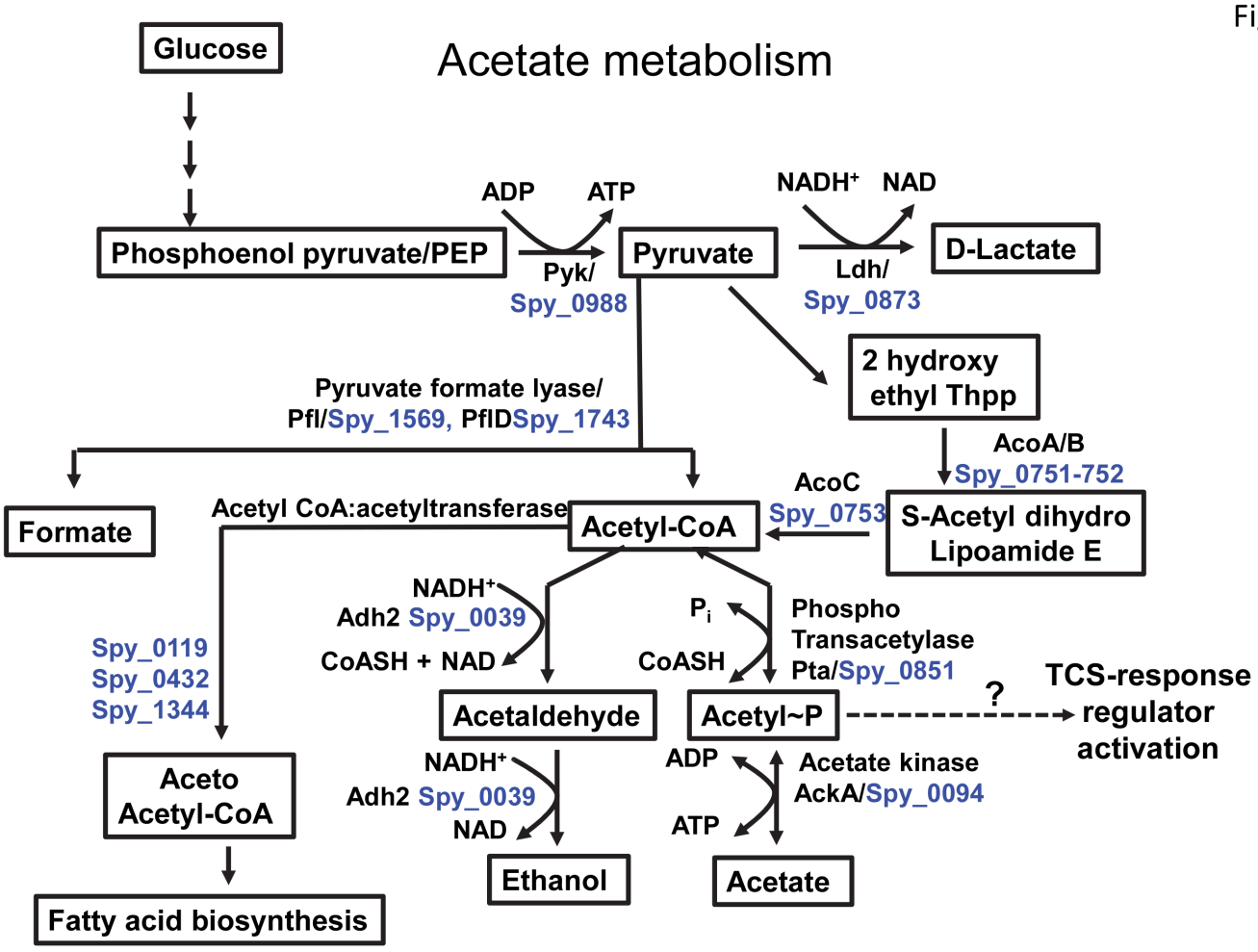


Figure 8: Acetate metabolism pathways for partially oxidized metabolites. Acetate activation pathways involve two key enzymes: Ack (acetate kinase) and Pta (phospho-transacetylase). These enzymes catalyze the formation of energy rich-acetyl-phosphate (Acetyl~P, ΔG^o = -43.3 kJ/mol) from acetate and acetyl-CoA, respectively. Acetyl-CoA is derived from pyruvate as the end product of the EMP pathway, either directly via pyruvate formate lyase (Pfl or PflD) or ApoABC enzymes. Acetate metabolism yields two molecules of NAD and one molecule of ATP and plays a crucial role in the initiation of fatty acid metabolism. Acetyl~P may participate in the activation of response regulators of two-component regulatory systems in the absence of an active cognate histidine kinase and may serve as a global signal. The Spy_xxxx assignments are based on the strain type M1 *S. pyogenes* MGAS5005 genome.

Unlike *S. aureus* and *Listeria*, *S. pyogenes* like *S. pneumoniae* lacks FabI (enoyl-ACP reductase), and instead encodes this activity encoded by a non-homologous gene *fabK (Spy_1492)*.

These genes contribute to lipid biosynthesis as follows: The first committed step in the fatty acid synthesis is the conversion of acetyl-CoA to malonyl-CoA, which is catalyzed by the acetyl-CoA carboxylase complex, consisting of the carboxyltransferase subunits AccD and AccA, along with biotin carboxylase (AccC) and the biotin carboxylase carrier protein (AccB). A unique, small, acidic, and highly soluble acyl carrier protein (ACP) (Spy_1493/AcpP, Spy_0021/AcpP.2; 74 and 80 aa, respectively) participates in fatty acid synthesis by helping to shuttle the growing acyl chain between enzymatic active sites. The 4'-phosphopantetheine prosthetic group is transferred from CoA to apo-ACP by the monomeric ACP synthase (Spy_1533) (Figure 9, Step-I). The acyl intermediates bind to Ser residues of ACP (Ser35 of AcpP or Ser39 of AcpP.2) through a thioester linkage mediated by acyl-ACP-thioesterase (Spy_0766). The latter promotes the transacylation of malonyl-CoA to ACP via FabD, which forms malonyl-ACP and adds a two-carbon acetyl unit to a nascent or growing fatty acyl chain

(Figure 9, Step-II). Subsequently, FabH or FabF catalyzes the initial irreversible condensation of malonyl-ACP with acetyl-CoA to form acetoacetyl-ACP (Figure 8, Step-II). The elongation of the acyl chains involves the participation of four enzymes. These enzymes participate in each iterative cycle of chain elongation. First, FabF adds two carbon units from malonyl-ACP to the growing acyl-ACP. This ketoester is then reduced by the NADPH-dependent β-ketoacyl-ACP reductase (FabG), and a water molecule is removed by β-hydroxyacyl-ACP dehydratase enzyme (FabZ). The last step is catalyzed by enoyl-ACP reductase (FabK) to form a saturated acyl-ACP, which then serves as the substrate for another condensation reaction (Figure 9, Step-III). Thus, for the second cycle, the product would be a butyryl group; likewise, the subsequent cycles would generate a hexanoyl group, an octanoyl group, a decanoyl group, a dodecanoyl group, and so on.

Lipid biosynthesis and its contribution to pathogenesis have been studied only in a small number of Gram-positive pathogens (Lu, et al., 2006), such as *Streptococcus pneumoniae* (Lu & Rock, 2006), *Staphylococcus aureus* (Balemans, et al., 2010; Parsons, et al., 2014a; Parsons, Frank, Jackson, Subramanian, & Rock, 2014b; Parsons, Frank, Rosch, & Rock, 2013; Parsons, Frank, Subramanian, Saenkham, & Rock, 2011), and *Listeria monocytogenes* (Sun & O'Riordan, 2010). For *S. pyogenes*, there is evidence from global transcriptome analyses of *S. pyogenes* mutants lacking either surface export of SDH/GAPDH (Spy_0233) (Jin, Agarwal, Agarwal, & Pancholi, 2011) or the gene encoding serine/threonine phosphatase (SP-STP) (Agarwal, Agarwal, Pancholi, & Pancholi, 2011), suggesting that increased lipid biosynthesis may be associated with a reduction of virulence. Both of these mutants are attenuated for virulence and display up-regulated genes involved in lipid biosynthesis. However, a biochemical or physiological link between these genes and lipid biosynthesis remains to be established. Fatty acid biosynthesis has been demonstrated to play an important role in the maintenance of the integrity of the ExPortal system, which is responsible for the export of several streptococcal secretory products (Rosch & Caparon, 2005; Rosch, Hsu, & Caparon, 2007; Vega, Port, & Caparon, 2013), and mutations in fatty acid biosynthesis genes and regulators are associated with an increased resistance to cationic antimicrobial peptides (Port, Vega, Nylander, & Caparon, 2014).

Since fatty acid biosynthesis plays an important role in bacterial virulence and the FASII complex found in bacteria differs significantly from the type I pathway that is involved in mammalian cell fatty acid biosynthesis, this process has been targeted for therapeutic interventions. In studies to date that have evaluated inhibitors of FASII in *S. aureus* and *Streptococcus agalactiae* (group B *Streptococcus*), the results have been mixed. While Gram-positive bacteria are susceptible to these lipid biosynthesis inhibitors under standard culture conditions *in vitro*, some species may be able to overcome the direct inhibition of FASII enzymes by importation and incorporation of host-derived fatty acids *in vivo*, defeating the action of lipid inhibitors (Brinster, et al., 2009; Parsons & Rock, 2011). There is little information available on fatty acid biosynthesis in *S. pyogenes*, and it is unknown if it can incorporate exogenously added fatty acids. Thus, whether lipid biosynthesis inhibitors are an effective therapeutic agent is debatable, at present (Parsons & Rock, 2011).

As described above, biotin or vitamin H plays an essential role in one of the early steps of lipid biosynthesis. It serves as an essential cofactor in carboxylation, decarboxylation, and transcarboxylation reactions. Many plants and prokaryotes synthesize biotin, including certain bacteria, archaea, and fungi (Lin & Cronan, 2011; Streit & Entcheva, 2003). However, certain firmicutes, including *S. pyogenes*, are naturally biotin-auxotrophic. As a result, biotin must be exogenously provided for *S. pyogenes* growth. In general, little is known about the mechanisms underlying the biotin transport in prokaryotic cells. Recently, bioinformatics-based functional genomic and experimental investigations have presented evidence that, unlike the fungal *bioYMN* biotin transporter, only *bioY* is widespread among bacterial genomes. However, even this gene is absent from many proteobacteria, including *E. coli* (Entcheva, Phillips, & Streit, 2002; Rodionov, et al., 2009; Rodionov, Hebbeln, Gelfand, & Eitinger, 2006; Rodionov, Mironov, & Gelfand, 2002). Homologs of fungal *bioMN* in prokaryotes share distinct similarities with the genes encoding CbiO and CbiQ, which are components of a prokaryotic cobalt transporter (Entcheva, Phillips, & Streit, 2002; Rodionov, et al., 2009). It has been proposed that these *bioMN* homologs may encode the components of a biotin transporter. The atypical nature of this putative prokaryotic biotin

transporter highlights the fact that it is not encoded by a typical *bioYMN*-type operon—instead, all three genes are distantly located in the genome. Based on recent reports (Hebbeln & Eitinger, 2004; Hebbeln, Rodionov, Alfandega, & Eitinger, 2007), biotin transport in *S. pyogenes* is likely to be mediated by a *bioYMN*-like module constituted by *bioY/Spy_0177* and homologs of cobalt transporter encoding genes *cbiQ/spy_1522/ecfT* and *cbiO/spy_1521/ecf1/ecf2* (see also Figure 14). Two other genes also share the function of these genes: *spy_1846/ecf1* and *spy_1845/ecf2*. Experimental evidence obtained from *Rhodobacter capsulatum* (Hebbeln, Rodionov, Alfandega, & Eitinger, 2007) suggests that BioY/CbiQ/CbiO ternary complexes are stable, but the BioY/CbiO complex is unstable for efficient biotin transport. Functional genomic analysis of these genes in *S. pyogenes* is required to understand the precise role of biotin transport in lipid biosynthesis and other metabolic activities affecting *S. pyogenes* virulence.

Aerobic metabolism and the salvage NAD biosynthesis pathway

The coenzyme NAD^+ is an essential growth factor that serves as a coenzyme in numerous biochemical reactions that involve NAD^+-hydrolyzing enzymes and hydride transfer-catalyzing enzymes. Typical *de novo* NAD^+ biosynthesis in microorganisms involves two enzymatic steps that convert L-aspartate to quinolinic acid (QA), via an unstable aspartate intermediate, using the enzymes L-aspartate oxidase (NadB) and quinolinic acid synthetase (NadA) (Figure 10). QA is then successively converted to nicotinate mononucleotide (NaMN) and nicotinate adenine dinucleotide (NaAD) by QA phosphoribosyl transferase (NadC) and NaMN adenyltransferase (NadD). NaAD is then converted to NAD^+ by NAD^+ synthetase (NadE). Genomic analyses of all *Streptococcus* species, including *S. pyogenes*, revealed that these organisms lack genes with obvious homology to those encoding the first two enzymes required for the *de novo* synthesis of NAD^+ (i.e., NadB and NadA). Thus, the organisms are likely to be dependent on the salvage of the exogenous pyridine precursors nicotinamide (NM) or nicotinic acid (NA) (namely, vitamin B3), which are transported to the cytoplasm via a family of niacin transporters (NiaX). The salvage pathway involves two enzymes, PncA (Spy_1511) and PncB (Spy_1358), which convert the transported NM to NA and the NA to NaMN, respectively (Figure 10). Sorci *et al.* recently reported that unlike other streptococcal species, *S. pneumoniae* and *S. pyogenes* uniquely possess QA phosphoribosyl transferase (NadC), which is encoded by an orphan gene (*Spy_0170*) and is not linked to any of the other NAD^+ biosynthetic genes involved in the last step of the *de novo* NAD^+ biosynthesis pathway (Sorci, et al., 2013) (Figure 10). This unique feature has led to two important discoveries: (i) *S. pyogenes* is not solely dependent on its NM/NA salvage pathway to synthesize NAD^+; and (ii) *S. pyogenes* can overcome its niacin (namely, NM/NA) autotrophy using NadC to salvage quinolate (QA) from the surrounding environment, even in the absence of *pncA* and *pncB*. The unique presence of NadC is likely to be advantageous, as in humans, QA is a product of tryptophan degradation that accumulates during many diseases, and likely also accumulates during infection and preceding ongoing sepsis in critically ill patients (El-Zaatari, et al., 2014; Hoshi, et al., 2012; Niño-Castro, et al., 2014; Pfefferkorn, 1984; Zeden, et al., 2010). In fact, tryptophan catabolism has been reported to restrict IFN-gamma-expressing neutrophils and *Clostridium difficile* immunopathology (El-Zaatari, et al., 2014). The product of NadC (NaMN) can then be converted into NAD^+, using NadD (Spy_0263) and NadE (Spy_1357) (Figure 10). This functional redundancy between NM/NA and QA salvage pathways, therefore, leaves two enzymes downstream of the common intermediate NaMN (namely, NadD and NadE) (Sorci, et al., 2013) as the potential drug targets.

Other aspects of aerobic metabolism

During the initial stages of infection subsequent to colonizing the skin or the oropharynx, *S. pyogenes* encounters an environment that is considerably higher in oxygen content. Certain surface proteins, including important adhesins, such as the fibronectin-binding protein (alternately known as Protein F or Sfb) (Futai, Noumi, & Maeda, 1989; Görke & Stülke, 2008), are expressed in response to oxygen stress (Gibson & Caparon, 1996;

Fig. 9

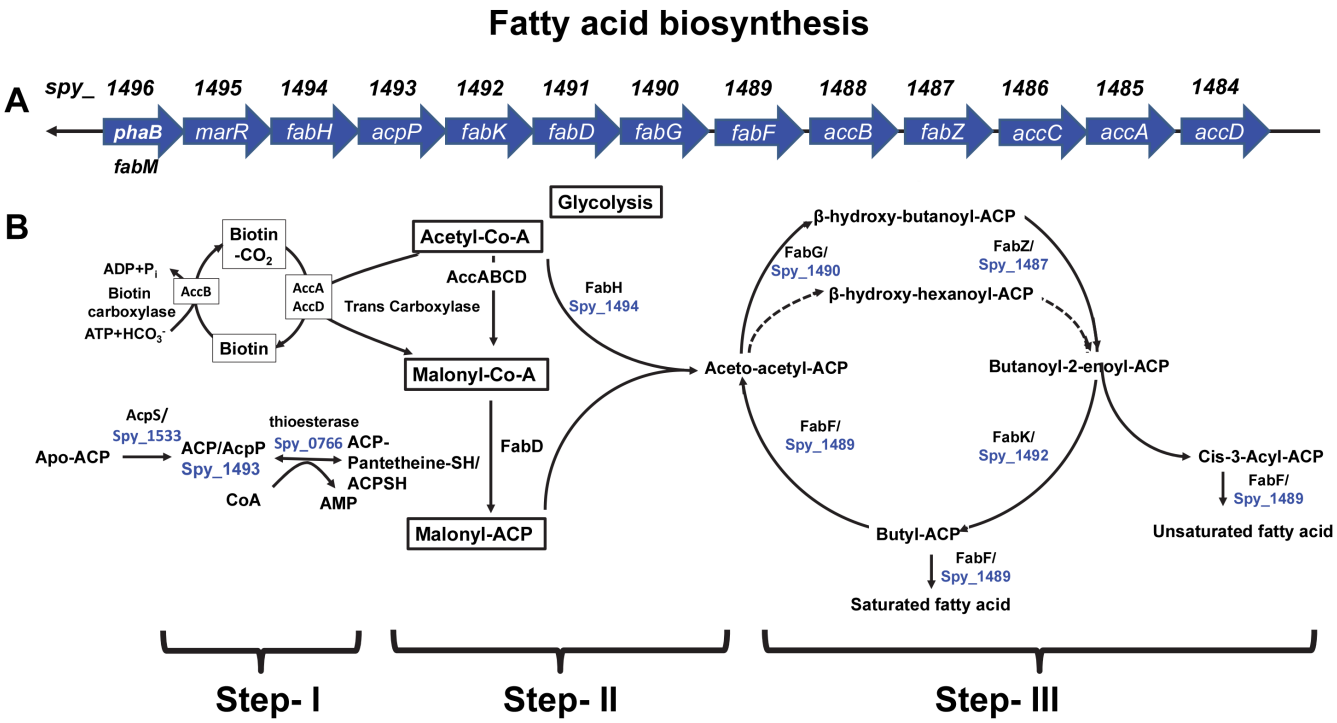


Figure 9: Fatty acid biosynthesis pathway in *S. pyogenes*. (A). The genomic organization of genes involved in fatty acid biosynthesis in *S. pyogenes*. (B). Fatty acid biosynthesis is divided in to three major steps involving several enzymes.
Step I. In this step, the acetyl-CoA carboxylase reaction is performed in two substeps to form malonyl-CoA. Biotin, which is covalently attached to the biotin carboxyl carrier protein (BCCP) (AccB), is carboxylated by the carboxylase subunit AccC. The heterodimeric trans-carboxylase (AccA and AccD) then transfers the CO_2 to acetyl-CoA, which forms malonyl-CA.
Step II. Step II involves the initiation of fatty acid synthesis. Malonyl-CoA binds to the acyl carrier protein (Acp). Fad (malonyl-CoA ACP transacylase) transfers the malonyl group from CoA to ACP, and B-ketoacyl-ACP synthase III (FabH) catalyzes the initial irreversible condensation of malonyl-ACP with acetyl-CoA to form acetoacetyl-ACP.
Step III. Step III involves a cycle of fatty acyl chain elongation or fatty acid condensation. As shown, all intermediates in fatty acid synthesis are shuttled through the cytosol as thioesters of the acyl carrier protein (ACP). β-ketoacyl-ACPreductase (FabG), β-hydroxyl-ACPdehydratase (FabZ), trans-2-enolyl-ACP reductase (FabK), and β-ketoacyl ACP synthase (FabF) act in a sequential fashion during elongation. Every cycle adds two carbon acetyl units. MarR is a predicted transcriptional regulator of fatty acid synthesis. FabM/phaB is involved in long fatty acid chain condensation. The Spy_xxxx assignments are based on the strain type M1 *S. pyogenes* MGAS5005 genome.

VanHeyningen, Fogg, Yates, Hanski, & Caparon, 1993). The latter stages of *S. pyogenes* infection are characterized by intense inflammation, during which the production of various bactericidal reactive oxygen species (ROS) from immune cells, such as neutrophils and monocytes, contributes to an important component of host defenses. Thus, despite its strict reliance on fermentation to generate energy, *S. pyogenes*, as a facultative anaerobe, contains a number of genes that promote its adaptation to aerobic environments and ROS-induced stress. The highly reactive, toxic byproducts of oxygen (ROS) include superoxide anion ($O_2^{\bullet -}$), hydroxyl radicals ($OH^{\bullet}$), singlet oxygen ($O_2^{\bullet}$), and hydrogen peroxide (H_2O_2), which are capable of damaging nucleic acid,

Fig. 14

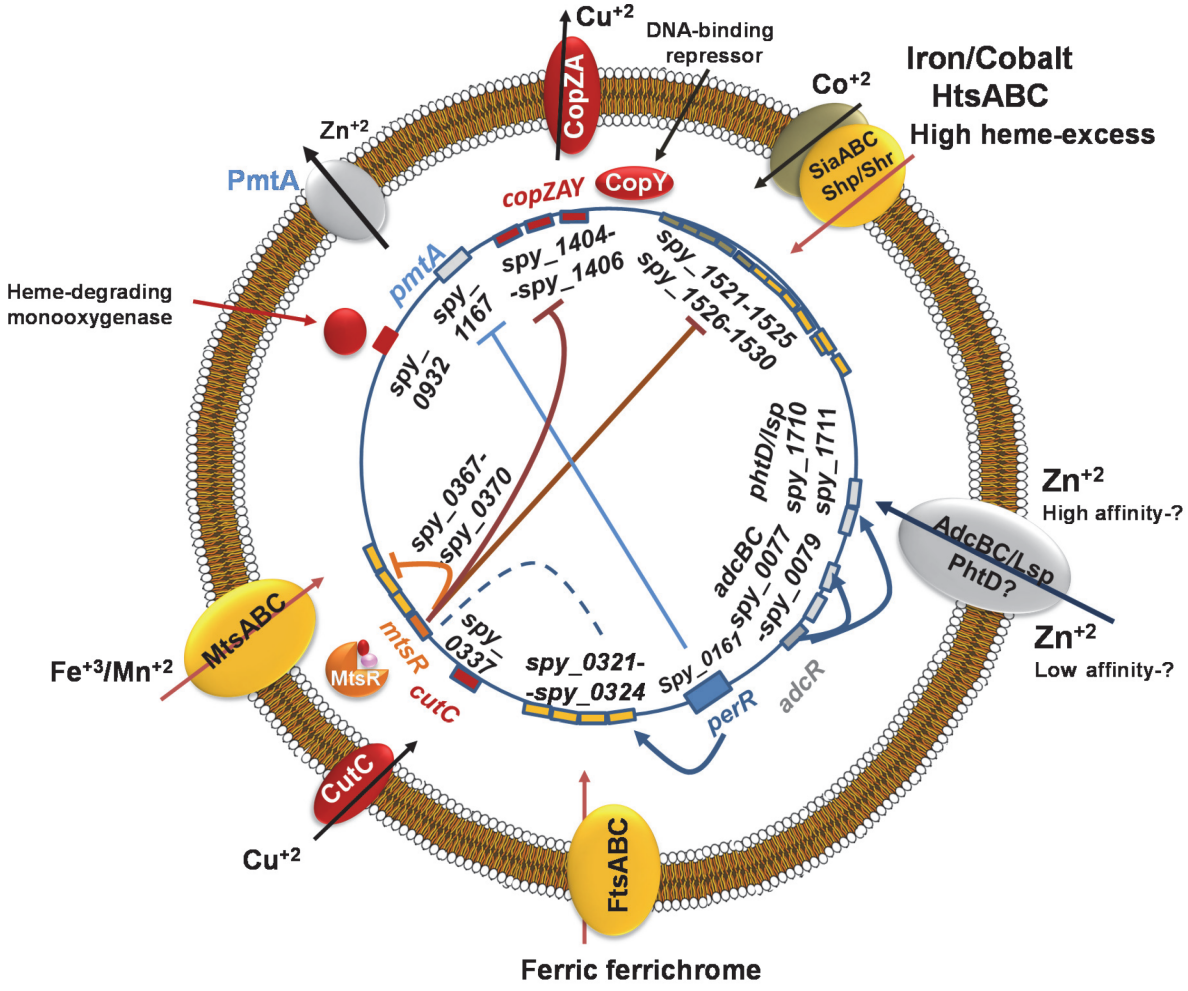


Figure 14: A schematic diagram of the involvement of genes and the corresponding proteins in the transport of various ions (Fe/Mn, heme, ferrichrome, Co, Cu, and Zn), and their regulation in *S. pyogenes*. Arrows indicate positive regulation. Blunt-ended arrows indicate repression/ negative control. The inner circle represents the relative location of iron transport genes in the *S. pyogenes* M5005 genome. The outer circle depicts the putative cell membrane location of ion transporters and their functions. Directions of arrows on these transporters indicate efflux or influx of the ions. The broken line arrow indicates a probable regulatory role. The Spy_xxxx assignments are based on the strain type M1 *S. pyogenes* MGAS5005 genome.

protein as well as cell membranes (Figure 11). Unlike many other bacteria, *S. pyogenes* contains a single superoxide dismutase (Spy_1145/SodA) and serves as an antioxidant (Gerlach, Reichardt, & Vettermann, 1998; Gibson & Caparon, 1996). It allows *S. pyogenes* to tolerate the accumulated toxic superoxide anion [$O_2^{\bullet -}$] during aerobic oxygen stress by converting it to hydrogen peroxide (H_2O_2) and O_2 (McCord & Fridovich, 1969). *S. pyogenes* mutant lacking SodA lose the ability to grow aerobically, although they still grow anaerobically (Gibson & Caparon, 1996). In addition, *S. pyogenes* is one of the few streptococcal species that accumulates significant levels of glutathione, a reductant; which, along with the enzyme glutathione reductase (Spy_0627), maintains reducing conditions in the bacterial cytoplasm. Glutathione also serves as a co-factor for glutathione (GSH) peroxidase (Spy_0503/GpoA), a selenoprotein oxidoreductase that detoxifies both inorganic and organic peroxides (Brenot, King, Janowiak, Griffith, & Caparon, 2004). Since the genome of *S. pyogenes* does not contain a gene with high homology to the enzymes responsible for the *de novo* synthesis of glutathione (such as γ-glutamyl cysteine synthase and glutamate-cysteine ligase), *S. pyogenes* likely salvages glutathione from its environment. Unlike SodA mutants, GpoA-deficient mutants can grow aerobically. While GpoA mutants are

Fig. 10

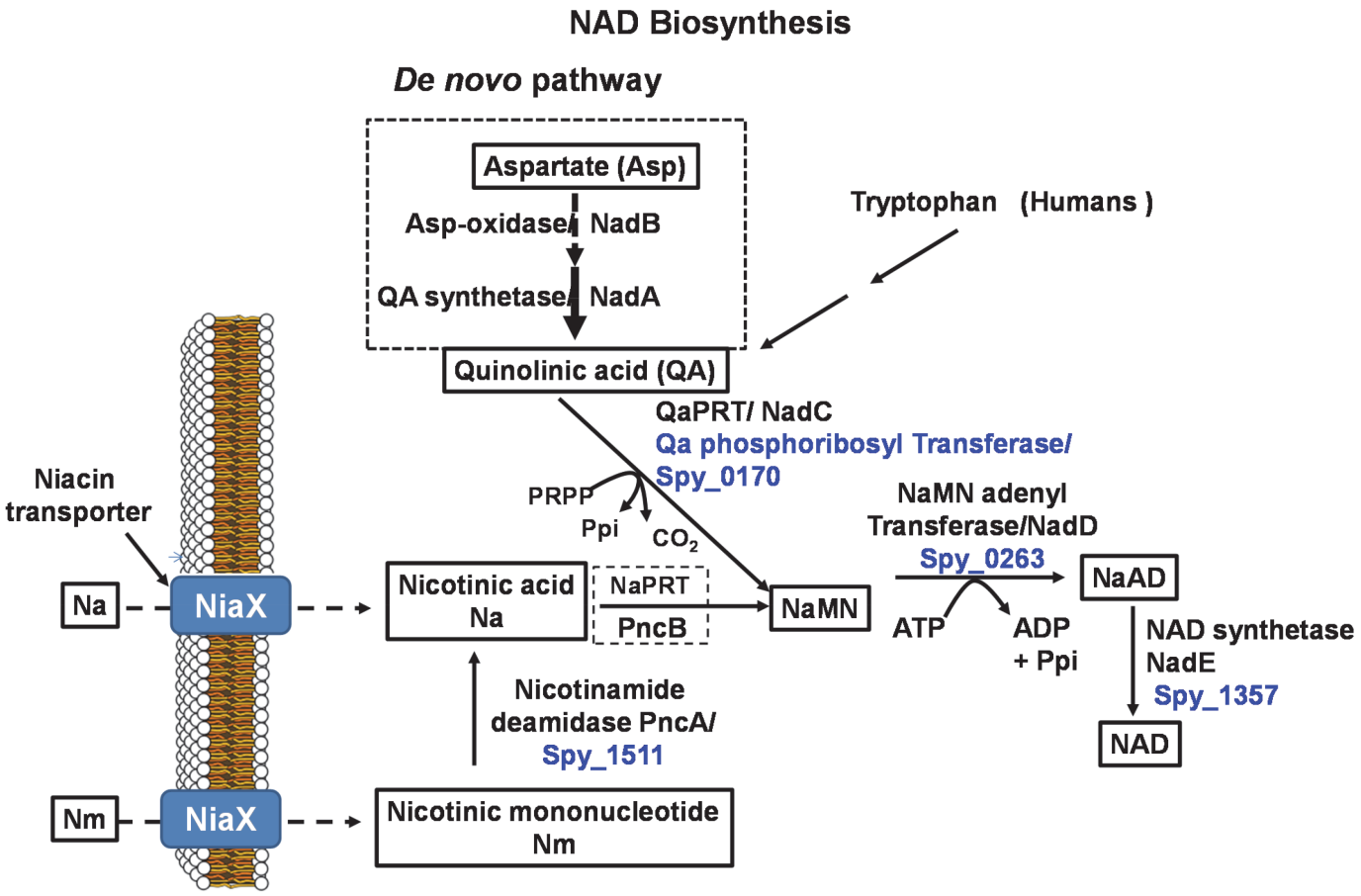


Figure 10: A schematic presentation of NAD biosynthesis in *S. pyogenes*. *S. pyogenes* lacks the NadA and NadB enzymes in the *de novo* NAD biosynthesis pathway (shown in the dotted rectangle). This organism also lacks the nicotinamide phosphoribosyl transferase (NaPRT/PncB). However, in the *de novo* pathway, the presence of the *nadC* gene that encodes quinolinate phosphoribosyltransferase may help *S. pyogenes* to utilize quinolinate (quinolinic acid) present in the microenvironment. The accumulation of quinolinate occurs in certain pathological conditions in humans as a byproduct of tryptophan degradation. The Spy_xxxx assignments are based on the strain type M1 *S. pyogenes* MGAS5005 genome.

fully virulent in a model of muscle infection in zebrafish that is notable for its lack of inflammation, they are attenuated in murine models of soft tissue and systemic infection that feature inflammation (Brenot, King, Janowiak, Griffith, & Caparon, 2004).

Because *S. pyogenes* cannot synthesize heme, it lacks cytochromes that are important for respiration in other bacteria, but can consume oxygen to enhance its aerobic growth. It also lacks catalase, a heme-containing peroxidase that plays an important role in resistance to H_2O_2 stress in many other bacterial species. However, in addition to GpoA, *S. pyogenes* contains several additional non-heme peroxidase and oxidase enzymes that contribute to its ability to resist oxidative stress and for the utilization of oxygen for growth under aerobic conditions. Two of these enzymes are NADH-consuming oxidases, Nox-1 (also known as AhpF, Spy_1769) that converts O_2 to H_2O_2 and the flavoprotein NOXase (also known as Nox-2, Spy_0872), which uses NADH to transfer 4 electrons to reduce O_2 directly to H_2O (Figure 11). Nox-1 along with AhpC (Spy_1768) are the two subunits that form alkyl hydroperoxide reductase, which uses the Nox-1 generated H_2O_2 as an electron acceptor for the AphC- catalyzed reduction of organic peroxides. AhpC mutants become sensitive to organic peroxide

stress, but also normally grow under aerobic conditions and are attenuated for virulence in the murine soft-tissue infection model (Brenot, King, & Caparon, 2005). In contrast, NOXase mutants of *S. pyogenes* grow poorly aerobically, but normally grow under anaerobic conditions (Gibson, Mallett, Claiborne, & Caparon, 2000). Under aerobic conditions, NOXase consumes oxygen and regenerates NAD^+ from the pool of NADH produced by the EMP pathway of glycolysis, and thus is likely contributing to aerobic growth under glucose-limited conditions by re-routing pyruvate from NAD^+ regeneration into other energy-generating and anabolic pathways. However, the restricted aerobic growth of NOXase mutants comes from their over-production of H_2O_2 to auto-intoxicating concentrations, which can be reversed by a complementation with a heterologous NADH peroxidase (NPXase) from *Enterococcus faecalis* (Gibson, Mallett, Claiborne, & Caparon, 2000). Interestingly, the *S. pyogenes* genome contains a gene encoding a putative NPXase (Spy_1378) that has not yet been characterized, but that may play a role in the inactivation of H_2O_2 generated as a byproduct of the SodA-catalyzed dismutation of superoxide. An additional H_2O_2-generating enzyme in *S. pyogenes* is lactate oxidase (Spy_0340/LctO) (Kietzman & Caparon, 2010; Seki, Iida, Saito, Nakayama, & Yoshida, 2004), which converts lactate into pyruvate using NADH to reduce O_2 to H_2O_2. Analysis of corresponding mutants has shown that LctO is responsible for the ability of *S. pyogenes* to produce auto-toxic concentrations of H_2O_2 during aerobic growth (Kietzman & Caparon, 2010; Watson, Nielsen, Hultgren, & Caparon, 2013). Since LctO can recover pyruvate and regenerate NAD^+, it likely contributes to growth under aerobic conditions when glucose is limiting, albeit at the expense of producing toxic H_2O_2. Not surprisingly, the transcription of *lctO* is tightly coupled to glucose concentrations by CcpA (Kietzman & Caparon, 2010; Kietzman & Caparon, 2011). Although H_2O_2 can be a potent toxin that targets both host cells and other bacteria, the contribution of LctO to pathogenesis is less clear, as only a subset of *S. pyogenes* strains is peroxogenic (Seki, Iida, Saito, Nakayama, & Yoshida, 2004) (Figure 11).

The cysteine and methionine residues of proteins are the most susceptible targets for oxidation by ROS, and *S. pyogenes* contains several proteins that protect against this type of damage. The oxidation of Met residues in proteins generates methionine sulfoxide (MetSo), which can induce conformational changes that lead to either the activation or inactivation of proteins (Drazic, et al., 2013; Drazic & Winter, 2014; Zeller & Klug, 2006). Although, the key extracellular reducing system constitutes a thioredoxin-like lipoprotein (Etrx1), its paralog Etrx2 and their redox partners Ccd1, Ccd2, and methionine sulfoxide reductase (MsrAB2) have recently been shown to be key players in pneumococcal pathogenesis and oxidative stress resistance (Saleh, et al., 2013). However, unlike the genome of *S. pneumoniae* D39, in which *etrx1* and *etrx2* are found in two separate operons (*spd_0570-0576* and *spd_0884-0889)*, the homologous proteins in *S. pyogenes* are encoded by a single operon (*spy_1282/msrAB*, *spy_1283*/*tlpA*/*etrx*, and *spy_1284* /*ccdA*/cytochrome C biogenesis protein), and are likely to function in a similar manner. However, the importance of this redox system in *S. pyogenes* is presently unknown.

The defenses of *S. pyogenes* against reactive oxygen species (ROS) are also partially coordinated by the peroxide stress response transcriptional regulator PerR (Spy_0161) (Brenot, King, & Caparon, 2005; King, Horenstein, & Caparon, 2000; Wen, et al., 2011). Under unstressed conditions, PerR, as a member of the metal-binding Fur family of DNA-binding proteins, binds zinc and functions to repress several genes that are involved in ROS resistance and membrane-associated metal transporter genes (Brenot, King, & Caparon, 2005; King, Horenstein, & Caparon, 2000; Wen, et al., 2011). PerR represses the expression of the non-membrane metal-binding protein, MrgA (also known as Dps or Dpr, Spy_1259). MrgA is a ferritin-like protein that contributes to ROS resistance by binding and sequestering excess free iron, and by binding to DNA to protect it from highly reactive hydroxyl radicals generated by iron via the Fenton reaction ($H_2O_2 + Fe^{2+} \rightarrow OH^{\bullet} + OH^- + Fe^{3+}$) (Stadtman & Berlett, 1991). MrgA mutants are hypersensitive to H_2O_2 stress, but are not attenuated in the murine soft tissue infection model (Brenot, King, & Caparon, 2005). Interestingly, derepression of the PerR regulon in a PerR mutant results in hyper-resistance to H_2O_2 stress *in vitro*; however, the mutants are highly attenuated *in vivo* (Brenot, King, & Caparon, 2005; Janulczyk, Ricci, & Björck, 2003; King, Horenstein, & Caparon, 2000), likely due to altered metal homeostasis resulting from dysregulation of metal transport (Brenot, Weston, & Caparon, 2007; Janulczyk, Ricci, & Björck, 2003). Metal transport will be described in detail in the latter section of this chapter.

In addition to enzymatic detoxification of ROS-mediated toxic effects, *S. pyogenes* also resists oxidative stress in a non-enzymatic fashion by orchestrating a repertoire of proteins and polysaccharides to counteract ROS produced by host cells. Those proteins include the M protein, hyaluronan capsule, and Mac-1 like proteins. Wild-type *S. pyogenes* strains that produce M or M-like proteins, in comparison to isogenic mutants lacking M proteins, cause an increased oxidative burst that is characterized by increased production of H_2O_2 upon their internalization by human neutrophils by inhibiting the fusion of azurophilic granules with phagosomes (Staali, Bauer, Mörgelin, Björck, & Tapper, 2006; Staali, Mörgelin, Björck, & Tapper, 2003). The M protein is also directly involved in activating neutrophils by triggering the release of heparin-binding protein and heme-dependent myeloperoxidase, a member of the XPO subfamily of peroxidases that produces hypochlorous acid (HOCl) from H_2O_2 and chloride anion (Cl^-) or another halide during the neutrophil's respiratory burst (Herwald, et al., 2004; Macheboeuf, et al., 2011; Soehnlein, et al., 2008). Hyaluronan capsule bearing *S. pyogenes* grow in aggregates, which take up oxygen at a slower rate and thus, resist H_2O_2 and other oxygen metabolites (Cleary & Larkin, 1979). Similar to this thwarting effect, *S. pyogenes* Mac-1-like proteins also seem to inhibit ROS production *ex vivo* (Lei, et al., 2001; Persson, Söderberg, Vindebro, Johansson, & von Pawel-Rammingen, 2015; Söderberg, Engström, & von Pawel-Rammingen, 2008; Söderberg & von Pawel-Rammingen, 2008; Voyich, et al., 2003). Although these surface molecules directly or indirectly participate in the production and inhibition of ROS, the underlying defined mechanism of their actions is presently unknown. It is likely that the observed effects are the outcomes of the down-stream effects of altered interactions of *S. pyogenes* with human innate immune cells.

Nucleotide Biosynthesis

The metabolic requirement for nucleotides is met by two classes of nitrogenous compounds: purines (i.e., adenine, guanine, hypoxanthine, and xanthine) and pyrimidines (i.e., cytosine, thymine, and uracil). Purines are heterocyclic organic compounds composed of a pyrimidine ring fused to an imidazole ring. Pyrimidines are aromatic six-membered heterocyclic organic compounds with two nitrogen atoms in the pyrimidine ring at positions 1 and 3. These compounds occur in the cell as nucleic acids (i.e., AMP, ADP, and ATP) and nucleotide-containing co-enzymes. The *S. pyogenes* genome contains the *pur* operon and the *pyr* genes, which encode the enzymes involved in purine and pyrimidine biosynthesis, respectively. However, the pathways for both purine and pyrimidine biosynthesis for the formation of RNA and DNA are not characterized in *S. pyogenes.*

Purine biosynthesis

In *S. pyogenes,* the *pur* operon is constituted by 11 genes (*spy_022–spy_0027*, and *spy_0029–Spy_0033*) that encode the enzymes involved in purine biosynthesis. This operon is interrupted by *Spy_0028* (KEGG, 2015), which encodes a putative autolysin (Figure 12), the functional significance of which is presently unknown. The synthesis of purine nucleotides begins with 5-phosphor-ribosyl 1-pyrophosphate (PRPP) and leads to the first fully formed nucleotide: inosine 5'-monophosphate (IMP). PRPP is synthesized from ribose-1-p or ADP-ribose and ribose-5 phosphate by phospho-pento(glucose)mutase (Spy_0696/DeoB or Spy_0938/PgmA) and ribose-phosphate pyrophosphokinase (Spy_0018/PrsA.2 and/or Spy_0845/PrsA.1) in the presence of ATP. The synthesis of IMP requires five moles of ATP, two moles of glutamine, one mole of glycine, one mole of CO_2, one mole of aspartate, and two moles of formate. PRPP is then converted to β-5 phosphoribosylamine (PRA) by amido phosphoribosyl transferase (Spy_0024/PurF) in the presence of glutamine. In the subsequent series of enzymatic reactions, PRA is converted to glycinamide ribotide (GAR), formyl-GAR (FGAR), formyl glycinamidine ribotide (FGAM), and 5-aminoimidazole ribotide (AIR) via PRA-glycine ligase (Spy_0029/PurD). PRglycinamide formyl transferase (Spy_0026/PurN), PRFG synthetase (Spy_0023), and PRaminoimidazole synthetase (Spy0025/PurM). The two subsequent reactions are catalyzed by PRAI carboxylase-ATPase (Spy_0031/PurK), PRAI-carboxylase (Spy_0030/PurE) and PRAI-succinocarboxamide synthase (Spy_0022/PurC) and convert AIR into 5-AI-4N-succinylcarboxamide-R (SAICAR) using one molecule each of ATP and aspartate. In the last three reactions, SAICAR is sequentially converted to 5-AI-carboxamide-R (AICAR), 5-forma-AICAR (FAICAR), and

Fig. 11

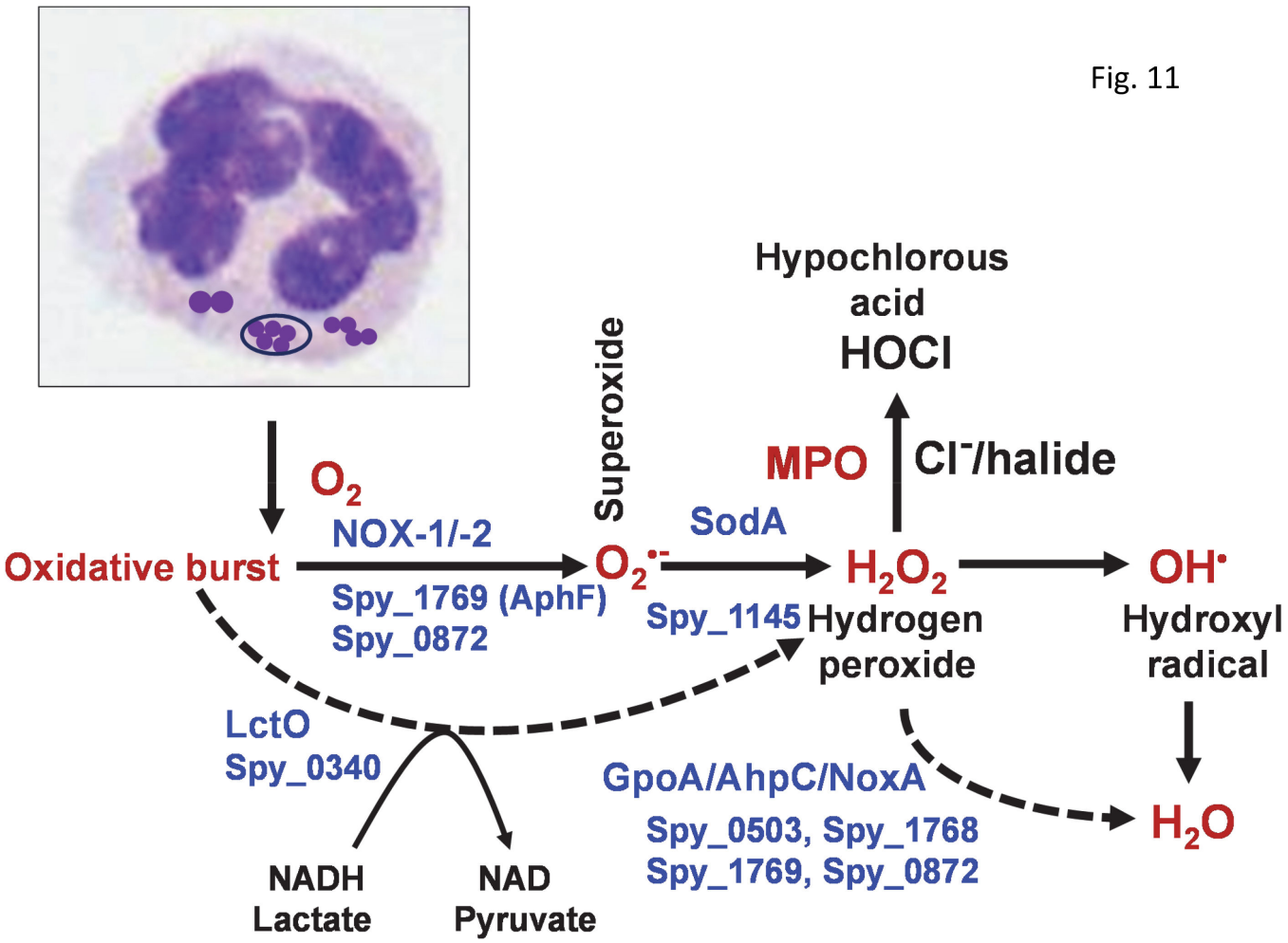


Figure 11: Aerobic metabolism of *S. pyogenes* and its role in thwarting deleterious effects of reactive oxygen intermediates (shown in red) generated by enzymes in human neutrophils during infection and phagocytosis. The schematic diagram shows the conversion of oxygen (O_2) to superoxide ion ($O_2^{\bullet-}$) following the activation of NOX in neutrophils. Superoxide is converted to H_2O_2 by the *S. pyogenes* SodA. Superoxide and hydrogen peroxide are converted to hydroxyl radicals ($OH^{\bullet}$) during the Fenton reaction. *S. pyogenes* iron chelators maintain iron homeostasis and thwart the toxic/bactericidal effect of hydroxyl radicals. During the oxidative burst, the neutrophils' enzyme, MPO, also catalyzes the production of hypochlorous acid (HOCl) from H_2O_2 in the presence of chloride (Cl^-) ion. Potential bactericidal activity of the H_2O_2-derived HOCl is prevented by detoxifying H_2O_2 by a number of enzymes as indicated. The Spy_xxxx assignments are based on the strain type M1 *S. pyogenes* MGAS5005 genome.

finally to IMP by adenylosuccinate lyase (Spy_0033/PurB) and PRAICF-transferase/IMPcyclohydrolase (Spy_0027/PurH), using one molecule of tetrahydrofolate (N-formyl THF). IMP serves as a branch point for purine biosynthesis because it can be converted to either AMP via adenylosuccinate [by PurA (Spy_0136) and PurB (Spy_0033)], or GMP via xanthine monophosphate [by GuaB (Spy_1857) and GuaA (Spy_0919)]. The AMP and GMP pathways require energy in the form of GTP and NAD/ATP, respectively. The down-regulation of the Pur operon was observed as one of the important implications of the deletion of serine/threonine kinase in *S. agalactiae* (Rajagopal, Vo, Silvestroni, & Rubens, 2005) and *S. pyogenes* (Pancholi, 2013) and is associated with the attenuation of bacterial virulence, possibly through effects on RNA synthesis. However, the mechanism underlying this attenuation is currently unknown.

Fig.12

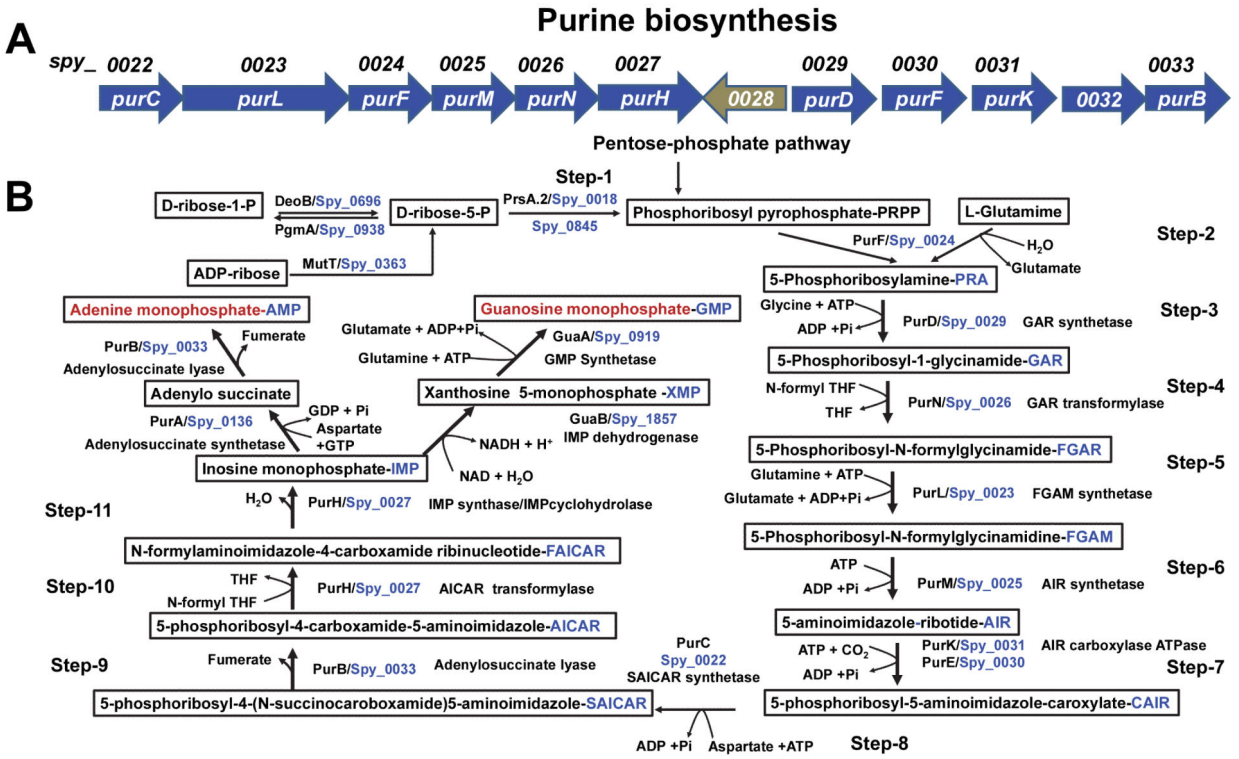


Figure 12: Schematic representation of the purine biosynthesis pathway in *S. pyogenes*. (A). The genomic organization of the 12-gene "*pur*" operon (*M5005_Spy_0022-0033*). 11 genes are involved in the purine biosynthesis. The role of *Spy_0028* is presently unknown. (B). The flow chart shows the steps in the biosynthesis of inosine monophosphate (IMP) and AMP or GMP using phosphoribosyl pyrophosphate (derived from the pentose phosphate pathway) and L-glutamine as starting substrates. The synthesis of IMP requires five moles of ATP, two moles of glutamine, one mole of glycine, one mole of CO_2, one mole of aspartate and two moles of formate, and is completed in 11 steps.

Step 1: PRPP synthesis from ribose-5-phosphate and ATP by ribose-5-phosphate pyrophosphokinase.

Step 2: 5-Phosphoribosyl-β-1-amine synthesis from α-PRPP, glutamine, and H_2O by glutamine phosphoribosylpyrophosphate amidotransferase.

Step 3: Glycinamide ribonucleotide (GAR) synthesis from glycine, ATP, and 5-phosphoribosyl-β-amine by glycinamide ribonucleotide synthetase.

Step 4: Formylglycinamide ribonucleotide synthesis from N10-formyl-THF and GAR by GAR transformylase.

Step 5: Formylglycinamidine ribonucleotide (FGAM) synthesis from FGAR, ATP, glutamine, and H_2O by FGAM synthetase (FGAR amidotransferase). The other products are ADP, Pi, and glutamate.

Step 6: 5-Aminoimidazole ribonucleotide (AIR) synthesis is achieved via the ATP-dependent closure of the imidazole ring, as catalyzed by FGAM cyclase (AIR synthetase).

Step 7: Carboxy aminoimidazole ribonucleotide (CAIR) synthesis occurs through two steps that involve CO_2, ATP, and AIR, and are catalyzed by the AIR carboxylase catalytic ATPase subunit-1 (PurK) and subunit-2 (PurE).

Step 8: N-succinylo-5-aminoimidazole-4-carboxamide ribonucleotide (SAICAR) synthesis from aspartate, CAIR, and ATP by SAICAR synthetase.

Step 9: 5-Aminoimidazole carboxamide ribonucleotide (AICAR) formation via the nonhydrolytic removal of a fumarate from SAICAR by adenylosuccinase.

Step 10: 5-Formylaminoimidazole carboxamide ribonucleotide (FAICAR) formation from AICAR and N10-formyl-THF by AICAR trans-formylase.

Step 11: the dehydration process then yields the authentic purine ribonucleotide IMP. The synthesis of AMP from IMP involves two reactions that are catalyzed by adenylosuccinate synthetase and adenylosuccinate lyase in the presence of aspartate and GTP. The formation of GMP from IMP occurs via xanthine monophosphate (XMP) and involves NAD, ATP and glutamine and the enzymes IMP dehydrogenase and GMP synthetase. The Spy_xxxx assignments are based on the strain type M1 *S. pyogenes* MGAS5005 genome.

Pyrimidine biosynthesis

Compared to purine biosynthesis, pyrimidine (i.e., cytosine, thymine, and uracil) biosynthesis is less complex due to the simple one heterocyclic ring structure of the pyrimidines (Figure 13). The basic pyrimidine biosynthesis pathway, which forms uridine monophosphate (UMP), is derived from 1 mole each of glutamine, ATP, CO_2, and aspartate. The first important precursor, carbamoyl phosphate, is formed by the heterodimeric enzyme carbamoyl phosphate synthetase (Spy_0642/CarA and Spy_0643/CarB) in the presence of ATP, glutamine and CO_2 (i.e., bicarbonate from the cytosol). The carbamoyl phosphate is converted to carbamoyl aspartate (CA) via a reaction that is catalyzed by aspartate transcarbamylase (ATcase, Spy_0641/PyrB) and a condensation with aspartate. CA is then dehydrated to dihydroorotate by CA-hydratase (Spy_709/PyrC). In the subsequent NAD-dependent dihydroorotate dehydrogenase reaction (Spy_1165/PyrD), the dihydroorotase is converted to orotic acid. The orotate phosphoribosyl transferase (Spy_0704/PyrE)-catalyzed enzymatic reaction requires a cytoplasmic source of phosphoribosylpyrophosphate (PRPP) to convert the orotic acid to orotidine monophosphate, which is then converted to UMP via a carboxylation reaction (orotidine-5-phosphate carboxylase, Spy_0703/PurF). The UMP is then doubly phosphorylated via UDP by some enzymes (Spy_0240, Spy_379/uridylate kinase, and/or Spy_0670/774/775 nucleotide/nucleoside diphosphate kinase) in the presence of ATP to form UTP (Figure 12). An additional one mole, each of glutamine and ATP, are required for the conversion of UTP to CTP by CTP synthase (Spy_1609-1610/PyrG). RNA synthesis from CTP and UTP is catalyzed by the RNA polymerase complex (Spy_0070/RpoA, Spy_0083/RpoB, Spy0084/RpoC, Spy_1340/ ΩRNAP, and Spy_1611/RpoE). Polynucleotide phosphorylase (Spy_1660) catalyzes a similar synthesis from CDP and UDP.

Ion transport

Trace metals, including iron, zinc, manganese, nickel, and copper participate in many structural and enzymatic functions in both eukaryotes and prokaryotes, including *S. pyogenes.* Thus, these metals influence physiology, metabolism, and pathogenesis. While the presence of these ions serves as an indispensable nutrient source for bacteria, excess amounts of these ions result in deleterious and even toxic effects, which make metal homeostasis essential to metabolism. The concentrations of these metals vary dynamically in the host environment. Thus as a successful pathogen, *S. pyogenes* is required to control the transport and utilization of these metals in a constantly changing host environment. *S. pyogenes* is endowed with a variety of mechanisms to tolerate and utilize available trace metals. The following discussion covers the established and putative ion transport and trace metal homeostasis mechanisms available for *S. pyogenes*, and is summarized in Figure 14.

Iron and Manganese

Similar to the *S. aureus* iron-regulated surface determinant (*isd*) system (Mazmanian, et al., 2003), *S. pyogenes* also acquires iron in the form of heme through a streptococcal iron acquisition relay system (i.e., the Sia system) that is encoded by a ten-gene operon (*Spy_1521-1530*) that includes the genes encoding the HtsABC ATP-binding cassette (ABC) type transporter (Bates, Montañez, Woods, Vincent, & Eichenbaum, 2003; Lei, et al., 2003; Ouattara, et al., 2010). In general, the protein components of these systems bind human hemoproteins, remove the heme molecule, and transport heme through the cell wall and plasma membrane for accumulation in the bacterial cytoplasm. Once inside the cell, the porphyrin ring of heme is degraded by the heme-degrading luciferase-like monooxygenase enzyme (Spy_0932), which leads to the formation of free iron for use by the bacterium as a nutrient source. Recently, the Shr protein of the Sia system (Spy_1530) was demonstrated to achieve heme binding through its unique N-terminal domain (NTD) and two distinct heme-binding near-iron transporters (NEAT) motifs (Ouattara, et al., 2013). In the Fe-excess environment (high heme/methemoglobin-metHb), the heme scavenged by the NEAT1 domain is rapidly transferred to the Shp protein (Spy_1529) for delivery into the SiaABC transporter (Spy_1528, Spy_1527, and Spy_1526). The heme acquired by the NEAT-2

Fig. 13

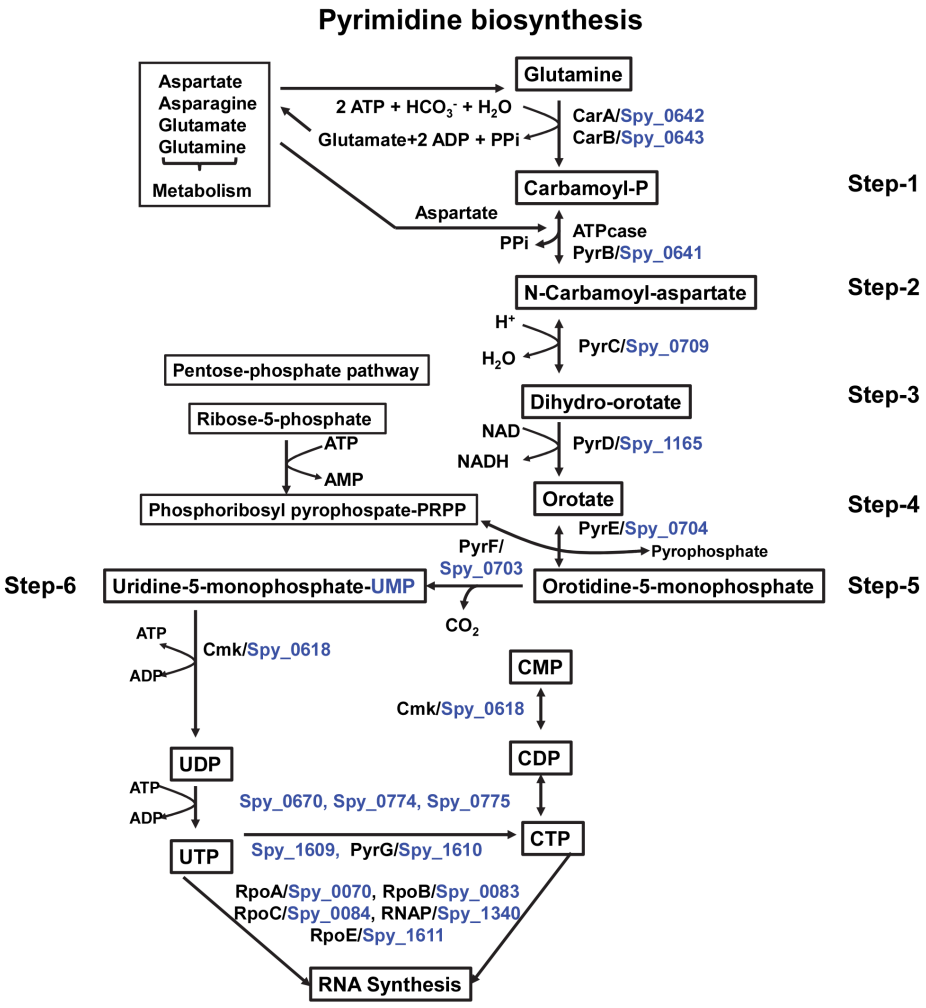


Figure 13: A schematic diagram of the pyrimidine biosynthesis pathway in *S. pyogenes*. This pathway involves six steps.
Step 1: Carbamoyl-P synthesis.
Step 2: The condensation of carbamoyl phosphate and aspartate to yield carbamoyl aspartate is catalyzed by aspartate transcarbamoylase or ATPcase.
Step 3: The intramolecular condensation is catalyzed by dihydroorotase, which results in the production of dihydroorotate (DHO).
Step 4: The oxidation of DHO by dihydroorotate dehydrogenase generates orotate in an NAD-dependent manner.
Step 5: PRPP provides the ribose-5-P moiety that converts orotate into orotidine 5'-monophosphate, which is a pyrimidine nucleotide.
Step 6: The decarboxylation of OMP by OMP decarboxylase yields UMP. The UMP then is converted into UDP and UTP in the presence of ATP. UTP is similarly converted to CTP. UTP and CTP are then utilized for RNA synthesis. The Spy_xxxx assignments are based on the strain type M1 *S. pyogenes* MGAS5005 genome.

domain of Shr (Spy_1530) is stored, transferred back to NEAT1 when the concentration of heme is limiting. The replenished source of heme on the NEAT1 domain is then transferred to Shp (Spy_1529). The remaining genes in this operon appear to be involved in cobalt ion transport, although no report is available to substantiate this predicted function (Figure 14). As described before, the putative cobalt-transporting gene may have a role in the transport of biotin required for lipid biosynthesis (Figure 9, Step I).

In addition to HtsABC, *S. pyogenes* also possesses genes that encode two additional ABC type metal transporters: MtsABC (Spy_0368, Spy_0369, and Spy_0370) and FtsABCD (Spy_0321/FhuG, Spy_0322/FhuB, Spy_0323/FhuD, and Spy_0324/FhuA) (Bates, Toukoki, Neely, & Eichenbaum, 2005; Hanks T. S., Liu, McClure, Fukumura, Duffy, & Lei, 2006; Hanks, Liu, McClure, & Lei, 2005). These transporters are responsible for the

uptake of Fe^{3+} and Mn^{2+} (Janulczyk, Ricci, & Björck, 2003) and ferric ferrichrome (Hanks, Liu, McClure, Fukumura, Duffy, & Lei, 2006), respectively. Studies are in progress to uncover the complexities and interactions of the homeostatic regulatory networks that balance expression of the transporters with the availability of certain metals. For example, MtsR (Spy_0367/scaR) is a Dtx family transcription repressor that itself is a metalloprotein that plays a key role in the regulatory network (Bates, Toukoki, Neely, & Eichenbaum, 2005; King, Horenstein, & Caparon, 2000). MtsR binds to DNA in the presence of both Fe^{3+} and Mn^{2+}, but does not regulate the expression of *ftsABCD*. In contrast, MtsR regulates both *mtsABC* and *htsABC*, but does so according to distinct patterns, as both Fe^{3+} and Mn^{2+} regulate *mtsABC*, but only Fe^{3+} regulates *htsABC* (King, Horenstein, & Caparon, 2000). The molecular basis of this discrimination and its functional significance has yet to be revealed, as, unlike PerR mutants, MtsR mutants are not attenuated in the murine soft tissue infection model (King, Horenstein, & Caparon, 2000).

Zinc

Zinc is the second most abundant transition metal found in human tissues, and like Fe^{2+}, it serves as a structural element or cofactor for several surface proteins, enzymes, and regulatory proteins. Similar to other metals, *S. pyogenes* precisely calibrates the expression of genes encoding proteins that are responsible for Zn^{2+} export and import to preserve homeostasis and to avoid any excess Zn^{2+} concentration-related cytotoxicity.. An interesting feature of the *S. pyogenes* Zn^{2+} regulatory network is that its genome lacks Zur, a transcriptional regulator that plays an important role in the regulation of Zn^{2+} homeostasis in many other Gram-positive pathogens (Hantke, 2005). Instead, the peroxide-stress response regulator PerR (a homolog of Zur) likely serves in a dual capacity by possessing an additional function as the regulator of Zn^{2+} homeostasis (Brenot, Weston, & Caparon, 2007). PerR regulates the expression of PmtA (Spy_1167), a putative Zn^{2+} efflux transporter. It also indirectly regulates several additional genes that are involved in Zn^{2+} metabolism, presumably because de-repression of PmtA results in unregulated Zn^{2+} efflux leading to Zn^{2+} starvation. Among the indirectly regulated genes are two operons regulated by the MarR-family transcription repressor AdcR (Spy_0077) that encode components of an ABC-family membrane transporter for Zn^{2+} import (Spy_0078/AdcC, Spy_0079/AdcB) and two genes in an unlinked operon encoding Lsp (Spy_1711) and PhtD (Spy_1710). The former is a cluster 9 family lipoprotein and the latter a putative histidine-triad family protein, both of which have homology to known Zn^{2+}-binding membrane proteins. Interestingly, the Adc operon is incomplete, as it encodes the ATPase subunit (AdcC) and permease subunit (AdcB), but lacks a gene for a putative Zn^{2+}-binding subunit. Analysis of Lsp mutants indicates that this protein is required for growth under conditions of Zn^{2+} starvation, which raises the intriguing possibility that Lsp functions as the Zn^{2+}-binding component of the Adc transporter. Should PhtD have a similar function, it is possible that Lsp and PhtD act as interchangeable high and low-affinity solute-binding components to fine-tune adaptor affinity to environmental Zn^{2+} concentrations (Elsner, et al., 2002; Weston, Brenot, & Caparon, 2009). Lsp-deficient mutants are highly attenuated in the murine soft tissue infection model, which indicates that the PerR/Zn^{2+} homeostasis network is critical for *S. pyogenes* virulence (Elsner, et al., 2002; Weston, Brenot, & Caparon, 2009) (Figure 14).

Copper

Like most lactic acid bacteria, the *S. pyogenes* genome does not contain any known copper-requiring enzyme (Ridge, Zhang, & Gladyshev, 2008). However, the acid produced by its fermentative metabolism can cause the release of copper from host enzymes, which may accumulate to toxic levels. Excess copper can damage cells by a number of mechanisms, including the generation of ROS by participation in the Fenton reaction. The principle mechanism by which Gram-positive bacteria protect themselves from copper toxicity is by removing copper from the cytosol via a dedicated P-type ATPase via a dedicated efflux pump, encoded by the *cop* operon. The function of this operon has been thoroughly characterized in *Enterococcus hirae* (Magnani & Solioz, 2005). However, the *S. pyogenes* operon most closely resembles the three-gene–containing *cop* operon that has recently

been described for *Streptococcus mutans* (Singh, Senadheera, & Cvitkovich, 2014; Singh, Senadheera, Lévesque, & Cvitkovich, 2015). In addition to a transcriptional regulator, (Spy_1406/CopY), the P-type ATPase (Spy_1405CopA) and a copper chaperone (Spy_1404/CopZ), the *S. pyogenes* genome also contains a gene (*spy_0337*/*cutC*) for an additional protein that may participate in copper homeostasis. The regulation of such homeostasis in streptococci is expected to follow a variation of the paradigm that has been developed for the more complex import/efflux system of *E. hirae* (Magnani & Solioz, 2005; Singh, Senadheera, & Cvitkovich, 2014). In the latter, an excess of free copper is recognized by the CopZ chaperone, which then donates copper to both the CopA efflux ATPase and the CopY DNA-binding repressor. Donation of copper to CopY results in the replacement of a molecule of Zn^{2+} and an allosteric change that reduces the affinity of CopY for DNA, which leads to derepression of the *cop* operon promoter and transcription of the *cop* operon genes (Magnani & Solioz, 2005) (Figure 14). To date, neither the details of this paradigm nor the functional significance of this operon for copper homeostasis and/or virulence has been investigated in *S. pyogenes*. However, in *S. mutans*, *cop* deletion mutants become hypersensitive to copper toxicity and fail to form biofilms (Singh, Senadheera, Lévesque, & Cvitkovich, 2015).

Sodium and protons (H^+) transport

Genomic analysis of *S. pyogenes* revealed the presence of two operons containing genes that encode energy-generating, proton-translocating, heteromultimeric protein complexes. The V_1V_0 Na^+-ATPase (V-ATPase) and the F_1F_0-ATPase (F-ATPase/synthase) complexes are structurally related, but have distinct ATPase-complexes that can be found in bacterial cytoplasmic membranes. These complexes are often referred to as proton pumps, because they couple the passive flux of protons (H^+) or sodium ions via the ATPase channel to the synthesis and/or hydrolysis of ATP, depending on the direction of ion flow: "out" is coupled to hydrolysis, whereas "in" is coupled to synthesis. However, unlike eukaryotes, the primary function of these complexes in bacteria is to extrude ions at the expense of ATP.

F-ATPase

Studies of F-ATPases were initially undertaken to study oxidative phosphorylation in mitochondria and photosynthesis in chloroplasts. These studies identified the F-ATP synthase in *E. coli*. Together, these studies demonstrated that the fundamental mechanism of biological energy transformation is similar among all organisms (Futai, Nakanishi-Matsui, Okamoto, Sekiya, & Nakamoto, 2012; Futai, Noumi, & Maeda, 1989; Muench, Trinick, & Harrison, 2011). The *S. pyogenes* genome contains an eight-gene operon (*Spy_0575 to Spy_0582*) that encodes proteins that are highly similar to F-ATPase proteins in *E. coli*, which allows the structure of the *S. pyogenes* F-ATPase to be predicted based on the structure described the *E. coli* complex (Futai, Nakanishi-Matsui, Okamoto, Sekiya, & Nakamoto, 2012) (Figure 14). The F_0 domain is integral in the membrane and is comprised of three proteins: the F_0-F_1 subunit C (65 aa), subunit A (238 aa), and subunit B (164 aa), with a composition of 12C:1A:2B. These proteins are encoded by *spy_0575/atpE*, *spy_0576/atpB*, and *spy_0577/atpF* genes respectively. The proteins of the F_1 domain are peripherally associated with the F_0 domain and are located on the cytoplasmic side of the membrane (Figure 15). The F_1 complex is formed by five polypeptides: α (502 aa), β (468 aa), γ (291 aa), δ (178 aa), and ε (138 aa), with a composition ratio of 3α:3β:1γ:1δ:1ε. These proteins are encoded by *spy_0579/atpA, spy_0581/atpD, spy_0580/atpG, spy-0578/atpH*, and *spy_0582/atpC* genes, respectively. During ATP synthesis, a transmembrane proton gradient is generated by the influx of protons across the membrane by the F-ATPase. The synthesis and hydrolysis of ATP are performed by the F_1 subunit (Figure 15), in which the three β subunits (Spy_581/AtpD) provide the active binding sites for the substrate, while the three α subunits (Spy_0579/AtpA) provide regulatory substrate binding sites. During the catalysis of ATP, the rotations of the central stalk (i.e., F_1-γ and F_1-ε; Spy_0580/AtpG and Spy_00582/AtpC) is counter-rotational to the H^+ gradient-driven movement of the F_0-C subunit (Spy_0575/AtpE). Thus, when the F_0-unit moves counter-clockwise, the F_1-unit moves clockwise to convert a chemical gradient into a mechanical

movement that can be harnessed for ATP synthesis. In contrast, the hydrolysis of ATP can drive the reverse movement of the subunits, which results in the expulsion of protons from the cell. This latter activity plays a critical role in correcting the pH imbalance imposed by acid stress, by pumping protons out of the cell against an H^+ gradient to prevent the cytoplasm from acidifying. Since the concentration of ATP can be a limiting factor under acid stress conditions, accessory metabolic pathways that can supplement the ATP pool can make important contributions to stress resistance. For example, citrulline enhances survival during acid stress by producing ATP via the arginine deiminase pathway (Cusumano & Caparon, 2015) (as described above). However, citrulline-mediated protection can be blocked by specific chemical inhibitors of the F_1F_0 ATPase, such as N, N'-dicyclohexylcarbodiimide (DCCD), which demonstrates that the ATP generated by citrulline catabolism is hydrolyzed for the expulsion of protons via the ATPase (Cusumano & Caparon, 2015).

V_1V_0 ATPase

The vacuolar ATPase or V-ATPase, which resembles the F-ATPase/F-ATP synthase, also functions as a proton pump. The structures and functions of these ATPases have been described in detail for *Enterococcus hirae* (Iwata, et al., 2004; Murata, Arechaga, Fearnley, Kakinuma, Yamato, & Walker, 2003; Murata, Yamato, Kakinuma, Leslie, & Walker, 2005b; Murata, et al., 2008; Murata, et al., 2002) and *Thermus thermophiles* (Maher, et al., 2009; Makyio, et al., 2005). The *E. hirae* V-ATPase transports Na^+, rather than H^+, under physiological conditions. Hence, this pump is also known as Na-ATPase. In *S. pyogenes*, the V-ATPase system is encoded by the Ntp operon, which consists of 7–8 genes (Figure 16). Like the F-ATPase, the V_1 domain of the V-ATPase is a peripheral complex that is responsible for ATP hydrolysis. The V_1 domain complex consists of six proteins: NtpA (Spy_0128, 591 aa), NtpB (Spy_0132, 471 aa), NtpC (Spy_0129, 332 aa), NtpD (Spy_0133, 208 aa), NtpE (Spy_0128, 194 aa) and NtpF (Spy_0130, 106 aa). Except for the *S. pyogenes* type M1 strain SF370, all other *S. pyogenes* genomes sequenced to date contain NtpF (Spy_0130). The physiological significance of the absence of *ntpF* in SF370 is currently unknown. The A and B subunits (Spy_0131 and Spy_0132) presumably participate in nucleotide binding with the catalytic site located in NtpA. Three copies of each subunit are presumably arranged around the central stalk, which is composed of a single copy of NtpD (Spy_0133). Based on studies of *E. hirae* V_1V_0 ATPase complex, the function of the central stalk is to rotate via a conformational change of the A subunit (Spy_0131) that is caused by the binding and hydrolysis of ATP. NtpE (Spy_0128) and NtpF (Spy_0130) may form a subcomplex that constitutes the peripheral stalk. The C subunit (Spy_0129) has no counterpart in F-ATPases and may connect the foot of the central stalk (Spy_0133/NtpD) to the membrane rotor ring of the V_0 complex.

The V_0 domain is a membrane-associated domain, and is responsible for Na^+ translocation across the membrane. Based on the *E. hirae* V_0 domain structure and the F-type AtpC protein (described in Figure13), the *S. pyogenes* V_0 domain also contains NtpK (Spy0127, 159 aa), which likely forms a ring with 10-fold symmetry and a single copy of NtpI (Spy_0126, 673 aa) (Figure 16). The composition of this system in *S. pyogenes* is simpler than that of *E. hirae*, as it lacks two additional components (namely, NtpG and NtpH). The function of this proton pump has been demonstrated to be similar to that of the F-type proton pump, although recent structural studies have demonstrated several variations in rotation patterns between the pumps of *E. hirae* and *T. thermophiles* (Maher, et al., 2009; Makyio, et al., 2005; Murata, Yamato, & Kakinuma, 2005a). While the predicted functions of individual components of the *S. pyogenes* F- and V-ATPases have not been investigated to date, global transcriptome analyses of several *S. pyogenes* mutants have revealed a differential expression of the corresponding genes, which implies that it plays an adaptive role.

Conclusion

The fact that *S. pyogenes* remains a successful pathogen, despite its susceptibility to most modern therapies, reflects its exquisite ability to adapt its metabolism to exploit a variety of adverse environments and host tissues. Since metabolism is intimately linked to virulence, it is a burgeoning question and a matter of important

Fig. 15

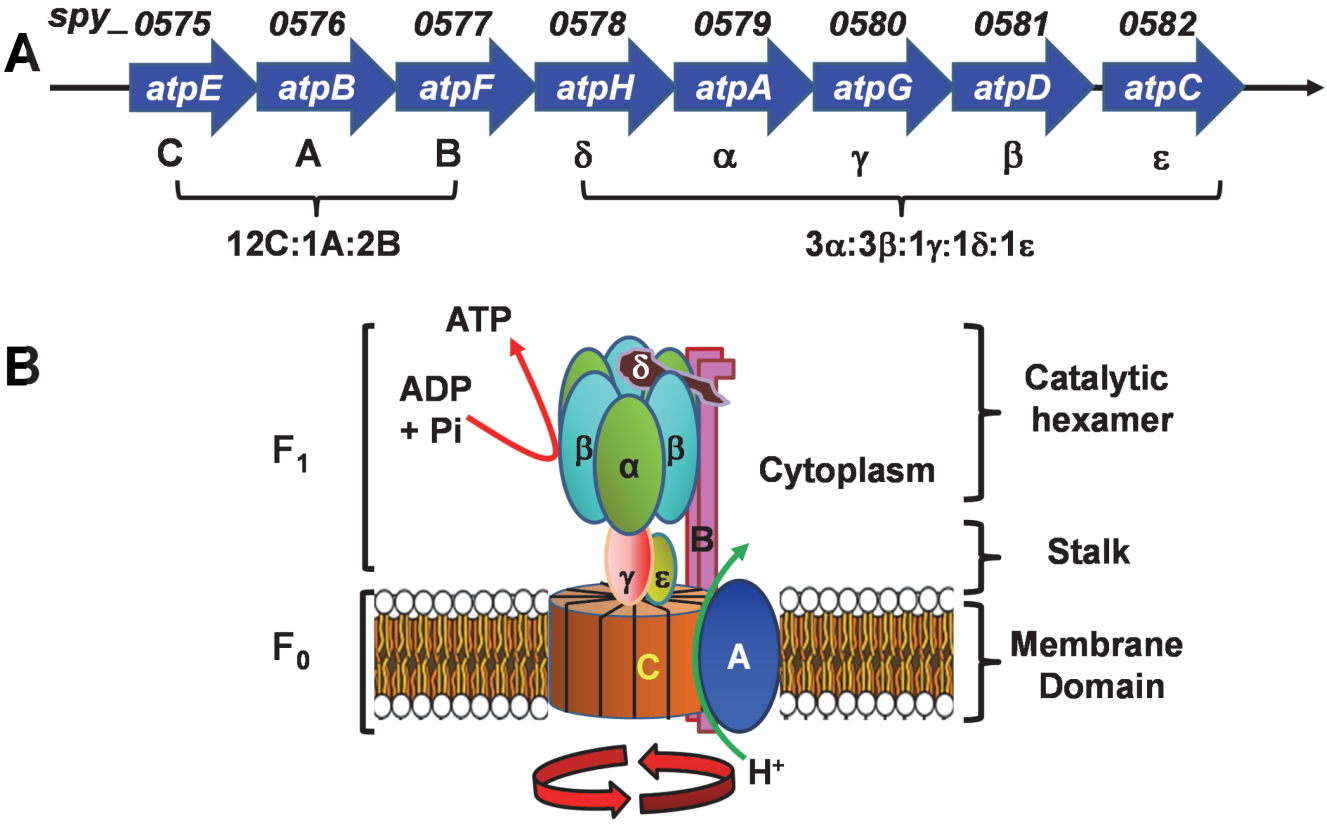


Figure 15: A schematic diagram of the predicted F_1-F_0 (F)-ATPase transport system in *S. pyogenes*. (A) The F-ATPase proton pump and its eight components are encoded by an operon constituted of eight genes: *M5005_SPy_0575-0582*. (B) A schematic diagram of the F_1-F_0 (F)-ATPase pump is based on the F-ATPase structure described for *Escherichia coli*. The F_0 components C and A in a composition of 12C:1A:2B are found associated with the cytoplasmic membrane. The F1 components are located peripherally and are composed of a catalytic hexamer (3α, 3β) and a stalk (1γ, 1ε) that are connected to the membrane-located F_0 proton motor C. The location of the small δ protein is predicted, based on the *E. coli* F_0-F_1 ATPase complex. The direction of the arrow indicates the anti-clockwise movement of the motor C, the energy for which is provided by the catalytic (α, β) hexamer. The Spy_xxxx assignments are based on the strain type M1 *S. pyogenes* MGAS5005 genome.

discussion whether a virulence-promoting alteration to its metabolism comes at too high a cost in fitness and is a non-adaptive side effect of traits required for superficial symptomatic infection (Wollein Waldetoft & Råberg, 2014). Cumulative evidence has provided unequivocal evidence that diverse metabolic activities enable *S. pyogenes* to survive successfully in the presence of a variety of stress conditions. The earlier interest in understanding the metabolism of *S. pyogenes* was driven by a need to investigate the biochemical basis for its growth requirements. The present chapter covers the knowledge obtained from subsequent reports of numerous investigators who used the established biochemical basis of metabolism to understand the underlying mechanisms of virulence as to how *S. pyogenes,* as a successful pathogen, senses its environment and changes its metabolic status to survive, persist, and proliferate in a broad range of host environments. It is also worth noting that early studies on *S. pyogenes* metabolism focused on various aspects of the pathogen's carbohydrate and amino acid metabolism. However, its lipid metabolism and membrane transport have received relatively limited attention, despite the fact that these processes play a crucial role in the secretion of many cellular products,

Fig. 16

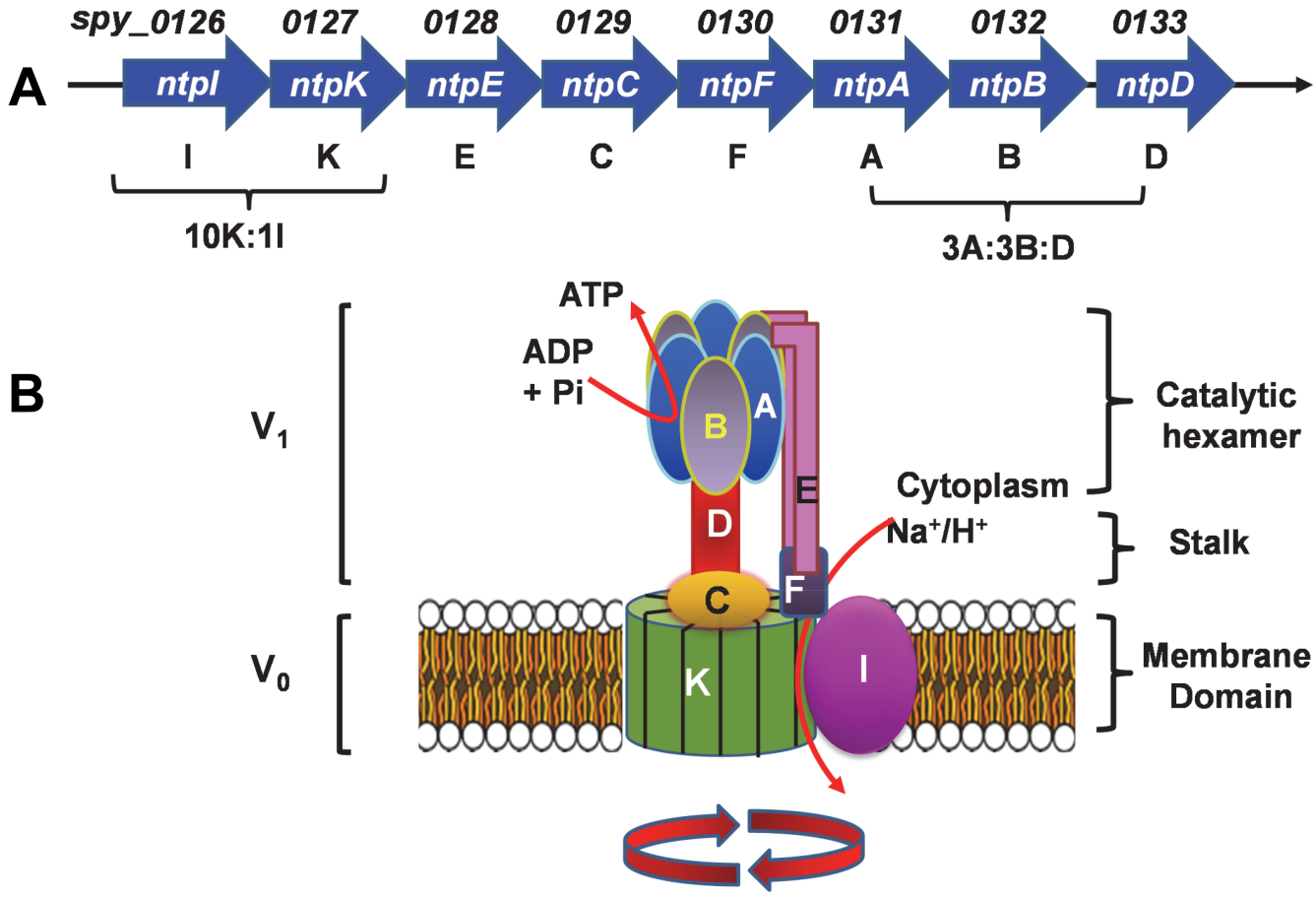


Figure 16: A schematic diagram of the predicted V_1-V_0 (V)-ATPase transport system in *S. pyogenes*. (A) The V_1V_0-ATPase complex is encoded by an operon that contains eight genes (*M5005_Spy_0126-0133*), and annotated as shown. (B) The diagram of the predicted structure of the V_1-V_0 ATPase is based on the *E. hirae* V_1-V_0 Na+/H+ ATPase complex. The basic architecture of this ATPase complex is similar to that of the F_1F_0-ATPase shown in Figure 15. The V_1 complex is located peripherally near the membrane and is composed of six protein components (NtpABCDEF). The 3A:3B:D hexameric complex hydrolyzes ATP. Components C and D together form a central stalk, and components E and F form the peripheral stalk and connect to the motor complex that is constituted of the K and I proteins, with a composition of 10K:1I. The Na^+/H^+ motor is predicted to rotate clockwise. The Spy_xxxx assignments are based on the strain type M1 *S. pyogenes* MGAS5005 genome.

including many virulence factors. As expected, genes that are often responsible for one metabolic function are arranged in tandem in the form of one operon. However, this paradigm does not apply to all metabolic functions. With few exceptions, when a defined structural complex is required to perform a metabolic function (such as proton transport or sugar transport), the responsible genes are typically arranged in one operon. It is not clear why the genes responsible for EMP pathways are found in tandem in some Gram-positive and negative bacteria such as *Staphylococcus aureus* and *E.coli,* but not in *S. pyogenes*. These two examples of metabolic functions, related to their corresponding genomic organization, suggest that metabolic pathways that are not dependent on a defined structure, even though their intermediary products are dependent on the preceding enzymatic products, are not necessarily organized in one operon. For the latter case, it is an open question whether the individual gene products form an arbitrary putative metabolon complex to conserve energy to carry out a cascade of reactions to achieve the final product in a timely fashion. Further research on such mechanistic aspects may help understand whether metabolic pathways are compartmentalized within the cytoplasm.

Another feature that this chapter highlights is the neofunctionalization of Lac.D1 activity for tagatose transport tagatose-6-phosphate metabolism. The presence of an enzymatically inactive/ nonessential *lac.1* operon and an functionally intact *lac.2* operon provides an example of selective advantage to *S. pyogenes* as a result of gene duplication. Since both LacD.1 and LacD.2 retain differential tagatose aldolase activity, despite their similar structures, but only LacD.1 possesses a unique regulatory function because of a truncated LacC.1 gene product, it is difficult to judge whether this is an example of subfunctionalization or neofunctionalization in the absence of similar paralogs. As compared to metazoans, examples of such neofunctionalization are relatively rare in prokaryotes. Although *S. pyogenes* possesses multiple copies of phosphoglycerate mutase (Spy_0497, Spy_1164, and Spy_1503), an important enzyme in the EMP pathway with no known other physiologically relevant significance, there is no report in *S. pyogenes* other than the duplicated *lac*-operon that highlights essential neofunctionalization as a process to provide a selective advantage in adapting to changing environments. It is likely that subfunctionalization may help preserve duplicated gene copies and serve as a transition state to neofunctionalization. Future studies on high-resolution analysis of genomes and SNPs observed in genomes of several strains isolated over long periods may resolve the relative contribution of this gene duplication-related neo/subfunctionalization of their products to the evolution of *S. pyogenes* virulence. It is also true that the functions of a substantial number of genes with defined open reading frames in the *S. pyogenes* have yet to be explored. Hence, future efforts towards high-resolution genome analysis may reveal other examples and allow us to fine-tune our understandings of the mechanisms of *S. pyogenes* pathogenesis.

High-throughput next-generation sequencing has greatly advanced our understanding of the impact of sequence variations on the global transcriptome at the individual-strain level; however, mRNA expression data and proteomic analyses do not tell the true story of what might be happening in a cell. In this regard, future streptococcal research endeavors in NMR and mass spectrometry-based metabolomics studies, which provide quantitative analyses of the dynamics of multiparametric metabolic profiles of living systems in response to pathophysiological stimuli or genetic modification, may give an instantaneous snapshot of the physiology or metabolic fingerprint of a bacterial cell of interest. However, one of the challenges of systems biology and functional genomics that may persist is the challenge of efficiently integrating proteomics, transcriptomics, and metabolomics information to provide a complete picture of living organisms.

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Streptococcus pyogenes Genomics

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Introduction

Outbreaks of epidemic infections by *Streptococcus pyogenes*, the group A streptococci, have been known for centuries to be a major cause of substantial human morbidity and mortality. Early researchers placed great emphasis on understanding the mechanisms of pathogenesis in order to prevent severe *S. pyogenes* diseases. The recent development of genome sequencing has facilitated bacterial research by providing significant information that could not be obtained from traditional research, which has often focused on phenotypic observations or manipulations of limited genes. As mentioned in a subsequent section of this chapter, genome-based studies have previously been carried out on *S. pyogenes*. These developments include understanding mechanisms of evolutional differentiation and prevailing gene content of individual strains that make up various epidemic lineages. This chapter highlights current approaches of comparative genomics in bacteriology, as well as major findings in *S. pyogenes* genomics. We review the main studies that have focused on a limited number of *S. pyogenes* lineages and describe the significance of molecular events in their evolution. In addition, future perspectives are discussed in relation to an overall understanding of *S. pyogenes* itself.

Comparative genomics in bacterial research

Comparative genomics is a growing field in bacterial genomic research because of the rapid appearance of many new publicly available genomes each year (Alföldi & Lindblad-Toh, 2013; Craddock, Harwood, Hallinan, & Wipat, 2008). Technical improvement in next-generation sequencing has facilitated extensive comparative genomic analyses in various bacterial species, as well as in non-bacterial organisms. Comparative genomics is also a powerful approach to understand the biological nature of bacteria, as it can provide information about which genes are present or absent and/or are modified in a particular bacterial genome. Applications of comparative genomics can be wide for bacterial populations, and can include geographic and time series comparisons, as well as phylogenetic delineation. For example, the emergence and spread of drug-resistant lineages was demonstrated in *Mycobacterium tuberculosis* through an extensive comparison of worldwide genomes (Galagan, 2014; Hershberg, et al., 2008). Pneumococcal strains were sequenced to demonstrate the significance of recombination on its evolution (Chewapreecha, et al., 2014), or to delineate phylogenetically close strains under high resolution (Harris, et al., 2010).

Genome sequences of *S. pyogenes* strains

Basic information of the *S. pyogenes* genome

The genome sequences of numerous *S. pyogenes* strains have been determined because of their clinical importance as human pathogens (Table 1). At the time of writing this article (April 1, 2015), the Genomes OnLine Database (Pagani, et al., 2012) recorded 23 complete genomes and 201 permanent draft genomes of *S. pyogenes*. From the selected 19 complete genomes, the basic information of an *S. pyogenes* genome is as follows:

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the genome is composed of a single chromosome that is approximately 1.8-Mbp long with a GC content of 38.5 ± 0.1% on average, and includes 1,826 ± 92 protein-coding regions (CDSs), 5 or 6 rRNA operons, and from 57 to 67 tRNA encoding genes. These values of genome size and GC content are usual for the phylum Firmicutes and are low in the Eubacterial domain (Bentley & Parkhill, 2004).

The core *S. pyogenes* genes based on strain SF370 were analyzed and mapped for metabolic pathways using KAAS (http://www.geno- me.jp/kaas-bin/; (Moriya, Itoh, Okuda, Yoshizawa, & Kanehisa, 2007), and were visualized using iPath v.2 (Yamada, Letunic, Okuda, Kanehisa, & Bork, 2011), as Figure 1 shows. In addition, the presence of known virulence genes was determined using BLASTP for all core genes against the virulence genes described in previous reports (Banks, Lei, & Musser, 2003; Sharma, 2010; Dashper, Seers, Tan, & Reynolds, 2011). A search for novel virulence gene candidates was accomplished using BLASTP against the Virulence Factor Database (VFDB; last accessed 25 November 2013; (Chen, Xiong, Sun, Yang, & Jin, 2012) and the microbial database of protein toxins, virulence factors and antibiotic resistance genes for biodefense applications (MvirDB; last accessed 21 March 2014; (Zhou, Smith, Lam, Zemla, Dyer, & Slezak, 2007). These results are shown in Supplemental Table 1.

Among the 1,343 core *S. pyogenes* genes in Supplemental Table 1, 243 and 543 genes were related to virulence, as determined from the VFDB and MvirDB database analyses, respectively. These observations indicate that many of the genes in a small genome, such as *S. pyogenes*, are involved in pathogenicity. Although Ferretti et al. (Ferretti, et al., 2001) showed only 34 putative virulence genes on the chromosome, the difference in the number of candidate genes is due to the difference of the threshold on the BLAST search (e-value < 1e-5) and the databases used, both of which were unavailable in 2001. Nevertheless, the results indicate that many more intrinsic genes related to virulence may exist in *S. pyogenes*. Although many virulence factors, including M-protein and part of the *mga* virulon, are thought to be found on a pathogenicity island, these factors are conserved among the 19 genomes sequenced and could be intrinsic and non-phage pathogenic islands acquired before *S. pyogenes* speciation (Panchaud, et al., 2009). As previously described (Chaussee, Somerville, Reitzer, & Musser, 2003; Ferretti, et al., 2001), some metabolic pathways present in the *S. pyogenes* genome include a complete glycolytic pathway, fatty acid synthesis, nucleotide synthesis and transport, and carbohydrate transport and metabolism. The absence of a complete tricarboxylic acid cycle pathway and its accompanying electron transport systems is consistent with its homofermentative metabolism and the facultative anaerobic environment in which this organism resides. Only a few amino acids are synthesized, in accordance with the fastidious growth requirements of the organism. These synthetic pathway deficiencies are offset by scavenging resources from the environment; that is, ABC transporters that have been identified as amino acid uptake systems in addition to the other transporter systems that might mediate the uptake of dipeptides and oligopeptides (Podbielski, et al., 1996; Podbielski & Leonard, 1998). The results are in accordance with the fact that *S. pyogenes* is a human-specific pathogen that relies on its host for both catabolic and anabolic substrates (Chaussee, Somerville, Reitzer, & Musser, 2003).

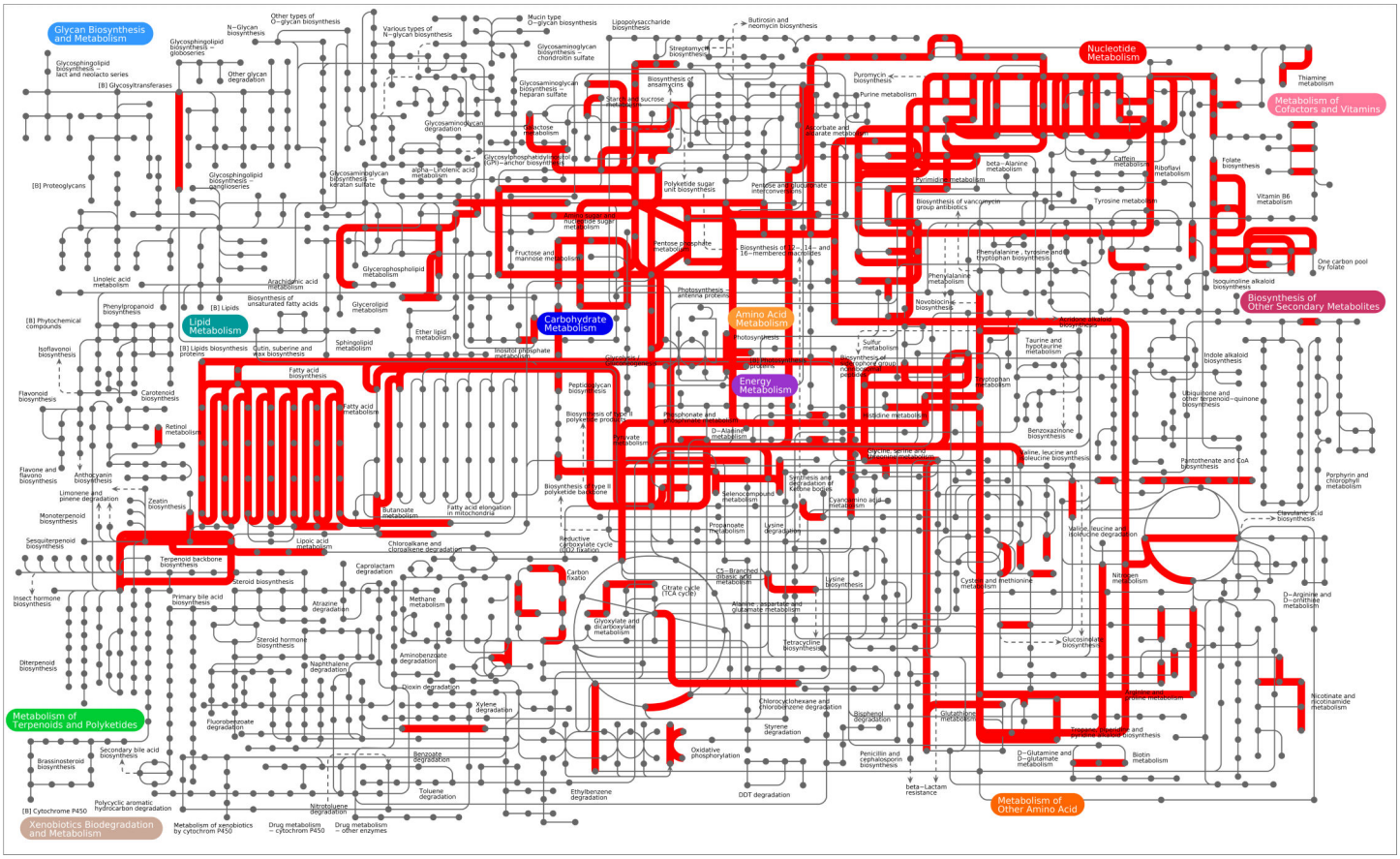

Figure 1. Conserved metabolic pathways in 19 *S. pyogenes* genomes.

Table 1. *S. pyogenes* strains with complete genome sequence

Strain	*emm* type	Genome size (bp)	GC content (%)	Number of CDS	Number of tRNA	Number of rRNA operon	NCBI accession no.	Reference
A20	1	1,837,281	38.5	1,808	67	6	NC_018936	(Zheng, et al., 2013)
M1-476	1	1,831,128	38.5	1,808	57	5	NC_020540	(Miyoshi-Akiyama, Watanabe, & Kirikae, 2012)
MGAS5005	1	1,838,554	38.5	1,811	67	6	NC_007297	(Sumby, et al., 2005)
SF370	1	1,852,441	38.5	1,811	60	6	NC_002737	(Ferretti, et al., 2001)
MGAS10270	2	1,928,252	38.4	1,913	67	6	NC_008022	(Beres, et al., 2006)
MGAS315	3	1,900,521	38.6	1,912	67	6	NC_004070	(Beres, et al., 2002)
SSI-1	3	1,894,275	38.6	1,917	57	5	NC_004606	(Nakagawa, et al., 2003)
STAB902	3	1892120	38.5	1925	59	5	NZ_CP007041.1	(Soriano, et al., 2014)
MGAS10750	4	1,937,111	38.3	1,908	67	6	NC_008024	(Beres, et al., 2006)
Manfredo	5	1,841,271	38.6	1,822	66	6	NC_009332	(Holden, et al., 2007)
MGAS10394	6	1,899,877	38.7	1,868	67	6	NC_006086	(Banks, et al., 2004)
MGAS2096	12	1,860,355	38.7	1,774	67	6	NC_008023	(Beres, et al., 2006)

Table 1. continued from previous page.

Strain	*emm* type	Genome size (bp)	GC content (%)	Number of CDS	Number of tRNA	Number of rRNA operon	NCBI accession no.	Reference
MGAS9429	12	1,836,467	38.5	1,782	67	6	NC_008021	(Beres, et al., 2006)
HSC5	14	1,818,351	38.5	1,785	67	6	NC_021807	(Port, Paluscio, & Caparon, 2013)
MGAS8232	18	1,895,017	38.5	1,908	67	6	NC_003485	(Beres, et al., 2002)
M23ND*	23	1,846,477	38.6	1,842	57	5	CP008695	(Bao, et al., 2014)
MGAS6180	28	1,897,573	38.4	1,864	67	6	NC_007296	(Green, et al., 2005)
STAB901	44	1795610	38.5	2029	67	6	CP007024.1	(Soriano, et al., 2014)
NZ131	49	1,815,785	38.6	1,769	66	6	NC_011375	(McShan, et al., 2008)
Alab49	53	1,827,308	38.6	1,791	67	6	NC_017596	(Bessen, et al., 2011)
MGAS1882	59	1,781,029	38.5	1,703	57	5	NC_017053	(Fittipaldi, et al., 2012)
MGAS15252	59	1,750,832	38.5	1,667	57	5	NC_017040	(Fittipaldi, et al., 2012)
ATCC19615	80	1844804	38.5	1788	67	6	NZ_CP008926.1	(Minogue, et al., 2014)
STAB1102	83	1709440	38.6	1624	66	6	NZ_CP007023.1	Unpublished
Mean ± SD		1,847,191 ± 55,447	38.5 ± 0.1	1,826 ± 92	64 ± 4	6 ± 0.4		

* The genomic information was not contained in the database until recently and not used for further analyses.

CDS homology and distribution among *S. pyogenes* strains

Beres and Musser (Beres & Musser, 2007) studied the variation in gene content among strains of *S. pyogenes* to examine the molecular processes that produce intraspecies genomic diversity and the contribution of specific genetic differences to host-pathogen interactions by comparative genomics. *S. pyogenes* is known to cause a wide variety of infections, and epidemiologic studies have clarified that certain types of M proteins are associated with particular human infections (Cunningham, 2000; Cunningham, 2008), which meant that a rich phenotypic and clinical framework was available for interpreting genome sequence information. In addition, the increase in number of reports describing the emergence of *S. pyogenes* strains resistant to antimicrobial agents from many countries required genome sequencing of additional strains (Wong & Yuen, 2012).

Comparative genomics is based on the analyses of both nucleic and amino acid information; thus, its methodology includes a variety of genetic analyses (Table 2). From the coding DNA sequence (CDS) of a number of strains in a particular bacterial species, the pan-genome size and the number of core genes can be estimated. The pan-genome size is expected to increase when the number of strains increases, while the number of core genes is expected to decrease. These two values were determined in the 19 complete *S. pyogenes* genomes by using GET_HOMOLOGUES following CDS clustering by a pan-genome analysis pipeline (PGAP). The PGAP was used with the default parameters to obtain CDS clusters based on protein homology by amino acid sequence similarity. Given that n is the estimated value and N is the number of strains, the estimated formulas are given as the following: $n = 1{,}807N^{0.1070}$ for pan-genome size, and $n = 1{,}713N^{-0.09209}$ for the number of core genes (Figure 2). The exponents in the former and the latter formula were between 0 and 1, and less than 1,

respectively, which indicates that *S. pyogenes* is an open pan-genome species (Tettelin, Riley, Cattuto, & Medini, 2008). This observation suggests that the acquisition of new genes is an additional factor that is responsible for *S. pyogenes* evolution.

The PGAP analysis identified both the core genes and the other accessory genes of the 19 *S. pyogenes* strains. The 34,621 genes in these 19 strains were clustered into 4,246 CDS clusters. Of these 4,246 clusters, 1,342 were core genes, including 33 multi-copy and 1,309 single-copy genes (Table 3, Table 4, Supplemental Table 2). There were a total of 2,129 strain-specific genes, with 112.1 ± 27.4 as an average ± standard deviation for a given strain (for details, see Supplemental Table 2). Thus, it was shown that, on average, each *S. pyogenes* strain had core genes and strain-specific genes as 73.7% and 6.1% of the total genes on the genome (1,342/1,822 and 112/1,822), respectively. Annotation information showed that at least 69.0% and 11.1% of the strain-specific genes encoded hypothetical and phage-related proteins (1,470/2,129 and 237/2,129), respectively.

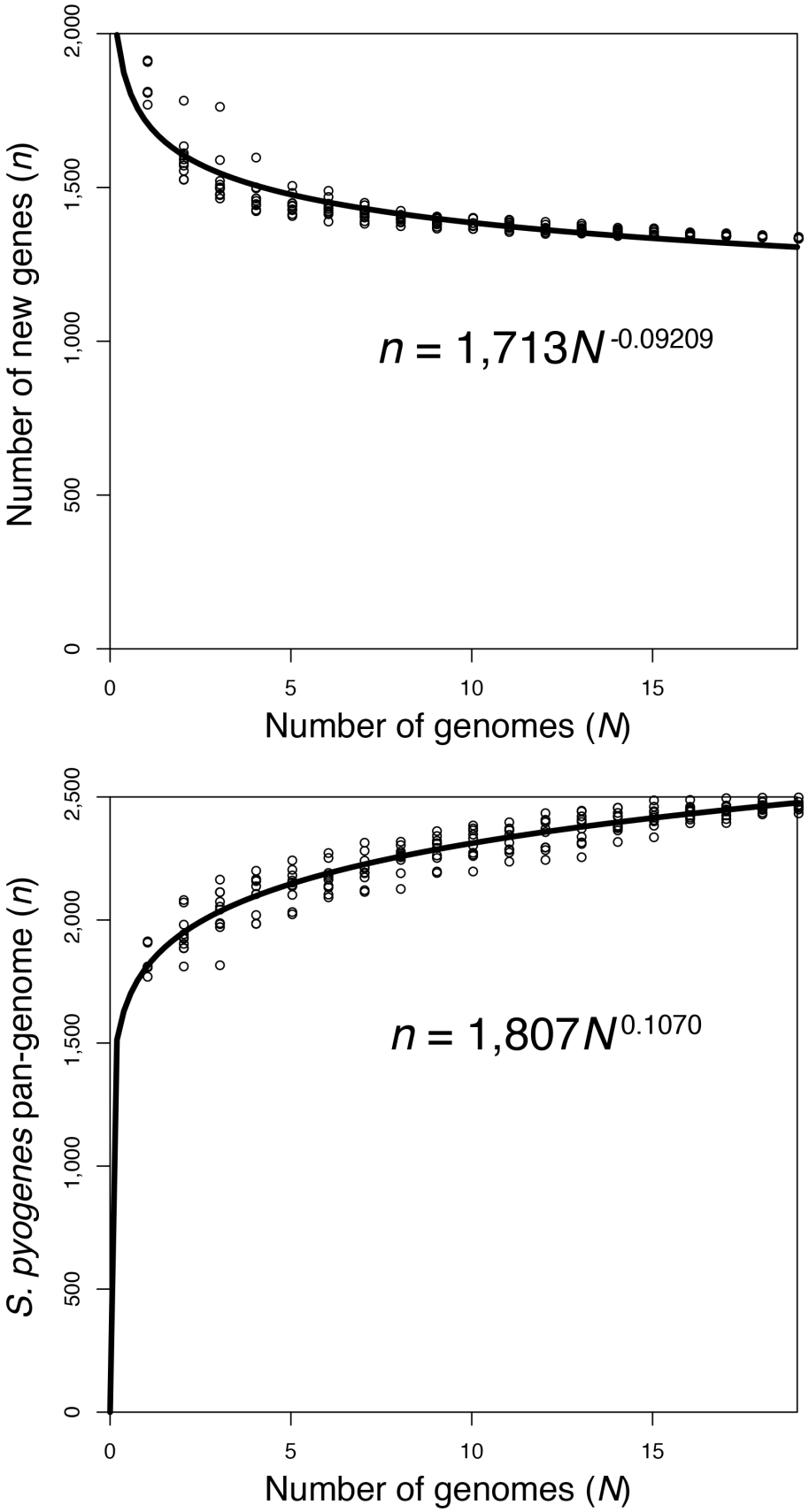

Figure 2. Estimation of pan-genome from 19 *S. pyogenes* genomes. The software GET_HOMOLOGUES was used to calculate the number of new genes (*n* in the upper graph) or pan-genome (*n* in the lower graph) in each number of genomes (*N*). Ten values of *n* were obtained in each *N*, and were used for estimation of exponential formulas that are shown in the graph areas.

Table 2. Available software for CG

Software	Examples of executable analyses	Reference
PGAP	Gene clustering, estimation of pan-genome size, SNP calling, evolutionary analysis, and functional analysis of orthologous clusters	(Zhao, Wu, Yang, Sun, Xiao, & Yu, 2012)
GET_HOMOLOGUES	Gene clustering, and estimation of pan-genome size	(Contreras-Moreira & Vinuesa, 2013)
MEGA	Sequence alignment, distance calculation, and construction of phylogenetic tree	(Tamura, Stecher, Peterson, Filipski, & Kumar, 2013)
RAxML	Construction of phylogenetic tree based on maximum likelihood method	(Stamatakis, 2006; Stamatakis, Ludwig, & Meier, 2005)
SAMtools	NGS read mapping onto a reference genome, and SNP calling	(Li, et al., 2009)
PAML	Comparison of phylogenetic trees, likelihood ratio tests, and estimation of dN/dS ratio	(Xu & Yang, 2013; Yang, 2007)

Table 3. Multi-copy core genes in 19 *S. pyogenes* strains

Annotation
Amino acid transport ATP-binding protein
Nucleoside-binding protein
Peptidoglycan hydrolase, Autolysin2
General stress protein, Gls24 family
ABC transporter ATP-binding protein
Butyrate-acetoacetate CoA-transferase subunit B
Ribose-phosphate pyrophosphokinase
Formate--tetrahydrofolate ligase
Positive transcriptional regulator, MutR family
Butyrate-acetoacetate CoA-transferase subunit A
Phosphate transport ATP-binding protein PstB (TC 3.A.1.7.1)
Competence-specific sigma factor ComX
Foldase protein prsA 1 precursor
Lactose phosphotransferase system repressor
Galactose-6-phosphate isomerase, LacA subunit
Galactose-6-phosphate isomerase, LacB subunit
Immunogenic secreted protein
Tagatose 1,6-bisphosphate aldolase
3-ketoacyl-CoA thiolase @ Acetyl-CoA acetyltransferase
UTP--glucose-1-phosphate uridylyltransferase
Oxaloacetate decarboxylase alpha chain
Mobile element protein
FIG01116026: hypothetical protein
Glycerol uptake facilitator protein
Replicative DNA helicase

Table 3. continued from previous page.

Annotation
Streptodornase B; Mitogenic factor 1
Single-stranded DNA-binding protein
Pleiotropic regulator of exopolysaccharide synthesis, competence and biofilm formation Ftr, XRE family
Quinolinate phosphoribosyltransferase [decarboxylating]
Malonate permease
FIG01114229: hypothetical protein
DNA-entry nuclease (Competence-specific nuclease)
Collagen-like surface protein

Table 4. Single-copy core genes in 19 *S. pyogenes* strains

Annotation
COG0488: ATPase components of ABC transporters with duplicated ATPase domains
Acid phosphatase
Signal peptidase I
ATPase component BioM of energizing module of biotin ECF transporter
D-beta-hydroxybutyrate permease
Isopentenyl-diphosphate delta-isomerase, FMN-dependent
Serine hydroxymethyltransferase
Glucosamine-6-phosphate deaminase
Transporter
ABC transporter permease protein
Alcohol dehydrogenase
V-type ATP synthase subunit E
Beta-glucosidase
Putrescine transport ATP-binding protein PotA (TC 3.A.1.11.1)
Ribosomal RNA small subunit methyltransferase C
Phosphate transport system regulatory protein PhoU
Lipase/Acylhydrolase family protein
LSU ribosomal protein L28p
Inosine-5'-monophosphate dehydrogenase
Transcriptional regulator, DeoR family
Translation initiation factor 3
Ribosome recycling factor
Ferrichrome transport ATP-binding protein FhuC (TC 3.A.1.14.3)
YpfJ protein, zinc metalloprotease superfamily
Ribosomal RNA large subunit methyltransferase N
Thiol:disulfide oxidoreductase associated with MetSO reductase

Table 4. continued from previous page.

Annotation
Transaldolase
Pyruvate formate-lyase activating enzyme
Dephospho-CoA kinase
COG2110, Macro domain, possibly ADP-ribose binding module
2-dehydro-3-deoxygluconate kinase
3'->5' exoribonuclease Bsu YhaM
GTP pyrophosphokinase
Thioredoxin reductase
Sucrose operon repressor ScrR, LacI family
Multiple sugar ABC transporter, membrane-spanning permease protein MsmG
Potassium efflux system KefA protein / Small-conductance mechanosensitive channel
Streptolysin S biosynthesis protein B (SagB)
Cell division protein FtsH
Glyoxalase family protein
Dihydroorotate dehydrogenase, catalytic subunit
Predicted regulator for deoxynucleoside utilization, GntR family
FIG01114764: hypothetical protein
Leucine rich protein
GTP-binding protein HflX
3-hydroxyacyl-[acyl-carrier-protein] dehydratase, FabZ form
Phage infection protein
Putative iron-sulfur cluster assembly scaffold protein for SUF system, SufE2
Aspartyl-tRNA(Asn) amidotransferase subunit B @ Glutamyl-tRNA(Gln) amidotransferase subunit B
hypothetical protein
Phenylalanyl-tRNA synthetase domain protein (Bsu YtpR)
Chorismate mutase I
4-oxalocrotonate tautomerase; Xylose transport system permease protein xylH
Hypothetical ATP-binding protein UPF0042, contains P-loop
Acetyl-coenzyme A carboxyl transferase alpha chain
Heat shock protein GrpE
Lactoylglutathione lyase
FIG01114175: hypothetical protein
Carbonic anhydrase
Competence-induced protein Ccs4
DNA repair protein RecN
ATP-dependent nuclease, subunit B
RNA methyltransferase, TrmA family

Table 4. continued from previous page.

Annotation
Large-conductance mechanosensitive channel
UPF0135 protein Bsu YqfO
Arginine pathway regulatory protein ArgR, repressor of arg regulon
Phosphoenolpyruvate-protein phosphotransferase of PTS system
Cell division protein FtsW
Predicted nucleoside phosphatase
Ferric iron ABC transporter, iron-binding protein
Phosphoribosylglycinamide formyltransferase
GTP-binding protein YqeH, required for biogenesis of 30S ribosome subunit
3-oxoacyl-[acyl-carrier protein] reductase
Methionyl-tRNA synthetase
ATP-dependent Clp protease, ATP-binding subunit ClpE
N-acetylmannosamine-6-phosphate 2-epimerase
Putative ABC transporter ATP-binding protein, spy1790 homolog
Phosphoribosylaminoimidazole carboxylase ATPase subunit
Alkyl hydroperoxide reductase protein C
Excinuclease ABC subunit A
KH domain RNA binding protein YlqC
Streptococcal histidine triad protein
PTS system, mannose-specific IID component
Glutamine synthetase type I
Glutamate 5-kinase
ATP synthase beta chain
Cell envelope-associated transcriptional attenuator LytR-CpsA-Psr, subfamily F2 (as in PMID19099556)
Export ABC transporter ATP-binding protein; Streptolysin S export protein (SagG)
FIG01114317: hypothetical protein
ribosomal protein L7Ae family protein
Guanylate kinase
COG1242: Predicted Fe-S oxidoreductase
SSU ribosomal protein S19p (S15e)
Cobalt-zinc-cadmium resistance protein CzcD
Glycine betaine ABC transport system, permease protein OpuAB / Glycine betaine ABC transport system, glycine betaine-binding protein OpuAC
LSU ribosomal protein L31p @ LSU ribosomal protein L31p, zinc-independent
Predicted transcriptional regulator of pyridoxine metabolism
DNA replication intiation control protein YabA
ABC transporter, predicted N-acetylneuraminate transport system permease protein 2

Table 4. continued from previous page.

Annotation
Transmembrane component of general energizing module of ECF transporters
Hypothetical protein VC0266 (sugar utilization related?)
Anaerobic ribonucleoside-triphosphate reductase
FIG001886: Cytoplasmic hypothetical protein
Two-component system histidine kinase
ABC transporter substrate-binding protein
3-dehydroquinate dehydratase I
Ribonucleotide reductase of class III (anaerobic), activating protein
DNA polymerase IV
Ribosomal small subunit pseudouridine synthase A
Chromosome segregation helicase
Methionine aminopeptidase
Manganese ABC transporter, periplasmic-binding protein SitA
Two-component response regulator, malate
Phosphatidate cytidylyltransferase
Deoxyribose-phosphate aldolase
ABC transporter, permease protein
RecA protein
Putative two-component responsible histidine kinase
hypothetical protein
Histone acetyltransferase Gcn5
Rhodanese domain protein UPF0176, Firmicutes subgroup
SSU ribosomal protein S11p (S14e)
5'-methylthioadenosine nucleosidase @ S-adenosylhomocysteine nucleosidase
Neutral endopeptidase O
Purine nucleoside phosphorylase; N-Ribosylnicotinamide phosphorylase ## possible
2-dehydropantoate 2-reductase
Type I restriction-modification system, DNA-methyltransferase subunit M
A/G-specific adenine glycosylase
FIG001960: FtsZ-interacting protein related to cell division
Lipid A export ATP-binding/permease protein MsbA
LSU ribosomal protein L11p (L12e)
Late competence protein ComGC, access of DNA to ComEA, FIG007487
Transporter associated with VraSR
Cysteine desulfurase
Redox-sensitive transcriptional regulator (AT-rich DNA-binding protein)
PTS system, galactose-specific IIC component

Table 4. continued from previous page.

Annotation
FIG011440: Integral membrane protein
Rhodanese-like domain protein
Acetylornithine deacetylase/Succinyl-diaminopimelate desuccinylase and related deacylases
Adenylate kinase
Non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase (NADP)
HMP-PP hydrolase (pyridoxal phosphatase) Cof, detected in genetic screen for thiamin metabolic genes (PMID:15292217)
tRNA pseudouridine synthase B
D-alanyl-D-alanine carboxypeptidase
tRNA (guanine46-N7-)-methyltransferase
Uracil phosphoribosyltransferase / Pyrimidine operon regulatory protein PyrR
DEAD-box ATP-dependent RNA helicase CshA
putative glycosyltransferase - possibly involved in cell wall localization and side chain formation of rhamnose-glucose polysaccharide
Segregation and condensation protein A
ABC transporter permease protein
Glycerol-3-phosphate dehydrogenase [NAD(P)+]
Putative deoxyribose-specific ABC transporter, permease protein
Hypothetical protein ywlG
D-alanine--poly(phosphoribitol) ligase subunit 1
3-oxoacyl-[acyl-carrier protein] reductase
NAD synthetase
Oligopeptide transport system permease protein OppB (TC 3.A.1.5.1)
tRNA dimethylallyltransferase
2-deoxy-D-gluconate 3-dehydrogenase
Similar to ribosomal large subunit pseudouridine synthase D, Bacillus subtilis YjbO type
LSU m3Psi1915 methyltransferase RlmH
Glycosyltransferase involved in cell wall biogenesis
Hydroxyacylglutathione hydrolase
SSU ribosomal protein S6p
Ribonuclease J2 (endoribonuclease in RNA processing)
FIG01115059: hypothetical protein
Protein:protein lipoyl transferase
NADH peroxidase
Integral membrane protein
Xanthine permease
Phosphate transport system permease protein PstA (TC 3.A.1.7.1)

Table 4. continued from previous page.

Annotation
2,3-butanediol dehydrogenase, S-alcohol forming, (R)-acetoin-specific / Acetoin (diacetyl) reductase
Catabolite control protein A
Pseudouridylate synthases, 23S RNA-specific
Membrane protein involved in the export of O-antigen, teichoic acid lipoteichoic acids
Lipoate-protein ligase A
Chaperone protein DnaJ
Pyruvate kinase
Urocanate hydratase
tRNA-specific adenosine-34 deaminase
Streptolysin S biosynthesis protein (SagF)
Uncharacterized secreted protein associated with spyDAC
Phosphopentomutase
Ribonucleotide reductase of class Ib (aerobic), beta subunit
Cysteinyl-tRNA synthetase related protein
periplasmic component of efflux system
Inosine-5'-monophosphate dehydrogenase / CBS domain
Preprotein translocase subunit SecE (TC 3.A.5.1.1)
LSU ribosomal protein L14p (L23e)
Amino acid ABC transporter, glutamine-binding protein/permease protein
TsaC protein (YrdC domain) required for threonylcarbamoyladenosine t(6)A37 modification in tRNA
ATP synthase F0 sector subunit b
FIG01113961: hypothetical protein
Lysyl-tRNA synthetase (class II)
3-oxoacyl-[acyl-carrier-protein] synthase, KASII
N-acetylmannosamine kinase
Chaperonin (heat shock protein 33)
PTS system, IIA component, putative
Dihydroxyacetone kinase family protein
1-phosphofructokinase
Plasmid stabilization system toxin protein
FIG011178: rRNA methylase
ABC transporter permease protein
SSU ribosomal protein S12p (S23e)
Surface exclusion protein
50S ribosomal subunit maturation GTPase RbgA (B. subtilis YlqF)
Signal recognition particle receptor protein
Phosphoribosylamine--glycine ligase

Table 4. continued from previous page.

Annotation
ABC-type multidrug transport system, ATPase component
23S rRNA (guanosine-2'-O-) -methyltransferase rlmB
PTS system, galactose-specific IIA component
Hypothetical protein DUF194, DegV family
Pyridoxamine 5'-phosphate oxidase
Aspartyl-tRNA(Asn) amidotransferase subunit B @ Glutamyl-tRNA(Gln) amidotransferase subunit B
Carbamoyl-phosphate synthase large chain
6-aminohexanoate-cyclic-dimer hydrolase
PTS system, mannose-specific IIC component
tRNA:m(5)U-54 MTase gid
Ribosome-associated heat shock protein implicated in the recycling of the 50S subunit (S4 paralog)
Adenylosuccinate synthetase
L-serine dehydratase, beta subunit
FIG000557: hypothetical protein co-occurring with RecR
Ribosomal protein L11 methyltransferase
Transcription accessory protein (S1 RNA-binding domain)
Transcriptional antiterminator of lichenan operon, BglG family
2-amino-4-hydroxy-6- hydroxymethyldihydropteridine pyrophosphokinase
Nicotinamidase
Cysteine synthase
Integral membrane protein
Lon-like protease with PDZ domain
Cobalt-zinc-cadmium resistance protein
Heme ABC transporter (Streptococcus), ATP-binding protein
Histidine protein kinase
Deoxyuridine 5'-triphosphate nucleotidohydrolase
UDP-N-acetylmuramoylalanyl-D-glutamyl-2,6- diaminopimelate--D-alanyl-D-alanine ligase
PTS system, cellobiose-specific IIC component
Phosphoglycerate mutase
FIG01114242: hypothetical protein
Helicase PriA essential for oriC/DnaA-independent DNA replication
FIG139598: Potential ribosomal protein
Substrate-specific component PdxU of predicted pyridoxine ECF transporter
Substrate-specific component BioY of biotin ECF transporter
Ribonuclease Z
Thioredoxin
ATP synthase alpha chain

Table 4. continued from previous page.

Annotation
Substrate-specific component RibU of riboflavin ECF transporter
LemA protein
Potassium uptake protein TrkH
ComF operon protein A, DNA transporter ATPase
Cell division protein FtsK
LSU ribosomal protein L13p (L13Ae)
FIG01116435: hypothetical protein
LSU ribosomal protein L16p (L10e)
DNA-directed RNA polymerase beta subunit
YheO-like PAS domain
putative choline binding protein
Hypothetical protein SPy0110
Transcriptional antiterminator with PTS regulation domain, SPy0181 ortholog
Adenylosuccinate lyase
Putative deoxyribonuclease YcfH
Transcriptional regulator in cluster with unspecified monosaccharide ABC transport system
FIG01116936: hypothetical protein
ATPase component of general energizing module of ECF transporters
hypothetical protein
RNA polymerase sigma factor RpoD
FIG001583: hypothetical protein, contains S4-like RNA binding domain
Peptide methionine sulfoxide reductase MsrA
putative N-acetyl-muramidase
3-keto-L-gulonate 6-phosphate decarboxylase
DNA polymerase III alpha subunit
FIG014387: Transcriptional regulator, PadR family
ABC transporter, permease protein EscB
Dipeptide transport system permease protein DppB (TC 3.A.1.5.2)
Heteropolysaccharide repeat unit export protein
Late competence protein ComGG, FIG068335
putative phosphomannomutase
Transcriptional regulator
Aspartate--ammonia ligase
Transport protein SgaT, putative
Transcriptional regulator, GntR family
Two component system response regulator CiaR
Transcriptional regulator, RofA-like Protein (RALP)

Table 4. continued from previous page.

Annotation
Para-aminobenzoate synthase, aminase component / Aminodeoxychorismate lyase
PTS system, N-acetylgalactosamine-specific IIB component
FIG01114628: hypothetical protein
Phosphoesterase, DHH family protein
Phenylacetic acid degradation protein PaaD, thioesterase
Substrate-specific component PanT of predicted pantothenate ECF transporter
Aspartate carbamoyltransferase
Translation elongation factor Tu
FIG01114171: hypothetical protein
Tagatose-6-phosphate kinase
Protein of unknown function DUF1447
LSU ribosomal protein L3p (L3e)
DNA gyrase subunit B
ABC transporter membrane-spanning permease - macrolide efflux
ABC-type multidrug transport system, permease component
Transcription antiterminator, BglG family
tRNA S(4)U 4-thiouridine synthase (former ThiI)
rhamnose-containing polysacharide translocation permease
Oligoendopeptidase F
Negative regulator of genetic competence MecA
Prolyl-tRNA synthetase, bacterial type
Hydrolases of the alpha/beta superfamily
hypothetical protein
RecD-like DNA helicase YrrC
Serine acetyltransferase
Lipoate-protein ligase A
Peroxide stress regulator PerR, FUR family
LSU ribosomal protein L17p
SSU ribosomal protein S3p (S3e)
DNA mismatch repair protein MutL
Transaldolase
Arginine/ornithine antiporter ArcD
Glycyl-tRNA synthetase beta chain
Ribonucleotide reductase of class Ib (aerobic), alpha subunit
Excinuclease ABC subunit C
FIG01114490: hypothetical protein
Cell division protein FtsQ

Table 4. continued from previous page.

Annotation
Cystine transport system permease protein
Excinuclease ABC subunit B
Lipoprotein signal peptidase
Phosphonate ABC transporter phosphate-binding periplasmic component (TC 3.A.1.9.1)
Acetoin dehydrogenase E1 component beta-subunit
Glutamine ABC transporter, periplasmic glutamine-binding protein (TC 3.A.1.3.2) / Glutamine transport system permease protein GlnP (TC 3.A.1.3.2)
DNA topoisomerase I
FIG01114029: hypothetical protein
DNA polymerase III delta subunit
Oligopeptide transport ATP-binding protein OppD (TC 3.A.1.5.1)
ATP-dependent Clp protease ATP-binding subunit ClpA
Maltose/maltodextrin ABC transporter, substrate binding periplasmic protein MalE
Signal recognition particle associated protein
FIG01115418: hypothetical protein
Mannose-6-phosphate isomerase
hydrolase, haloacid dehalogenase-like family
Regulation of D-alanyl-lipoteichoic acid biosynthesis, sensor histidine kinase
Helicase loader DnaB
Protein serine/threonine phosphatase PrpC, regulation of stationary phase
Transcriptional regulators, LysR family
Malate Na(+) symporter
Conjugation pore forming protein EbsA
membrane protein, related to Actinobacillus protein (1944168)
L-ribulose-5-phosphate 4-epimerase
SSU ribosomal protein S7p (S5e)
Colicin V production protein
Ribosomal RNA small subunit methyltransferase D
Ferredoxin
PTS system, maltose and glucose-specific IIC component / PTS system, maltose and glucose-specific IIB component / PTS system, maltose and glucose-specific IIA component
Uridine kinase [C1]
PTS system, mannose-specific IIC component
Dihydroorotase
Iron-sulfur cluster assembly protein SufB
Streptolysin S biosynthesis protein D (SagD)
Tellurite resistance protein

Table 4. continued from previous page.

Annotation
Glucose-6-phosphate isomerase
ABC-type multidrug transport system, ATPase component
Diacylglycerol kinase
Nicotinate-nucleotide adenylyltransferase
ABC transporter, ATP-binding protein
UDP-N-acetylglucosamine 1-carboxyvinyltransferase
PTS system, lactose-specific IIA component
YfaA
16S rRNA processing protein RimM
RNA methyltransferase, TrmA family
Acetylornithine deacetylase/Succinyl-diaminopimelate desuccinylase and related deacylases
Methionine biosynthesis and transport regulator MtaR, LysR family
Putative amidotransferase similar to cobyric acid synthase
hypothetical protein
3-dehydroquinate synthase
hypothetical protein
Transcriptional regulator pfoR
Late competence protein ComGA, access of DNA to ComEA
Segregation and condensation protein B
Chromosome replication initiation protein DnaD
Glycerophosphoryl diester phosphodiesterase
GAF domain-containing proteins
DNA repair protein RadC
Phosphate acetyltransferase
Ferrichrome transport system permease protein FhuG
Multimodular transpeptidase-transglycosylase
Alkyl hydroperoxide reductase protein F
Histidine triad (HIT) nucleotide-binding protein, similarity with At5g48545 and yeast YDL125C (HNT1)
Uracil phosphoribosyltransferase
Tyrosyl-tRNA synthetase
Ribosomal silencing factor RsfA (former Iojap)
Glucokinase
PTS system, N-acetylgalactosamine-specific IID component
Adenine-specific methyltransferase
S-ribosylhomocysteine lyase / Autoinducer-2 production protein LuxS
Type I restriction-modification system specificity subunit

Table 4. continued from previous page.

Annotation
Phage shock protein C, putative; stress-responsive transcriptional regulator
Glutathione peroxidase family protein
Leucyl-tRNA synthetase
Glucan 1,6-alpha-glucosidase
D-alanyl-D-alanine carboxypeptidase
Phosphate:acyl-ACP acyltransferase PlsX
Lactate 2-monooxygenase
Two-component response regulator SA14-24
dTDP-glucose 4,6-dehydratase
Transcription termination protein NusA
internalin, putative
Streptococcal NAD glycohydrolase inhibitor
Holliday junction DNA helicase RuvB
Amidophosphoribosyltransferase
Lipid A export ATP-binding/permease protein MsbA
PhnO protein
Isoleucyl-tRNA synthetase
tRNA (adenine37-N(6))-methyltransferase TrmN6
tRNA uridine 5-carboxymethylaminomethyl modification enzyme GidA
LSU ribosomal protein L5p (L11e)
Lipoteichoic acid synthase LtaS Type IIc
Phosphopantetheine adenylyltransferase
Putative sugar uptake protein SpyM3_1856/SPs1852
Cadmium efflux system accessory protein
Integral membrane protein
Deoxyadenosine kinase / Deoxyguanosine kinase
possible permease
FIG009439: Cytosolic protein containing multiple CBS domains
Aspartyl-tRNA synthetase
Transketolase
Heme transporter IsdDEF, lipoprotein IsdE
Arsenate reductase
Dihydrofolate synthase @ Folylpolyglutamate synthase
Recombination protein RecR
UDP-N-acetylglucosamine--N-acetylmuramyl- (pentapeptide) pyrophosphoryl-undecaprenol N-acetylglucosamine transferase
Chloride channel protein

Table 4. continued from previous page.

Annotation
3'-to-5' exoribonuclease RNase R
Pneumococcal vaccine antigen A homolog
Ubiquinone/menaquinone biosynthesis methyltransferase UbiE
LSU ribosomal protein L1p (L10Ae)
FIG005935: membrane protein
Dihydrolipoamide dehydrogenase of acetoin dehydrogenase
ATP synthase epsilon chain
Glycosyltransferase LafA, responsible for the formation of Glc-DAG
Uridine phosphorylase
dTDP-4-dehydrorhamnose reductase
Ribosomal small subunit pseudouridine synthase A
Preprotein translocase secY subunit (TC 3.A.5.1.1)
tRNA-specific 2-thiouridylase MnmA
Aquaporin (Major Intrinsic Protein Family)
Exodeoxyribonuclease VII large subunit
Biotin carboxyl carrier protein of acetyl-CoA carboxylase
FIG004453: protein YceG like
FIG005686: hypothetical protein
L-proline glycine betaine binding ABC transporter protein ProX (TC 3.A.1.12.1) / Osmotic adaptation
GTP pyrophosphokinase
GTP-binding and nucleic acid-binding protein YchF
FIG001721: Predicted N6-adenine-specific DNA methylase
DNA polymerase III delta prime subunit
ABC transporter, periplasmic spermidine putrescine-binding protein PotD (TC 3.A.1.11.1)
LSU ribosomal protein L23p (L23Ae)
Dolichyl-phosphate mannoosyltransferase, involved in cell wall biogenesis
Transcriptional regulator RegR, rpressor of hyaluronate and KDG utilization
Negative transcriptional regulator-copper transport operon
Ribosome small subunit-stimulated GTPase EngC
Transcriptional regulators, LysR family
1-acyl-sn-glycerol-3-phosphate acyltransferase
ABC transporter permease protein
Histidine ammonia-lyase
Galactosamine-6-phosphate isomerase
Ribonuclease J1 (endonuclease and 5' exonuclease)
FIG01114161: hypothetical protein
DNA-damage-inducible protein J

Table 4. continued from previous page.

Annotation
Transcriptional regulator of fatty acid biosynthesis FabT
Unsaturated glucuronyl hydrolase
NADH peroxidase
DNA primase
tmRNA-binding protein SmpB
FIG01116176: hypothetical protein
Cell division transporter, ATP-binding protein FtsE (TC 3.A.5.1.1)
Aspartate aminotransferase
Aminopeptidase C
Glycyl-tRNA synthetase alpha chain
LSU ribosomal protein L21p
D-alanyl-D-alanine carboxypeptidase
Aspartate aminotransferase
Beta-glucoside bgl operon antiterminator, BglG family
Substrate-specific component BL0695 of predicted ECF transporter
Two-component response regulator yesN, associated with MetSO reductase
tRNA-dependent lipid II-Ala--L-alanine ligase
Transcription termination protein NusB
Arginine/ornithine antiporter ArcD
FIG01113973: possible membrane protein
Substrate-specific component QueT (COG4708) of predicted queuosine-regulated ECF transporter
Multidrug resistance protein B
FIG01114068: hypothetical protein
Hypoxanthine-guanine phosphoribosyltransferase
Trk system potassium uptake protein TrkA
Ascorbate-specific PTS system, EIIB component
HMP-PP hydrolase (pyridoxal phosphatase) Cof, detected in genetic screen for thiamin metabolic genes (PMID:15292217)
putative cationic amino acid transporter protein
FIG015389: hypothetical membrane associated protein
Lanthionine biosynthesis protein LanM
4-alpha-glucanotransferase (amylomaltase)
Cold shock protein CspA
Sensory transduction protein kinase
Transcriptional regulator, TetR family
Kup system potassium uptake protein
ABC transporter ATP-binding protein YvcR

Table 4. continued from previous page.

Annotation
Peptide chain release factor 1
Transcriptional regulator, LuxR family
Transcriptional regulator, MarR family
Arginine/ornithine antiporter ArcD
Transcription regulator [contains diacylglycerol kinase catalytic domain]
Phosphoenolpyruvate carboxylase
DNA-3-methyladenine glycosylase
Ribosomal-protein-S18p-alanine acetyltransferase
Group B streptococcal surface immunogenic protein
Putative ABC transporter (ATP-binding protein), spy1791 homolog
Long-chain-fatty-acid--CoA ligase
ABC transporter ATP-binding protein
Heat shock protein 60 family chaperone GroEL
Phenylalanyl-tRNA synthetase beta chain
FIG01114562: hypothetical protein
Histidine kinase of the competence regulon ComD
FIG01114660: hypothetical protein
Inositol-1-monophosphatase
Epoxyqueuosine (oQ) reductase QueG
putative transport accessory protein
V-type ATP synthase subunit I
Trehalose-6-phosphate hydrolase
Cell division protein FtsL
Transcriptional regulator SpxA1
Acyl-ACP thioesterase
ATP synthase gamma chain
NAD-dependent glyceraldehyde-3-phosphate dehydrogenase # Plasmin(ogen) receptor
Class B acid phosphatase precursor
LSU ribosomal protein L18p (L5e)
FIG01114729: hypothetical protein
Ribonuclease HIII
Zinc ABC transporter, inner membrane permease protein ZnuB
putative ATP-dependent Clp proteinase (ATP-binding subunit)
Methionine ABC transporter ATP-binding protein
Topoisomerase IV subunit B
GTP-binding protein EngA
COG1939: Ribonuclease III family protein

Table 4. continued from previous page.

Annotation
tRNA-guanine transglycosylase
Similar to ribosomal large subunit pseudouridine synthase D, Bacillus subtilis YhcT type
Alpha-mannosidase
Thymidylate kinase
Manganese-dependent inorganic pyrophosphatase
Arginyl-tRNA synthetase
Thymidylate synthase
DNA-directed RNA polymerase delta subunit
FIG01114438: possible membrane protein
ABC transporter ATP-binding protein
FIG01116163: hypothetical protein
MutT/nudix family protein
GTP cyclohydrolase I type 1
V-type ATP synthase subunit B
FIG01055109: hypothetical protein
Biotin operon repressor / Biotin-protein ligase
Peptide deformylase
Manganese ABC transporter, inner membrane permease protein SitD
6-phospho-beta-glucosidase
TsaE protein, required for threonylcarbamoyladenosine t(6)A37 formation in tRNA
Biotinyl-lipoyl attachment domain protein, GcvH-like
Peptidoglycan N-acetylglucosamine deacetylase
RopB; Rgg-like transcription regulator
LacI family regulatory protein
hypothetical protein
Tagatose-6-phosphate kinase
membrane protein, putative
Integral membrane protein
Arginine pathway regulatory protein ArgR, repressor of arg regulon
Single-stranded-DNA-specific exonuclease RecJ
Alkaline shock protein
UPF0246 protein YaaA
S-layer homology domain / putative murein endopeptidase
MORN motif family protein
Acyltransferase family
hypothetical protein BH3604
Prolipoprotein diacylglyceryl transferase

Table 4. continued from previous page.

Annotation
Hydroxymethylglutaryl-CoA synthase
Topoisomerase IV subunit A
Translation elongation factor P
peptidase, U32 family large subunit [C1]
SSU ribosomal protein S20p
Transporter, MFS superfamily
SSU ribosomal protein S8p (S15Ae)
COG4478, integral membrane protein
Dipeptide transport ATP-binding protein DppF (TC 3.A.1.5.2)
Endonuclease III
Thioredoxin reductase
Aspartyl-tRNA(Asn) amidotransferase subunit C @ Glutamyl-tRNA(Gln) amidotransferase subunit C
Thiamin pyrophosphokinase
PTS system, cellobiose-specific IIA component
Hypothetical protein SP1558
HMP-PP hydrolase (pyridoxal phosphatase) Cof, detected in genetic screen for thiamin metabolic genes (PMID:15292217)
Fructose-bisphosphate aldolase class II
FIG007079: UPF0348 protein family
Streptococcal lipoprotein rotamase A; Peptidyl-prolyl cis-trans isomerase
FIG01114188: hypothetical protein
Transcriptional regulator, TetR family
FIG01114378: hypothetical protein
Transcriptional regulator, CrP/Fnr family
Cytidylate kinase
FIG01116327: hypothetical protein
Putative cysteine desulfurase, associated with tRNA 4-thiouridine synthase
Rossmann fold nucleotide-binding protein Smf possibly involved in DNA uptake
Transcriptional regulator OrfX
SSU ribosomal protein S14p (S29e) @ SSU ribosomal protein S14p (S29e), zinc-independent
L-serine dehydratase, alpha subunit
FIG014356: hypothetical protein
Magnesium and cobalt efflux protein CorC
Deoxyguanosinetriphosphate triphosphohydrolase
Transcriptional regulator SpxA2
Nicotine adenine dinucleotide glycohydrolase (NADGH)
Sucrose-6-phosphate hydrolase (EC 3.2.1.B3)

Table 4. continued from previous page.

Annotation
DNA recombination protein RmuC
Inner membrane protein translocase component YidC, OxaA protein
ATP synthase F0 sector subunit c
unknown domain / Nucleoside 5-triphosphatase RdgB (dHAPTP, dITP, XTP-specific)
ADP-ribose pyrophosphatase
Phosphopantothenoylcysteine decarboxylase
Pyrrolidone-carboxylate peptidase
DNA-binding response regulator, AraC family
Duplicated ATPase component BL0693 of energizing module of predicted ECF transporter
Pyrroline-5-carboxylate reductase
FIG01114177: hypothetical protein
Phosphoglycerate mutase family 5
5'-nucleotidase
Peptide methionine sulfoxide reductase MsrB
Flavodoxin
Succinyl-CoA synthetase, alpha subunit-related enzymes
Chaperone protein DnaK
SSU ribosomal protein S2p (SAe)
UDP-N-acetylmuramoylpentapeptide-lysine N(6)-alanyltransferase
Type I restriction-modification system, restriction subunit R
D-tyrosyl-tRNA(Tyr) deacylase (EC 3.6.1.n1)
DNA recombination and repair protein RecF
Zn-dependent hydrolase YycJ/WalJ, required for cell wall metabolism and coordination of cell division with DNA replication
Hyaluronoglucosaminidase
Lipoteichoic acid synthase LtaS Type IIc
Phospho-N-acetylmuramoyl-pentapeptide-transferas e
Octaprenyl diphosphate synthase / Dimethylallyltransferase / (2E,6E)-farnesyl diphosphate synthase / Geranylgeranyl diphosphate synthase
Transcriptional repressor AdcR for Zn(2+)-responsive expression
Alpha-glycerophosphate oxidase
Transcriptional regulator, MerR family
Glycerate kinase
Alanine racemase
Valyl-tRNA synthetase
Bactoprenol glucosyl transferase
oxidoreductase of aldo/keto reductase family, subgroup 1

Table 4. continued from previous page.

Annotation
DNA polymerase III beta subunit
Cell division protein DivIC (FtsB), stabilizes FtsL against RasP cleavage
Heat shock protein HtpX
Transcriptional regulator, TetR family
Short chain dehydrogenase
Two-component response regulator, associated with ferric iron transporter, SPy1062 homolog
Orotate phosphoribosyltransferase
FIG01115301: hypothetical protein
DNA-directed RNA polymerase omega subunit
ATPase component of general energizing module of ECF transporters
FIG001553: Hydrolase, HAD subfamily IIIA
Membrane-bound protease, CAAX family
Glycine/D-amino acid oxidases family
Peptide deformylase
FIG000325: clustered with transcription termination protein NusA
DNA-directed RNA polymerase beta' subunit
Phosphoribosylformylglycinamidine cyclo-ligase
tRNA (Guanine37-N1) -methyltransferase
D-alanine--D-alanine ligase
FtsK/SpoIIIE family
Cell division protein FtsH
Transcription antitermination protein NusG
Membrane protein, putative
ATP synthase delta chain
Two-component sensor kinase, associated with ferric iron transporter, SPy1061 homolog
Laminin-binding surface protein
SAM-dependent methyltransferase, MraW methylase family
Dipeptide-binding ABC transporter, periplasmic substrate-binding component DppA (TC 3.A.1.5.2)
Arginine pathway regulatory protein ArgR, repressor of arg regulon
Putative membrane protein precursor SPs0273
Glycerol kinase
PhnO protein
Shikimate kinase I
DNA repair protein RadA
FIG01114335: hypothetical protein
Heme ABC transporter (Streptococcus), permease protein
Pyridoxal kinase

Table 4. continued from previous page.

Annotation
ABC transporter permease protein
DNA polymerase I
FIG005986: HD family hydrolase
Transcriptional regulator CtsR
LSU ribosomal protein L2p (L8e)
Ribonuclease M5
Lysyl aminopeptidase
tRNA and rRNA cytosine-C5-methylases
Metal-dependent hydrolase YbeY, involved in rRNA and/or ribosome maturation and assembly
Cystine ABC transporter, periplasmic cystine-binding protein FliY
Oxygen-insensitive NAD(P)H nitroreductase / Dihydropteridine reductase
Mn-dependent transcriptional regulator MntR
Adenylate cyclase
Transmembrane component BL0694 of energizing module of predicted ECF transporter
Hypothetical radical SAM family enzyme in heat shock gene cluster, similarity with CPO of BS HemN-type
Transcriptional regulator, XRE family
Ribosome-binding factor A
Cell division protein FtsA
Streptolysin S self-immunity protein (SagE)
Ascorbate-specific PTS system, EIIA component
SSU ribosomal protein S9p (S16e)
Translation initiation factor 2
periplasmic component of efflux system
Septation ring formation regulator EzrA
Para-aminobenzoate synthase, amidotransferase component
Peptidyl-prolyl cis-trans isomerase
Tryptophanyl-tRNA synthetase
Ribonuclease P protein component
Aminopeptidase YpdF (MP-, MA-, MS-, AP-, NP- specific)
Translation elongation factor Ts
DUF124 domain-containing protein
6-phosphofructokinase
Iron-sulfur cluster assembly ATPase protein SufC
PhnO protein
FIG01118158: hypothetical protein
Multiple sugar ABC transporter, substrate-binding protein

Table 4. continued from previous page.

Annotation
Streptopain inhibitor
Uracil permease
Alpha-L-Rha alpha-1,3-L-rhamnosyltransferase
membrane protein, putative
Na+ dependent nucleoside transporter NupC
HMP-PP hydrolase (pyridoxal phosphatase) Cof, detected in genetic screen for thiamin metabolic genes (PMID:15292217)
Late competence protein ComGD, access of DNA to ComEA, FIG038316
SSU ribosomal protein S13p (S18e)
GTP-binding protein EngB
SSU ribosomal protein S1p
Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein OppA (TC 3.A.1.5.1)
Deoxyribodipyrimidine photolyase
Cell surface protein
FIG01115412: hypothetical protein
GTPase and tRNA-U34 5-formylation enzyme TrmE
Spermidine Putrescine ABC transporter permease component PotB (TC 3.A.1.11.1)
Ribonucleotide reductase of class Ib (aerobic), alpha subunit
FIG013576: hypothetical protein
Pyruvate formate-lyase
Histidine transport protein (permease)
Substrate-specific component ThiT of thiamin ECF transporter
Na+ driven multidrug efflux pump
FIG001621: Zinc protease
Glutamate formiminotransferase @ Glutamate formyltransferase
FIG01115625: hypothetical protein
Preprotein translocase subunit YajC (TC 3.A.5.1.1)
Xanthine phosphoribosyltransferase
Mutator mutT protein (7,8-dihydro-8-oxoguanine-triphosphatase)
Xaa-Pro dipeptidyl-peptidase
PTS system, cellobiose-specific IIB component
Secreted antigen GbpB/SagA/PcsB, putative peptidoglycan hydrolase
Substrate-specific component PdxU2 of predicted pyridoxin-related ECF transporter
6-phospho-beta-galactosidase
Trans-2,cis-3-Decenoyl-ACP isomerase
Late competence protein ComGF, access of DNA to ComEA, FIG012620
Protein YidD

Table 4. continued from previous page.

Annotation
Integral membrane protein
Ribosomal subunit interface protein
Chromosome partition protein smc
Signal peptidase I
Undecaprenyl diphosphate synthase
Multi antimicrobial extrusion (MATE) family transporter
Putative deoxyribose-specific ABC transporter, permease protein
Dihydrofolate synthase @ Folylpolyglutamate synthase
Mutator mutT protein (7,8-dihydro-8-oxoguanine-triphosphatase)
Ferrichrome-binding periplasmic protein precursor (TC 3.A.1.14.3)
Capsule biosynthesis protein capA
Deoxyribonucleoside regulator DeoR (transcriptional repressor)
L-Cystine ABC transporter, periplasmic cystine-binding protein TcyA
Arsenate reductase
D-beta-hydroxybutyrate dehydrogenase
Formamidopyrimidine-DNA glycosylase
putative ABC transporter (ATP-binding protein)
Streptolysin S export transmembrane permease (SagH)
Two-component system response regulator
V-type ATP synthase subunit K
RNA-binding protein Jag
Methionine ABC transporter substrate-binding protein
Phosphomevalonate kinase
FIG01113992: hypothetical protein
Formiminotetrahydrofolate cyclodeaminase
Sensor histidine kinase VraS
PTS system, beta-glucoside-specific IIB component / PTS system, beta-glucoside-specific IIC component / PTS system, beta-glucoside-specific IIA component
Manganese ABC transporter, ATP-binding protein SitB
Transcriptional repressor of the fructose operon, DeoR family
4-diphosphocytidyl-2-C-methyl-D-erythritol kinase
Oxygen-insensitive NAD(P)H nitroreductase / Dihydropteridine reductase
Acetyl-coenzyme A carboxyl transferase beta chain
Sialic acid utilization regulator, RpiR family
ABC transporter ATP-binding protein
Cationic amino acid transporter - APC Superfamily
PurR: transcription regulator associated with purine metabolism

Table 4. continued from previous page.

Annotation
N-acetylglucosamine-1-phosphate uridyltransferase / Glucosamine-1-phosphate N-acetyltransferase
PTS system, mannose-specific IIA component / PTS system, mannose-specific IIB component
FIG002813: LPPG:FO 2-phospho-L-lactate transferase like, CofD-like
ABC transporter ATP-binding protein
Hypothetical protein, Spy1939 homolog
Lead, cadmium, zinc and mercury transporting ATPase; Copper-translocating P-type ATPase
Cell division protein FtsX
Exodeoxyribonuclease III
hypothetical protein
Cell division trigger factor
Helicase loader DnaI
Ribonuclease HII
FIG01114077: hypothetical protein
Uridine phosphorylase
Conserved protein
Streptococcal cell surface hemoprotein receptor Shr
Threonyl-tRNA synthetase
INTEGRAL MEMBRANE PROTEIN (Rhomboid family)
peptidase, U32 family small subunit [C1]
INTEGRAL MEMBRANE PROTEIN (Rhomboid family)
Methionyl-tRNA formyltransferase
LSU ribosomal protein L22p (L17e)
Phosphoribosylaminoimidazole-succinocarboxamide synthase
SWF/SNF family helicase
LSU ribosomal protein L27p
ABC transporter ATP-binding protein
Polyribonucleotide nucleotidyltransferase
Cell division protein GpsB, coordinates the switch between cylindrical and septal cell wall synthesis by re-localization of PBP1
Cell division protein FtsZ
ATP-dependent nuclease, subunit A
Oligohyaluronate lyase
Sodium/glycine symporter GlyP
Gamma-glutamyl phosphate reductase
Glycine betaine ABC transport system, ATP-binding protein OpuAA
Ribonucleotide reductase of class III (anaerobic), large subunit
FIG027054: hypothetical protein

Table 4. continued from previous page.

Annotation
Probable L-ascorbate-6-phosphate lactonase UlaG (L-ascorbate utilization protein G)
Promiscuous sugar phosphatase YidA, haloacid dehalogenase-like phosphatase family
UDP-N-acetylglucosamine 1-carboxyvinyltransferase
FIG053235: Diacylglucosamine hydrolase like
Multimodular transpeptidase-transglycosylase
ATP-dependent Clp protease ATP-binding subunit ClpX
FIG009210: peptidase, M16 family
CDP-diacylglycerol--glycerol-3-phosphate 3-phosphatidyltransferase
FIG01120689: hypothetical protein
FIG009886: phosphoesterase
DNA polymerase III subunits gamma and tau
Multiple sugar ABC transporter, ATP-binding protein
COG2740: Predicted nucleic-acid-binding protein implicated in transcription termination
Enolase
LSU ribosomal protein L9p
Proline dipeptidase
Ribonucleotide reduction protein NrdI
Transcriptional regulator, TetR family
Dipeptide transport system permease protein DppC (TC 3.A.1.5.2)
Cell division protein YlmG/Ycf19 (putative), YggT family
Carbamoyl-phosphate synthase small chain
UDP-glucose 6-dehydrogenase
Hypothetical protein in cluster with Ecs transporter (in Streptococci)
Competence protein F homolog, phosphoribosyltransferase domain; protein YhgH required for utilization of DNA as sole source of carbon and energy
tRNA nucleotidyltransferase
IMP cyclohydrolase / Phosphoribosylaminoimidazolecarboxamide formyltransferase
SSU ribosomal protein S10p (S20e)
membrane protein, putative
Dipeptidase
S1 RNA binding domain
Two component system sensor histidine kinase CiaH
LSU ribosomal protein L30p (L7e)
Carbamate kinase
rRNA small subunit methyltransferase I
Transcriptional regulator, GntR family
putative arylalkylamine n-acetyltransferase

Table 4. continued from previous page.

Annotation
DNA mismatch repair protein MutS
DNA-directed RNA polymerase alpha subunit
Immunoglobulin G-endopeptidase (IdeS) / Mac/ Secreted immunoglobulin binding protein (Sib38)
SSU ribosomal protein S15p (S13e)
Daunorubicin resistance transmembrane protein
LSU ribosomal protein L19p
Purine nucleoside phosphorylase
Membrane-associated zinc metalloprotease
SSU ribosomal protein S5p (S2e)
Triosephosphate isomerase
Transcriptional regulator DegU, LuxR family
Phosphoglycerate kinase
Ascorbate-specific PTS system, EIIC component
Cysteine desulfurase, SufS subfamily
LSU ribosomal protein L10p (P0)
Lipid A export ATP-binding/permease protein MsbA
Transmembrane histidine kinase CsrS
UDP-N-acetylmuramate--alanine ligase
Succinate-semialdehyde dehydrogenase [NAD]; Succinate-semialdehyde dehydrogenase [NAD(P)+]
Cell envelope-associated transcriptional attenuator LytR-CpsA-Psr, subfamily F1 (as in PMID19099556)
Phosphoglucomutase
Holliday junction DNA helicase RuvA
Dihydrolipoamide acyltransferases
Phosphatidylglycerophosphatase B
Mevalonate kinase
rRNA small subunit 7-methylguanosine (m7G) methyltransferase GidB
FIG00525014: hypothetical protein
Chloride channel protein
FIG01280259: hypothetical protein
Endoribonuclease L-PSP
Non-specific DNA-binding protein Dps / Iron-binding ferritin-like antioxidant protein / Ferroxidase
Trans-acting positive regulator
NAD-dependent protein deacetylase of SIR2 family
Ribonuclease BN
FIG01115860: hypothetical protein
Diadenylate cyclase spyDAC; Bacterial checkpoint controller DisA with nucleotide-binding domain
NAD-dependent malic enzyme

Table 4. continued from previous page.

Annotation
Probable NADH-dependent flavin oxidoreductase in cluster with COG2110
S-adenosylmethionine:tRNA ribosyltransferase-isomerase
Shikimate 5-dehydrogenase I alpha
D-alanine--poly(phosphoribitol) ligase subunit 2
Oligopeptide transport system permease protein OppC (TC 3.A.1.5.1)
Substrate-specific component CbrT of predicted cobalamin ECF transporter
V-type ATP synthase subunit D
aminoglycoside phosphotransferase family protein
D-alanyl transfer protein DltB
DNA gyrase subunit A
FIG004454: RNA binding protein
TPR-repeat-containing protein
Tyrosine recombinase XerC
Glycerol dehydrogenase
Transcriptional repressor for NAD biosynthesis in gram-positives
Metal-dependent hydrolase
Lipid A export ATP-binding/permease protein MsbA
Tripeptide aminopeptidase
Lantibiotic salivaricin A
Serine/threonine protein kinase PrkC, regulator of stationary phase
Hypothetical protein YggS, proline synthase co-transcribed bacterial homolog PROSC
UDP-N-acetylmuramoylalanyl-D-glutamate--L- lysine ligase
Late competence protein ComEC, DNA transport
Transcriptional repressor of arabinoside utilization operon, GntR family
SSU rRNA (adenine(1518)-N(6)/adenine(1519)-N(6))- dimethyltransferase
FIG01114299: hypothetical protein
Peptide methionine sulfoxide reductase MsrA / Peptide methionine sulfoxide reductase MsrB
Two-component sensor histidine kinase, malate
4-hydroxy-2-oxoglutarate aldolase @ 2-dehydro-3-deoxyphosphogluconate aldolase
Imidazolonepropionase
Malonyl CoA-acyl carrier protein transacylase
DegV family protein in cluster with TrmH family tRNA/rRNA methyltransferase YacO
FIG001614: Membrane protein
Biotin carboxylase of acetyl-CoA carboxylase
Serine protease, DegP/HtrA, do-like
DinG family ATP-dependent helicase YoaA
Nicotinate phosphoribosyltransferase

Table 4. continued from previous page.

Annotation
Tributyrin esterase
Dihydrolipoamide acetyltransferase component (E2) of acetoin dehydrogenase complex
2-haloalkanoic acid dehalogenase
SSU ribosomal protein S18p @ SSU ribosomal protein S18p, zinc-independent
GTP-binding protein Era
Hydrolase (HAD superfamily)
Transcriptional regulator, GntR family
LSU ribosomal protein L20p
PTS system, mannose/fructose family IIB component
Beta-galactosidase
FIG01115289: hypothetical protein
Phosphoribosylaminoimidazole carboxylase catalytic subunit
Glycosyltransferase LafB, responsible for the formation of Gal-Glc-DAG
FIG01115071: hypothetical protein
Putative stomatin/prohibitin-family membrane protease subunit YbbK
SSU ribosomal protein S17p (S11e)
Trehalose operon transcriptional repressor
ABC transporter, predicted N-acetylneuraminate transport system permease protein 1
Transcription-repair coupling factor
Methylenetetrahydrofolate dehydrogenase (NADP+) / Methenyltetrahydrofolate cyclohydrolase
Transcriptional regulator ArcR essential for anaerobic expression of the ADI pathway, Crp/Fnr family
FIG009688: Thioredoxin
LSU m5C1962 methyltransferase RlmI
Membrane-associated phospholipid phosphatase
Holo-[acyl-carrier protein] synthase
FIG000605: protein co-occurring with transport systems (COG1739)
DEAD-box ATP-dependent RNA helicase CshB
FIG01114159: hypothetical protein
V-type ATP synthase subunit C
FIG01115348: hypothetical protein
Cystathionine beta-lyase
Dipeptidase
C3-degrading proteinase
tRNA pseudouridine synthase A
Transcriptional regulator, RofA
Multidrug resistance efflux pump PmrA
NAD-dependent oxidoreductase

Table 4. continued from previous page.

Annotation
PTS system, mannose-specific IID component
UDP-N-acetylenolpyruvoylglucosamine reductase
DNA recombination and repair protein RecO
Hypothetical protein SPy1656
Glucose-1-phosphate thymidylyltransferase
putative repressor - phage associated
FIG01114855: hypothetical protein
Hydroxymethylpyrimidine phosphate kinase ThiD
Cytoplasmic copper homeostasis protein CutC
FIG107367: tRNA-binding protein
Alpha-L-Rha alpha-1,2-L-rhamnosyltransferase/alpha-L-Rha alpha-1,3-L- rhamnosyltransferase
proposed amino acid ligase found clustered with an amidotransferase
DNA polymerase III epsilon subunit
Copper-translocating P-type ATPase
DNA ligase
Ribosomal-protein-S5p-alanine acetyltransferase
Response regulator CsrR
Nucleoside diphosphate kinase
Transcriptional regulator, MarR family
Hypothetical mga-associated protein
FIG01114333: hypothetical protein
Chorismate synthase
Multidrug resistance protein B
FIG01116174: hypothetical protein
Ribosomal large subunit pseudouridine synthase B
Exodeoxyribonuclease VII small subunit
FIG01114118: hypothetical protein
Putative membrane protein YeiH
Protein-N(5)-glutamine methyltransferase PrmC, methylates polypeptide chain release factors RF1 and RF2
Probable aromatic ring hydroxylating enzyme, evidenced by COGnitor; PaaD-like protein (DUF59) involved in Fe-S cluster assembly
Hemolysin III
Enoyl-[acyl-carrier-protein] reductase [FMN]
Histidyl-tRNA synthetase
ABC transporter, ATP-binding protein EcsA
Zinc transport protein ZntB

Table 4. continued from previous page.

Annotation
Exfoliative toxin A
FIG01114865: hypothetical protein
L-proline glycine betaine ABC transport system permease protein ProV (TC 3.A.1.12.1)
hypothetical protein
Uridine monophosphate kinase
Transaldolase
Ribulose-phosphate 3-epimerase
S1 RNA binding domain
UDP-N-acetylmuramoylalanine--D-glutamate ligase
Peptidyl-tRNA hydrolase
Dipeptide transport ATP-binding protein DppD (TC 3.A.1.5.2)
Recombination inhibitory protein MutS2
Glutamate racemase
GMP reductase
Glutamyl-tRNA synthetase @ Glutamyl-tRNA(Gln) synthetase
5'-nucleotidase
L-asparaginase
Acetate kinase
Alcohol dehydrogenase, zinc-containing
ABC transporter, substrate-binding protein
Glucosamine--fructose-6-phosphate aminotransferase [isomerizing]
C5a peptidase
Glutaminyl-peptide cyclotransferase
Universal stress protein family
Immunodominant antigen A
C3 family ADP-ribosyltransferase
Phosphohydrolase (MutT/nudix family protein)
Ribonuclease III
Candidate zinc-binding lipoprotein ZinT
FIG028593: membrane protein
ATP synthase F0 sector subunit a
PTS system, trehalose-specific IIB component / PTS system, trehalose-specific IIC component / PTS system, trehalose-specific IIA component
Calcium-transporting ATPase
Chromosomal replication initiator protein DnaA
Fibronectin/fibrinogen-binding protein
PTS system, cellobiose-specific IIA component

Table 4. continued from previous page.

Annotation
Transcriptional regulator, Cro/CI family
Dicarboxylate/amino acid:cation (Na+ or H+) symporter
TsaB protein, required for threonylcarbamoyladenosine (t(6)A) formation in tRNA
Archaeal S-adenosylmethionine synthetase
PTS system IIB component
FIG01115635: hypothetical protein
Molybdopterin binding motif, CinA N-terminal domain / C-terminal domain of CinA type S
FIG00755240: hypothetical protein
Transcription elongation factor GreA
3-oxoacyl-[acyl-carrier-protein] synthase, KASIII
Oligoendopeptidase F
Undecaprenyl-diphosphatase
Glutamyl aminopeptidase
Transcriptional regulator in cluster with beta-lactamase, GntR family
Cysteine and methionine metabolism regulator CmbR, LysR family
Major facilitator:Oxalate:Formate Antiporter
N-acetylglucosamine-6-phosphate deacetylase
Streptolysin S export transmembrane permease (SagI)
Acyl carrier protein
Toxin to DNA-damage-inducible protein J
Ornithine carbamoyltransferase
3-ketoacyl-CoA thiolase @ Acetyl-CoA acetyltransferase
Thymidine kinase
Phosphate transport system permease protein PstC (TC 3.A.1.7.1)
DNA polymerase III alpha subunit
Manganese superoxide dismutase
Maltodextrin phosphorylase
Heat shock protein 60 family co-chaperone GroES
N-acetylneuraminate lyase
Dihydroneopterin aldolase
Putative regulator of the mannose operon, ManO
Hydrolase (HAD superfamily), YqeK
Translation elongation factor G
Signal recognition particle, subunit Ffh SRP54 (TC 3.A.5.1.1)
L-xylulose 5-phosphate 3-epimerase
PTS system, galactose-specific IIB component
Cysteine ABC transporter, permease protein

Table 4. continued from previous page.

Annotation
Streptokinase
V-type ATP synthase subunit F
Response regulator of the competence regulon ComE
Maltose/maltodextrin ABC transporter, permease protein MalF
NAD kinase
FIG01114318: hypothetical protein
2-keto-3-deoxy-D-arabino-heptulosonate-7- phosphate synthase I beta
Transcriptional regulator
ABC transporter permease protein
Phosphoglucosamine mutase
Additional lipoprotein component of predicted cobalamin ECF transporter
FIG01114555: hypothetical protein
Shikimate/quinate 5-dehydrogenase I beta
Streptolysin S biosynthesis protein C (SagC)
ABC transporter permease protein
Uracil-DNA glycosylase, family 1
LSU ribosomal protein L6p (L9e)
Two-component sensor kinase YesM
Sortase A, LPXTG specific
Salt-stress induced protein
Heat-inducible transcription repressor HrcA
FIG01115320: hypothetical protein
ATP-dependent Clp protease proteolytic subunit
Permease
Short chain dehydrogenase
HIT family protein
RecU Holliday junction resolvase
rRNA small subunit methyltransferase H
ABC-type Fe3+-siderophore transport system, permease component
FIG145533: Methyltransferase
FIG006988: Lipase/Acylhydrolase with GDSL-like motif
FIG01114091: hypothetical protein
Copper chaperone
Phosphopantothenoylcysteine synthetase
Thiol-activated cytolysin # pneumolysin
Ribose 5-phosphate isomerase A
FIG01115531: hypothetical protein

Table 4. continued from previous page.

Annotation
PTS system, sucrose-specific IIB component / PTS system, sucrose-specific IIC component / PTS system, sucrose-specific IIA component
Low temperature requirement C protein
GTP-binding protein Obg
FIG01114374: hypothetical protein
16S rRNA (cytosine(967)-C(5))-methyltransferase
Substrate-specific component NiaX of predicted niacin ECF transporter
Diphosphomevalonate decarboxylase
Peptide chain release factor 2; programmed frameshift-containing
PTS system, N-acetylgalactosamine-specific IIC component
LSU ribosomal protein L24p (L26e)
hypothetical protein
Tyrosine recombinase XerD
Pantothenate kinase
Serine endopeptidase ScpC
Regulation of D-alanyl-lipoteichoic acid biosynthesis, DltR
Dihydrofolate reductase
Cell division initiation protein DivIVA
Putative metallopeptidase (Zinc) SprT family
Beta-glucosidase
tRNA-dependent lipid II--L-alanine ligase @ tRNA-dependent lipid II--L-serine ligase
Acetoin dehydrogenase E1 component alpha-subunit
Hypothetical NagD-like phosphatase
predicted 4-deoxy-L-threo-5-hexosulose-uronate ketol-isomerase
Low molecular weight protein tyrosine phosphatase
DNA binding protein, FIG046916
Hypothetical DUF1027 domain protein
Aspartyl-tRNA(Asn) amidotransferase subunit A @ Glutamyl-tRNA(Gln) amidotransferase subunit A
Two-component sensor kinase SA14-24
hypothetical protein
Cadmium resistance protein
putative esterase
Peptide chain release factor 3
Methionine ABC transporter ATP-binding protein
Hypothetical, related to broad specificity phosphatases COG0406
FIG002344: Hydrolase (HAD superfamily)
Competence protein CoiA

Table 4. continued from previous page.

Annotation
dTDP-4-dehydrorhamnose 3,5-epimerase
Pyruvate formate-lyase
O-methyltransferase family protein [C1]
Asparaginyl-tRNA synthetase
GMP synthase [glutamine-hydrolyzing], amidotransferase subunit / GMP synthase [glutamine-hydrolyzing], ATP pyrophosphatase subunit
Magnesium and cobalt transport protein CorA
COG1399 protein in cluster with ribosomal protein L32p, Firmicutes subfamily
Undecaprenyl-phosphate N-acetylglucosaminyl 1-phosphate transferase
Hydroxymethylglutaryl-CoA reductase
Putative tRNA-m1A22 methylase
FIG01114027: hypothetical protein
Alanyl-tRNA synthetase
PTS system, lactose-specific IIB component / PTS system, lactose-specific IIC component
Spermidine Putrescine ABC transporter permease component potC (TC_3.A.1.11.1)
Cytochrome c-type biogenesis protein CcdA homolog, associated with MetSO reductase
bacterial seryl-tRNA synthetase related
ATP-dependent DNA helicase UvrD/PcrA
Maltose/maltodextrin ABC transporter, permease protein MalG
FIG01114293: hypothetical protein
Phage lysin, glycosyl hydrolase, family 25
ABC transporter, predicted N-acetylneuraminate-binding protein
Putative deoxyribose-specific ABC transporter, ATP-binding protein
DegV family protein
Late competence protein ComGB, access of DNA to ComEA
Competence-associated EpuA protein
Short chain fatty acids transporter
Ribosyl nicotinamide transporter, PnuC-like
Ribonucleotide reductase transcriptional regulator NrdR
ABC transporter membrane-spanning permease - glutamine transport
Pyruvate,phosphate dikinase
Branched-chain amino acid aminotransferase
FIG01114845: hypothetical protein
SSU ribosomal protein S4p (S9e)
Pyruvate formate-lyase activating enzyme
tRNA (cytidine(34)-2'-O)-methyltransferase
DegV family protein

Table 4. continued from previous page.

Annotation
Para-aminobenzoate synthase, amidotransferase component
Two component transcriptional regulator VraR
tRNA(Ile)-lysidine synthetase
Cardiolipin synthetase
Phosphate ABC transporter, periplasmic phosphate-binding protein PstS (TC 3.A.1.7.1)
GTP pyrophosphokinase, (p)ppGpp synthetase I
Late competence protein ComEA, DNA receptor
FIG002958: hypothetical protein
NAD(FAD)-utilizing dehydrogenases
Inner membrane protein translocase component YidC, short form OxaI-like
ATP-dependent DNA helicase RecG
5-Enolpyruvylshikimate-3-phosphate synthase
tRNA dihydrouridine synthase B
N-acetylmannosamine kinase
Transcription regulator [contains diacylglycerol kinase catalytic domain]
L-lactate dehydrogenase
Alpha-D-GlcNAc alpha-1,2-L-rhamnosyltransferase
ABC-transporter (ATP-binding protein) -possibly involved in cell wall localization and side chain formation of rhamnose-glucose polysaccharide
FIG146085: 3'-to-5' oligoribonuclease A, Bacillus type
putative L-glutamate ligase
FIG001802: Putative alkaline-shock protein
Ribosomal large subunit pseudouridine synthase D
FIG04612: Integral membrane protein (putative)
GTP-sensing transcriptional pleiotropic repressor codY
4-diphosphocytidyl-2-C-methyl-D-erythritol kinase
RNA binding methyltransferase FtsJ like
Maltose operon transcriptional repressor MalR, LacI family
FIG01115002: hypothetical protein
FIG01114110: hypothetical protein
FIG01116268: hypothetical protein
FIG015049: hypothetical protein
Alcohol dehydrogenase; Acetaldehyde dehydrogenase
FIG01114225: hypothetical protein
Phenylalanyl-tRNA synthetase alpha chain
Phosphinothricin N-acetyltransferase
Amino acid ABC transporter, amino acid-binding protein

Table 4. continued from previous page.

Annotation
Oligopeptide transport ATP-binding protein OppF (TC 3.A.1.5.1)
Ribosomal RNA small subunit methyltransferase E
ATP-dependent RNA helicase YfmL
TsaD/Kae1/Qri7 protein, required for threonylcarbamoyladenosine t(6)A37 formation in tRNA
FIG01115656: hypothetical protein
Late competence protein ComC, processing protease
COG2827: putative endonuclease containing a URI domain
hypothetical protein
putative esterase
Oleate hydratase
Cystathionine beta-lyase
Phosphocarrier protein of PTS system
Poly(glycerophosphate chain) D-alanine transfer protein DltD
FIG01114677: hypothetical protein
Beta-phosphoglucomutase
Methionine ABC transporter permease protein
Riboflavin kinase / FMN adenylyltransferase
Fructokinase
Multiple sugar ABC transporter, membrane-spanning permease protein MsmF
Ribonucleotide reduction protein NrdI
[Citrate [pro-3S]-lyase] ligase
Pullulanase
GTP-binding protein TypA/BipA
Formiminoglutamase
GlpG protein (membrane protein of glp regulon)
Two-component sensor kinase yesM, associated with MetSO reductase
Glycerol-3-phosphate transporter
Orotidine 5'-phosphate decarboxylase
FIG01114549: hypothetical protein
DNA-binding protein HBsu
Zinc ABC transporter, ATP-binding protein ZnuC
D-alanyl-D-alanine carboxypeptidase
Voltage-gated chloride channel family protein
Outer surface protein of unknown function, cellobiose operon
Uncharacterized conserved protein, contains double-stranded beta-helix domain
Acyl-phosphate:glycerol-3-phosphate O-acyltransferase PlsY
FIG01115226: hypothetical protein

Table 4. continued from previous page.

Annotation
Protein export cytoplasm protein SecA ATPase RNA helicase (TC 3.A.5.1.1)
Guanine-hypoxanthine permease
FIG01114610: hypothetical protein
Exodeoxyribonuclease III
HPr kinase/phosphorylase
FIG01114725: hypothetical protein
Adenine phosphoribosyltransferase
Hypothetical protein DUF901, similar to C-terminal domain of ribosome protection-type Tc-resistance proteins
CTP synthase
PTS system, cellobiose-specific IIB component
PTS system, hyaluronate-oligosaccharide-specific IIA component
Iron-sulfur cluster assembly protein SufD
Cytidine deaminase
Strepotococcal cysteine protease (Streptopain) / Streptococcal pyrogenic exotoxin B (SpeB)
Immunoreactive protein Se23.5 (Fragment)
FIG01115477: hypothetical protein
Cell division protein FtsI [Peptidoglycan synthetase]
FIG01115236: hypothetical protein
Putative Holliday junction resolvase YggF
PTS system, cellobiose-specific IIC component
Cysteinyl-tRNA synthetase
dCMP deaminase; Late competence protein ComEB
Sodium-dependent phosphate transporter
FIG007491: hypothetical protein YeeN
PTS system, fructose-specific IIA component / PTS system, fructose-specific IIB component / PTS system, fructose-specific IIC component
hypothetical protein
Arginine deiminase
Multimodular transpeptidase-transglycosylase / Penicillin-binding protein 1A/1B (PBP1)
V-type ATP synthase subunit A
S-adenosylmethionine synthetase
FIG01114264: hypothetical protein
Seryl-tRNA synthetase
LSU ribosomal protein L15p (L27Ae)
Polysaccharide deacetylase
FIG01114304: hypothetical protein

Table 4. continued from previous page.

Annotation
V-type ATP synthase subunit G
Ribonucleotide reductase of class Ib (aerobic), beta subunit
Glutathione reductase
Luciferase-like monooxygenase
Dihydropteroate synthase
Hypothetical similar to thiamin biosynthesis lipoprotein ApbE
SSU ribosomal protein S16p
FIG00553873: hypothetical protein
Phosphoribosylformylglycinamidine synthase, synthetase subunit / Phosphoribosylformylglycinamidine synthase, glutamine amidotransferase subunit
Bacteriocin-like peptide M BlpM
FIG01116077: hypothetical protein
Preprotein translocase subunit SecG (TC 3.A.5.1.1)

S. pyogenes genome structure

Bacterial evolution is driven by changes in genome structure, such as rearrangements, recombination either in a genome or between a genome and exogenous DNA, and occurrence of single nucleotide polymorphisms (SNPs) (Fraser, Hanage, & Spratt, 2007; Hughes, 2000; Wilson, 2012). The predominant changes in genome structure are different for each bacterial species; frequent recombinations are evident in *Helicobacter pylori* and *Porphyromonas gingivalis* (Suerbaum, et al., 1998; Watanabe, Nozawa, Aikawa, Amano, Maruyama, & Nakagawa, 2013b), while the periodontal bacterium *Tannerella forsythia* exhibits a stable genome structure without intricate rearrangements (Endo, et al., 2015). By using the Mauve alignment program (Darling, Mau, Blattner, & Perna, 2004; Darling, Mau, & Perna, 2010), the structural differences between 19 *S. pyogenes* genomes show a conserved structure for the *S. pyogenes* genome (Figure 3). In this figure, conserved regions among strains can be seen as local colinear blocks (LCBs), which are shown as colored rectangles. The LCBs are linked by a single line beyond strains if they belong to the same LCB cluster (Darling, Mau, Blattner, & Perna, 2004; Darling, Mau, & Perna, 2010). Considering that complex genome rearrangements were not observed, even in insertions sequences (IS), the hot spots for recombination in *S. agalactiae* (Rosinski-Chupin, et al., 2013), it would appear that *S. pyogenes* is more likely to conserve its genome structure. However, large-scale genome rearrangements were reported to derive a new prophage region in *S. pyogenes* (Nakagawa, et al., 2003), and the significance of recombination is inferred as an inter-species event for evolution of the genus Streptococcus (Lefébure & Stanhope, 2007). This may also be the case for the *S. pyogenes* genome, in which rearrangement and recombination are driving forces of bacterial evolution—even though they are not the principal mechanism of genomic structural changes.

Phylogenetic delineation of *S. pyogenes* strains

To delineate strains in a particular bacterial species, typing methods have been developed that are widely used for various species (Foxman, Zhang, Koopman, Manning, & Marrs, 2005; Sabat, et al., 2013). The pioneering work of Lancefield and colleagues established the M protein as a phenotype-based typing method for *S. pyogenes* (Gooder, 1961; Widdowson, Maxted, & Grant, 1970). The M protein is a protein exposed on the surface of the cell and is an antigen that is targeted by host antibodies. It was observed that differences exist among M proteins,

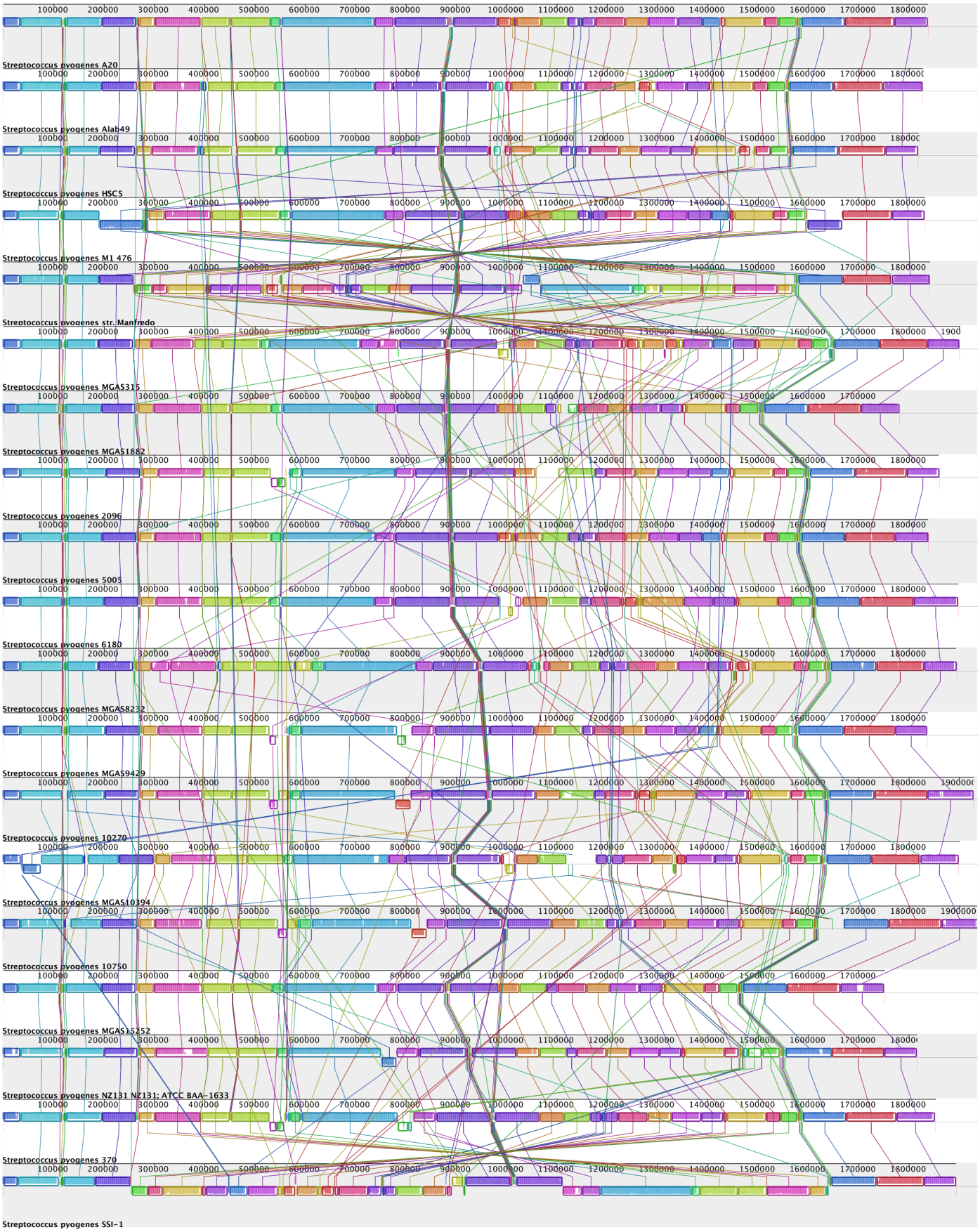

Figure 3. Alignment of 19 *S. pyogenes* genomes. Complete genome sequences of 19 *S. pyogenes* strains were aligned by the software Mauve, using progressive Mauve mode. Each colored rectangle indicates local colinear blocks (LCBs), which are the conserved regions among strains.

as they showed significant antigenic shift and drift, as compared to other surface proteins of *S. pyogenes* such as T protein, R protein, capsular polysaccharides, and peptidoglycan.

The serological method for M typing was based on the antigenic diversity of the M protein, and this method served as the major typing mechanism for *S. pyogenes* for many years. However, technical difficulties such as the need for new antiserum with the increasing number of new M protein types discovered prevented the widespread use of this M typing method. In 2000, serological M protein typing was replaced with *emm* sequence typing, which was developed as a comparison of nucleotide sequence of the M protein and was shown to correlate well with the serologic method (Facklam, et al., 1999; Beall, Gherardi, Lovgren, Facklam, Forwick, & Tyrrell, 2000). It has been reported that certain relationships exist between *emm* types and tissue sites where *S. pyogenes* infects (Bessen, et al., 2011; McGregor, et al., 2004), and that particular *emm* types are responsible for the epidemic prevalence of *S. pyogenes* (Creti, et al., 2007; Lamagni, et al., 2008).

Multilocus sequence typing (MLST) is a sequence-based genotyping method that is widely used for various bacterial species. MLST in *S. pyogenes* uses nucleotide sequences of seven gene loci (glucose kinase, *gki*; glutamine transporter protein, *gtr*; glutamate racemase, *murI*, DNA mismatch repair protein, *mutS*; transketolase, *recP*; xanthine phosphoribosyl transferase, *xpt*; and acetyl coenzyme A acetyltransferase, *yqiL*) to compare nucleotide differences by sequence alignment (Maiden, et al., 1998; Maiden, 2006). MLST provides a higher resolution and delineation than pulsed-field gel electrophoresis (PFGE), a method that is based on fragment length polymorphisms viewed in an electrophoresis image (Sabat, et al., 2013). An advantage of MLST is that the actual method can be generalized because of the use of nucleotide sequences, as compared to PFGE, in which band image resolution is sometimes distorted. For *S. pyogenes*, sequence data of previous MLST analyses are publicly available online (http://www.mlst.net).

In recent years, phylogenetic delineation based on whole genome sequences has advanced, because of the emergence of next-generation sequencing technology. Genome-based methods can identify nucleotide regions and sites with unique phylogenetic signals with a more accurate delineation than other methods, such as MLST, in which the precise analytic information is limited. A phylogenetic tree of 19 *S. pyogenes* strains was constructed that employed sets of its core genes by RAxML analysis. The 1,342 core genes were tested by the Phi test to exclude those areas in which rearrangement events were possible. This test removed sites that were insignificant for tree construction, which resulted in 689 core genes. In the constructed tree, some strains were clustered at the end of a branch; and when *emm* type information was considered, relationships between the clusters and *emm* type were more apparent (Figure 4).

Genetic position of *S. pyogenes* in the genus Streptococcus

Members of the genus *Streptococcus* are widespread among various environments, including human, animal, and natural environments (Köhler, 2007). *S. pyogenes* is phenotypically distinguished from the other *Streptococcus* species by its colony size and hemolytic activity. Lancefield employed eighteen groups of specific carbohydrates to classify *Streptococcus* species into groups A to O, and the Lancefield group A corresponded to *Streptococcus pyogenes*, which led to its designation as the group A streptococci (Lancefield, 1962). The complete genome sequences of the genus *Streptococcus* in the NCBI nucleotide database currently includes organisms that were originally designated into 27 species, according to bacterial nomenclature, as well as 2 unnamed species (as of August 2014). By using 300 core genes in 123 complete genomes of the 29 *Streptococcus* species in the NCBI database, a maximum likelihood phylogenetic tree was constructed (Figure 5, Table 5). The tree construction resulted in the delineation of the 29 species into several genetic lineages, including pyogenic, mitis, and salivarius groups, as reported in the analysis with 16S rRNA gene sequences in the previous study (Thompson, Emmel, Fonseca, Marin, & Vicente, 2013). *S. pyogenes* is shown to be genetically closest to *Streptococcus dysgalactiae* subsp. *equisimilis*, but can certainly be distinguished from that species, as supported by an extremely high bootstrap value (100% support in 100 bootstrap iterations). Recently, Richards et al. (Richards, et al., 2014) reported a similar phylogenomic tree using 44 species with similar methods. Although the phylogeny is

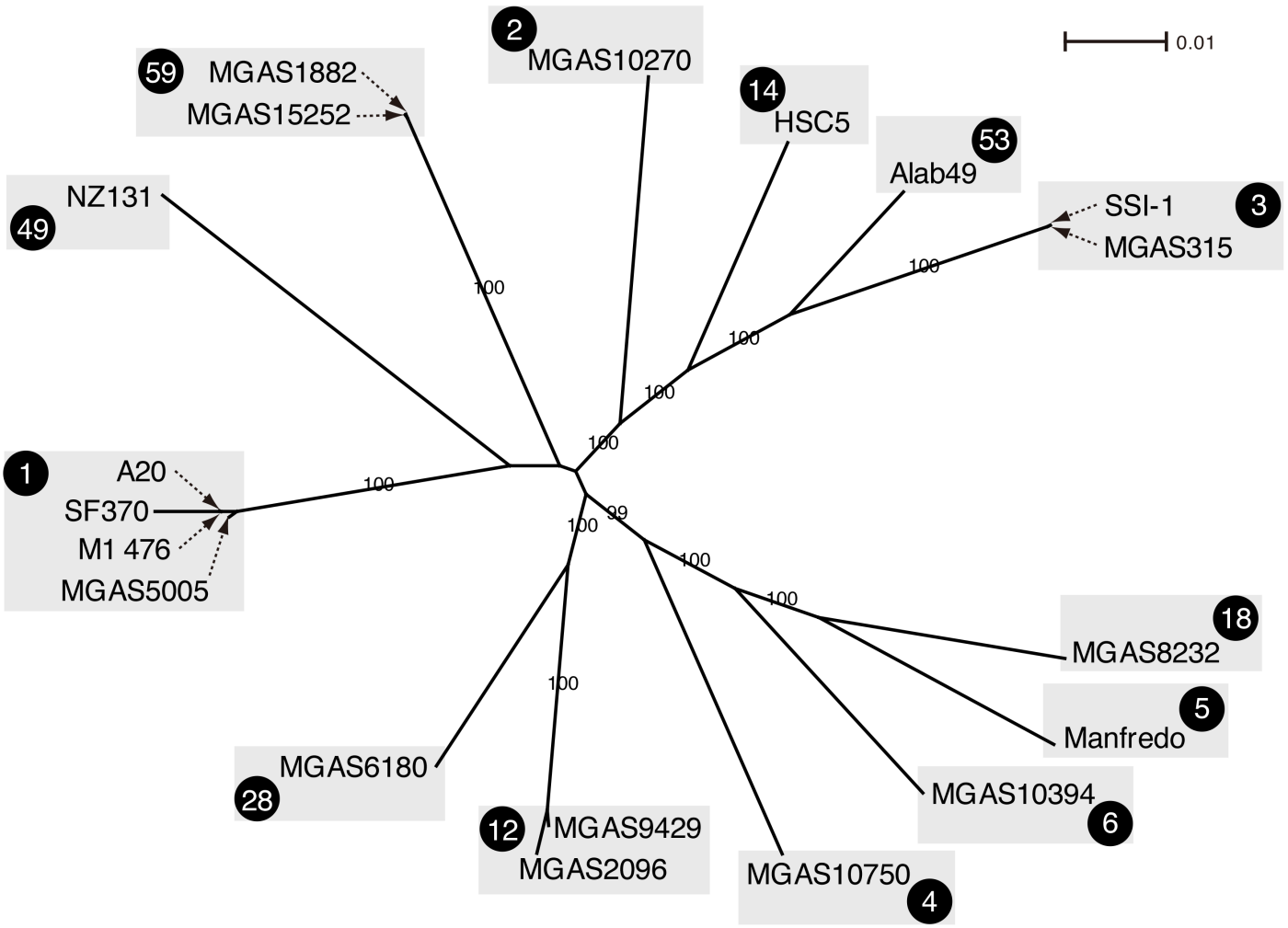

Figure 4. Maximum likelihood-based phylogenetic tree of 19 *S. pyogenes* genomes. Among the 19 *S. pyogenes* genomes, the core genes were 1,342 genes, and 689 out of 1,342 were tested and found to be free from rearrangement in the gene. The software RAxML was used for amino acid sequences of 689 genes to construct a maximum likelihood-based phylogenetic tree under the amino acid substitution model JTT and 100-times bootstrap iteration. Only the bootstrap values over 95% are shown along the tree branches. The *emm* type of each strain is shown as the number in the black circle.

consistent with that shown in Figure 5, the bootstrap value is lower, which results from a limitation of the draft genomes with a shorter core region compared to the complete genome, and which indicates that a determination of more complete genomes for each species is required to create an accurate phylogenetic tree.

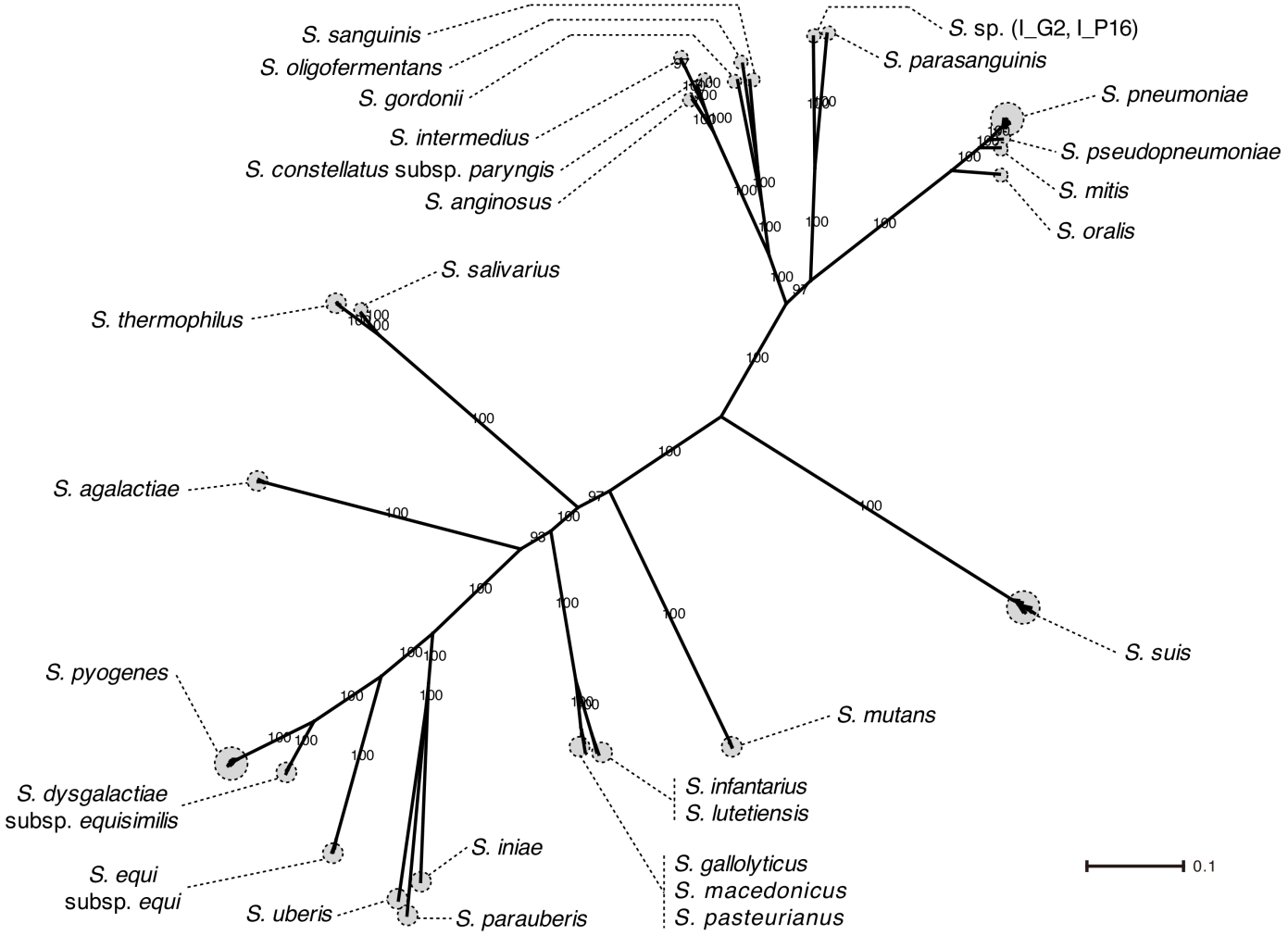

Figure 5. Maximum likelihood-based phylogenetic tree of 123 *Streptococcus* genomes. As well as in Figure 3, we determined 506 core genes among 123 *Streptococcus* genes, and 300 out of 506 were free from rearrangement in the gene. A maximum likelihood-based phylogenetic tree was constructed under the amino acid substitution model WAG and a 100-times bootstrap iteration. In the tree, the names of strains are omitted, and the species names are shown for the corresponding strains by surrounding the broken lines. Only the bootstrap values over 95% are shown along the tree branches.

Table 5. Strains of Streptococcus species with complete genome sequence (*S. pyogenes* strains in Table 1 not included)

Species	Strain	Genome size (bp)	Number of CDS	Number of tRNA	Number of rRNA operon	NCBI accession no.	Reference
Streptococcus sp.	I-G2	1,992,567	1,853	59	10	NC_022584	Unspecified
Streptococcus sp.	I_P16	2,023,580	1,952	84	13	NC_022582	Unspecified
Streptococcus agalactiae	09mas018883	2,138,694	2,086	80	21	NC_021485	(Zubair, et al., 2013)
Streptococcus agalactiae	2603V/R	2,160,267	2,134	80	21	NC_004116	(Tettelin, et al., 2002)
Streptococcus agalactiae	2-22	1,838,867	1,885	72	17	NC_021195	(Rosinski-Chupin, et al., 2013)
Streptococcus agalactiae	A909	2,127,839	2,089	80	21	NC_007432	(Tettelin, et al., 2005)

Table 5. continued from previous page.

Species	Strain	Genome size (bp)	Number of CDS	Number of tRNA	Number of rRNA operon	NCBI accession no.	Reference
Streptococcus agalactiae	GD201008-001	2,063,112	2,011	77	20	NC_018646	(Liu, Zhang, & Lu, 2012)
Streptococcus agalactiae	ILRI005	2,109,759	2,149	80	21	NC_021486	(Zubair, de Villiers, Younan, Andersson, Tettelin, & Riley, 2013)
Streptococcus agalactiae	ILRI112	2,029,198	2,069	79	21	NC_021507	(Zubair, de Villiers, Younan, Andersson, Tettelin, & Riley, 2013)
Streptococcus agalactiae	NEM316	2,211,485	2,157	80	21	NC_004368	(Sitkiewicz, Green, Guo, Bongiovanni, Witkin, & Musser, 2009)
Streptococcus agalactiae	SA20-06	1,820,886	1,842	79	21	NC_019048	(Pereira, et al., 2013)
Streptococcus anginosus	C1051	1,911,706	1,872	58	12	NC_022244	(Olson, et al., 2013)
Streptococcus anginosus	C238	2,233,640	2,214	58	12	NC_022239	(Olson, et al., 2013)
Streptococcus constellatus subsp. paryngis	C1050	1,991,156	2,012	59	12	NC_022238	(Olson, et al., 2013)
Streptococcus constellatus subsp. paryngis	C232	1,935,414	1,962	59	12	NC_022236	(Olson, et al., 2013)
Streptococcus constellatus subsp. paryngis	C818	1,935,662	1,964	59	12	NC_022245	(Olson, et al., 2013)
Streptococcus dysgalactiae subsp. equisimilis	167	2,076,397	2,111	57	15	NC_022532	(Watanabe, Kirikae, & Miyoshi-Akiyama, 2013a)
Streptococcus dysgalactiae subsp. equisimilis	AC-2713	2,179,445	2,146	57	15	NC_019042	Unspecified
Streptococcus dysgalactiae subsp. equisimilis	ATCC 12394	2,159,491	2,144	57	15	NC_017567	(Suzuki, et al., 2011)
Streptococcus dysgalactiae subsp. equisimilis	GGS_124	2,106,340	2,113	57	15	NC_012891	(Shimomura, et al., 2011)
Streptococcus dysgalactiae subsp. equisimilis	RE378	2,151,145	2,090	56	15	NC_018712	(Okumura, et al., 2012)
Streptococcus equi subsp. equi	4047	2,253,793	2,237	66	18	NC_012471	(Holden, et al., 2009b)
Streptococcus equi subsp. zooepidemicus	ATCC 35246	2,167,264	2,121	57	15	NC_017582	(Ma, et al., 2011)
Streptococcus equi subsp. zooepidemicus	MGCS10565	2,024,171	1,987	57	15	NC_011134	(Beres, et al., 2008)

Table 5. continued from previous page.

Species	Strain	Genome size (bp)	Number of CDS	Number of tRNA	Number of rRNA operon	NCBI accession no.	Reference
Streptococcus equi subsp. zooepidemicus	H70	2,149,868	2,126	57	15	NC_012470	(Holden, et al., 2009a)
Streptococcus gallolyticus	ATCC 43143	2,362,241	2,295	60	15	NC_017576	(Lin, et al., 2011)
Streptococcus gallolyticus	ATCC BAA-2069	2,356,444	2,287	80	21	NC_015215	(Hinse, et al., 2011)
Streptococcus gallolyticus	UCN34	2,350,911	2,273	71	18	NC_013798	(Rusniok, et al., 2010)
Streptococcus gordonii	Challis substr. CH1	2,196,662	2,092	59	12	NC_009785	(Vickerman, Iobst, Jesionowski, & Gill, 2007)
Streptococcus infantarius	CJ18	1,988,420	1,989	68	18	NC_016826	(Jans, Follador, Lacroix, Meile, & Stevens, 2012)
Streptococcus iniae	SF1	2,149,844	2,094	45	13	NC_021314	(Zhang, Zhang, & Sun, 2014a)
Streptococcus intermedius	B196	1,996,214	1,915	60	12	NC_022246	(Olson, et al., 2013)
Streptococcus intermedius	C270	1,960,728	1,900	60	12	NC_022237	(Olson, et al., 2013)
Streptococcus intermedius	JTH08	1,933,610	1,873	67	12	NC_018073	Unspecified
Streptococcus lutetiensis	033	1,975,547	1,975	60	18	NC_021900	(Jin, et al., 2013)
Streptococcus macedonicus	ACA-DC 198	2,130,034	2,148	70	18	NC_016749	(Papadimitriou, Ferreira, Papandreou, Mavrogonatou, Supply, & Tsakalidou, 2012)
Streptococcus mitis	B6	2,146,611	2,033	61	12	NC_013853	(Denapaite, et al., 2010)
Streptococcus mutans	GS-5	2,027,088	1,928	65	15	NC_018089	(Biswas & Biswas, 2012)
Streptococcus mutans	LJ23	2,015,626	1,926	65	15	NC_017768	(Aikawa, et al., 2012)
Streptococcus mutans	NN2025	2,013,587	1,924	65	15	NC_013928	(Maruyama, et al., 2009)
Streptococcus mutans	UA159	2,032,925	1,952	65	15	NC_004350	(Ajdić, et al., 2002)
Streptococcus oligofermentans	AS 1.3089	2,142,100	2,142	50	12	NC_021175	(Tong, Shang, Liu, Wang, Cai, & Dong, 2013)
Streptococcus oralis	Uo5	1,958,690	1,845	61	12	NC_015291	(Reichmann, et al., 2011)
Streptococcus parasanguinis	ATCC_15912	2,153,652	1,968	61	12	NC_015678	Unspecified
Streptococcus parasanguinis	FW213	2,171,609	2,020	61	12	NC_017905	(Geng, et al., 2012)
Streptococcus parauberis	KCTC 11537	2,143,887	2,235	60	15	NC_015558	(Nho, et al., 2011)

Table 5. continued from previous page.

Species	Strain	Genome size (bp)	Number of CDS	Number of tRNA	Number of rRNA operon	NCBI accession no.	Reference
Streptococcus pasteurianus	ATCC_43144	2,100,077	2,038	60	15	NC_015600	(Lin, et al., 2011)
Streptococcus pneumoniae	670-6B	2,240,045	2,317	58	12	NC_014498	(Donati, et al., 2010)
Streptococcus pneumoniae	70585	2,184,682	2,257	58	12	NC_012468	(Donati, et al., 2010)
Streptococcus pneumoniae	A026	2,091,879	2,109	58	13	NC_022655	(Sui, et al., 2013)
Streptococcus pneumoniae	AP200	2,130,580	2,175	54	9	NC_014494	(Camilli, et al., 2011)
Streptococcus pneumoniae	ATCC 700669	2,221,315	2,255	58	12	NC_011900	(Croucher, et al., 2009)
Streptococcus pneumoniae	CGSP14	2,209,198	2,228	58	12	NC_010582	(Ding, et al., 2009)
Streptococcus pneumoniae	D39	2,046,115	2,070	58	12	NC_008533	(Lanie, et al., 2007)
Streptococcus pneumoniae	G54	2,078,953	2,099	58	12	NC_011072	(Dopazo, et al., 2001)
Streptococcus pneumoniae	Hungary19A_6	2,245,615	2,306	58	12	NC_010380	(Donati, et al., 2010)
Streptococcus pneumoniae	INV104	2,142,122	2,209	58	12	NC_017591	(Donati, et al., 2010)
Streptococcus pneumoniae	INV200	2,093,317	2,120	58	12	NC_017593	(Donati, et al., 2010)
Streptococcus pneumoniae	JJA	2,120,234	2,149	58	12	NC_012466	(Donati, et al., 2010)
Streptococcus pneumoniae	OXC141	2,036,867	2,105	58	12	NC_017592	(Donati, et al., 2010)
Streptococcus pneumoniae	P1031	2,111,882	2,160	58	10	NC_012467	(Donati, et al., 2010)
Streptococcus pneumoniae	R6	2,038,615	2,062	58	12	NC_003098	(Hoskins, et al., 2001)
Streptococcus pneumoniae	SPN032672	2,131,190	2,196	58	12	NC_021003	(Donati, et al., 2010)
Streptococcus pneumoniae	SPN033038	2,133,496	2,229	58	12	NC_021004	(Donati, et al., 2010)
Streptococcus pneumoniae	SPN034156	2,024,476	2,085	58	12	NC_021006	(Donati, et al., 2010)
Streptococcus pneumoniae	SPN034183	2,037,254	2,114	58	12	NC_021028	(Donati, et al., 2010)
Streptococcus pneumoniae	SPN994038	2,026,239	2,103	58	12	NC_021026	(Donati, et al., 2010)

Table 5. continued from previous page.

Species	Strain	Genome size (bp)	Number of CDS	Number of tRNA	Number of rRNA operon	NCBI accession no.	Reference
Streptococcus pneumoniae	SPN994039	2,026,505	2,103	58	12	NC_021005	(Donati, et al., 2010)
Streptococcus pneumoniae	SPNA45	2,129,934	2,232	58	12	NC_018594	(Donati, et al., 2010)
Streptococcus pneumoniae	ST556	2,145,902	2,176	58	12	NC_017769	(Li, et al., 2012)
Streptococcus pneumoniae	TCH8431/19A	2,088,772	2,118	58	12	NC_014251	(Nelson, et al., 2010)
Streptococcus pneumoniae	TIGR4	2,160,842	2,178	58	12	NC_003028	(Tettelin, et al., 2001)
Streptococcus pneumoniae	Taiwan19F-14	2,112,148	2,140	58	12	NC_012469	(Donati, et al., 2010)
Streptococcus pneumoniae	gamPNI0373	2,064,154	2,123	57	10	NC_018630	(Williams, et al., 2012)
Streptococcus pseudopneumoniae	IS7493	2,190,731	2,248	41	3	NC_015875	(Shahinas, et al., 2011)
Streptococcus salivarius	57.I	2,138,805	1,925	68	18	NC_017594	(Geng, Huang, Li, Hu, & Chen, 2011)
Streptococcus salivarius	CCHSS3	2,217,184	1,987	68	18	NC_015760	(Delorme, et al., 2011a)
Streptococcus salivarius	JIM8777	2,210,574	1,946	68	18	NC_017595	(Guédon, et al., 2011)
Streptococcus sanguinis	SK36	2,388,435	2,298	61	12	NC_009009	(Xu, et al., 2007)
Streptococcus suis	05ZYH33	2,096,309	2,022	56	12	NC_009442	(Chen, et al., 2007)
Streptococcus suis	98HAH33	2,095,698	2,012	56	12	NC_009443	(Chen, et al., 2007)
Streptococcus suis	A7	2,038,409	1,938	56	11	NC_017622	(Zhang, et al., 2011b)
Streptococcus suis	BM407	2,146,229	2,055	56	12	NC_012926	(Holden, et al., 2009a)
Streptococcus suis	D12	2,183,059	2,092	56	12	NC_017621	(Zhang, et al., 2011b)
Streptococcus suis	D9	2,177,656	2,097	54	11	NC_017620	(Zhang, et al., 2011b)
Streptococcus suis	GZ1	2,038,034	1,943	49	10	NC_017617	(Ye, et al., 2009)
Streptococcus suis	JS14	2,137,435	2,048	62	12	NC_017618	(Hu, et al., 2011)
Streptococcus suis	P1/7	2,007,491	1,908	56	12	NC_012925	(Holden, et al., 2009b)
Streptococcus suis	S735	1,980,887	1,872	56	12	NC_018526	(Boyle, Vaillancourt, Bonifait, Charette, Gottschalk, & Grenier, 2012)
Streptococcus suis	SC070731	2,138,568	2,044	56	12	NC_020526	(Wu, et al., 2014)
Streptococcus suis	SC84	2,095,898	2,003	56	12	NC_012924	(Holden, et al., 2009b)
Streptococcus suis	SS12	2,096,866	2,021	59	12	NC_017619	(Zhang, et al., 2011b)
Streptococcus suis	ST1	2,034,321	1,986	58	12	NC_017950	(Zhang, et al., 2011b)
Streptococcus suis	ST3	2,028,815	1,959	54	11	NC_015433	(Hu, et al., 2011)

Table 5. continued from previous page.

Species	Strain	Genome size (bp)	Number of CDS	Number of tRNA	Number of rRNA operon	NCBI accession no.	Reference
Streptococcus suis	T15	2,240,234	2,157	55	11	NC_022665	Unspecified
Streptococcus suis	TL13	2,038,146	1,948	56	11	NC_021213	(Wang, Yao, Lu, & Chen, 2013b)
Streptococcus suis	YB51	2,043,655	1,970	56	12	NC_022516	(Wang, Chen, Yao, & Lu, 2013a)
Streptococcus thermophilus	CNRZ1066	1,796,226	1,900	67	18	NC_006449	(Bolotin, et al., 2004)
Streptococcus thermophilus	JIM 8232	1,929,905	1,982	67	17	NC_017581	(Delorme, et al., 2011b)
Streptococcus thermophilus	LMD-9	1,856,368	1,946	67	18	NC_008532	(Makarova, et al., 2006)
Streptococcus thermophilus	LMG_18311	1,796,846	1,898	67	18	NC_006448	(Bolotin, et al., 2004)
Streptococcus thermophilus	MN-ZLW-002	1,848,520	1,945	57	15	NC_017927	(Kang, Ling, Sun, Zhou, Zhang, & Sheng, 2012)
Streptococcus thermophilus	ND03	1,831,949	1,939	57	15	NC_017563	(Sun, et al., 2011)
Streptococcus uberis	0140J	1,852,352	1,822	58	15	NC_012004	

Factors producing individuality in *S. pyogenes*

The *S. pyogenes* genome is remarkable for its content of prophages, streptococcal phage-like chromosomal islands (SpyCIs), and other mobile genetic elements (MGEs), such as integrative and conjugative elements (ICEs) (Bessen, McShan, Nguyen, Shetty, Agrawal, & Tettelin, 2015). This book includes a separate chapter about streptococcal bacteriophages (see also (Brüssow & Hendrix, 2002; Frost, Leplae, Summers, & Toussaint, 2005), as well as a separate chapter about its acquired immunity system of clustered regularly interspaced short palindromic repeats (CRISPRs) (Bhaya, Davison, & Barrangou, 2011; Dupuis, Villion, Magadán, & Moineau, 2013; Fabre, et al., 2012; Labrie, Samson, & Moineau, 2010; Mruk & Kobayashi, 2014; Maruyama, et al., 2009). The CRISPR-associated (Cas) proteins, and specifically the Cas9 enzyme system of *S. pyogenes*, is also used for genome editing in eukaryotes based on sequence-specific DNA cleavage (Mali, Esvelt, & Church, 2013; Sander & Joung, 2014; Zhang, Wen, & Guo, 2014b).

In *S. pyogenes*, prophage regions have been shown to encode virulence factors such as exotoxins (Ferretti, et al., 2001; Banks, Lei, & Musser, 2003). In addition, it has been reported that genome rearrangements can generate a new prophage in an M3 strain (Nakagawa, et al., 2003). Integrative conjugative elements (ICEs) are also responsible for the transfer of DNA through integration and conjugation (Frost, Leplae, Summers, & Toussaint, 2005). It is known that prophages and ICEs are present on most *S. pyogenes* genomes (Nozawa, et al., 2011). These elements produce intra-strain diversity of *S. pyogenes* (see Supplemental Table 2) and produce an open–pan-genome (Figure 2). Other than the SNPs described later in this chapter and outside of the prophage and other mobile elements, most parts of *S. pyogenes* genomes are essentially colinear with the corresponding parts in other genomes—with the exception of two regions that consistently show great variation and confer much of the individuality of a given strain: the ~73-kbp region and the FCT region, both of which were previously reported (McShan, et al., 2008). The ~73-kbp region is known as a center of virulence of *S. pyogenes*, where the gene for the major antiphagocytic M protein (*emm*) and many other virulence-associated genes (*vir* regulon) are

present. The FCT region harbors many genes that encode surface matrix binding proteins, such as protein F and the streptococcal pilus or T antigen (McShan, et al., 2008). Bessen et al. have summarized these differences nicely (Bessen, McShan, Nguyen, Shetty, Agrawal, & Tettelin, 2015). As described in this chapter, a strong genetic linkage to the *emm* pattern group is observed for several other genes that have an established role in virulence or another important biological function and tropism. Accessory genes whose presence/absence are strongly linked to their *emm* pattern E (e.g., *sof*, *sfbX*, *spn/ nga*, *sse*, *grab*, *ralp3*) might play a dual role in infection of both the skin and throat epithelial tissue sites (Bessen, McShan, Nguyen, Shetty, Agrawal, & Tettelin, 2015). These important findings suggest that future studies should include the consideration of isolation sites to understand the barrier of horizontal gene transfer between different *S. pyogenes* types and the mechanism of creating variation in these two genomic positions.

Comparative genomic studies in *S. pyogenes*

Comparative genomic studies have been conducted in various bacterial species to investigate evolutionary history and strain differences (Chewapreecha, et al., 2014; Galagan, 2014; Hershberg, et al., 2008; Harris, et al., 2010). The expansion and evolutionary derivation styles of epidemic lineages are serious and elusive issues in a medical field. From this perspective, *S. pyogenes* can represent and serve as an appropriate research model of a pathogen for comparative genomic studies, because they continue to cause severe cases of worldwide human infection. Additionally, evolutionary events are spatially limited, because human tissue is the only natural host of *S. pyogenes*, which can lead to the exclusion of any other environment as a field of evolutionary change (Bessen, et al., 2011). The M1 type of *S. pyogenes* is one of the most studied lineages, as previous studies showed that M1 type strains were prevalent in several epidemic periods (Nasser, et al., 2014). The first report of a complete *S. pyogenes* genome sequence was the M1 strain SF370, and its genome sequence has served as a reference and is widely used in *S. pyogenes* research (Ferretti, et al., 2001). A subsequent study of over 60 M1 strains revealed that a contemporary strain, MGAS5005, was genetically different from the reference strain SF370 in terms of its gene and prophage content (Sumby, et al., 2005). The analyzed M1 strains were delineated by SNPs into SF370-like or MGAS5005-like strain lineages. Moreover, it was shown that an M12 lineage was suggested to be a donor of a 36-kbp chromosomal region to the current M1 lineages, which included toxic proteins, such as NAD^+-glycohydrolase (NADase) and streptolysin O (SLO). These findings were supported by a subsequent genome sequencing study that used 3,615 M1 strains, including those isolated from the 1920s to 2013 (Nasser, et al., 2014). The authors proposed that the evolutionary pathway to the current M1 strains with increased virulence occurred in a step-wise manner, with the initial event being the acquisition of a phage that encodes the SpeA1 variant of the streptococcal pyrogenic exotoxin A. In addition, a single nucleotide change resulted in the SpeA2 variant of the M1 progenitor strain, after which the M12-like 36-kbp region was acquired in a single cell in the early 1980s, and which carried the 2.6-kbp region that is located between the *slo* and *metB* genes and is highly divergent from the SF370 type.

The data from the massive sequencing effort was able to demonstrate the progression of polymorphisms that are primarily located in the 36 kbp region, which accumulated in the newly emerged hypervirulent strain in the early 1980s. These impressive studies also showed that the contemporary M1 strain was more virulent in an animal model of pharyngitis and necrotizing fasciitis than the pre-epidemic reference strain, which demonstrates how comparative genomics can track evolutionary events in the same or similar lineages. Similar findings were recently described by Turner et al. (Turner, et al., 2015) who found a dramatic shift in early M89 populations to the modern M89 strains that appeared in the 1990s by acquiring an NADase and streptolysin O locus, possibly from an M12 strain. This new variant had an enhanced expression of NADase and streptolysin O that resulted in a potentially more pathogenic strain with a selective advantage over other M89 variants, as well as a change in M89 epidemiology.

In a similar manner to the type M1 *S. pyogenes* strain lineages, type M59 strains have been shown to be prevalent in the last decade, though the type M59 strains were an uncommon cause of human disease (Fittipaldi, et al.,

2012; Luca-Harari, et al., 2009; Steer, Law, Matatolu, Beall, & Carapetis, 2009). By employing 601 type M59 strains, including those from a Canadian epidemic and from other countries, the prevalent lineage in Canada was shown to be phylogenetically distinct from the strains in other countries (Fittipaldi, et al., 2012). The genome of an invasive type M23 strain in Japan in 1965 also carried distinct characteristics from other known strains (Bao, et al., 2014). The M23 strain is phylogenetically distant from M1 or M59 strains and shares a common ancestry with M5, M6, and M18 strains. This M23 strain has unique genomic rearrangements and an asymmetric replicore that make it different than other sequenced *S. pyogenes* strains. From the date of its isolation and phylogenetic divergence, some key factors for an invasive phenotype of *S. pyogenes* may be explained by the number and variety of prophages that carry virulence genes and two-component systems (TCSs), as described below.

Significance of nucleotide polymorphisms in two-component regulatory systems and two highly variable regions for *S. pyogenes* invasiveness and pathogenesis

A subtle alteration in a bacterial genome can be a significant cause of dramatic changes of a phenotype, as previous studies have demonstrated that SNPs in the genes that encode components of TCSs are closely associated with the invasiveness of particular *S. pyogenes* lineages (Churchward, 2007). CovRS (also known as CsrRS) is a TCS in *S. pyogenes* and plays a central role in gene regulatory networks (Graham, et al., 2002). A frameshift mutation in *covS* was shown to be exclusively present among invasive strains (Sumby, Whitney, Graviss, DeLeo, & Musser, 2006). In these particular cases, the mutation caused a loss of the ability to repress expression of genes that encode virulence factors, such as streptolysin O (SLO) and a serine protease, ScpC, which led to overproduction of these factors and an impairment of neutrophil migration (Ato, Ikebe, Kawabata, Takemori, & Watanabe, 2008). In another study, a 1-bp mutation in *covS* was found to be present in hypervirulent invasive *S. pyogenes* strains (Li, et al., 2013). Neutrophils in mice were used to select *S. pyogenes* cells that carry this 1-bp mutation, which maximizes the potential to evade neutrophil responses (Li, et al., 2014). Another inactivating mutation in *covS* was reported to disturb neutrophil function by exhibiting a potent binding of host complement inhibitors, which leads to a minimal deposition of complement on *S. pyogenes* cells and a weak killing of the bacteria by neutrophils (Agrahari, Liang, Mayfield, Balsara, Ploplis, & Castellino, 2013). Abrogation of production of the protease SpeB has been found among hypervirulent strains that harbor *covS* mutations; however, a recent report has suggested that this abrogation of production was not necessary for the emergence of invasive *S. pyogenes* strains (Flores, et al., 2014). Similar to *covS*, mutations were also found in *covR*, the gene that encodes another component of the CovRS system, in invasive *S. pyogenes* strains; and a single alteration of an amino acid in CovR was shown to affect both interaction between the protein and DNA, as well as the structure of the protein (Horstmann, et al., 2011).

These findings suggest that a mechanism exists in *S. pyogenes* strains to acquire invasiveness; namely, by the overproduction of multiple virulence factors that are caused by inactivation of the repressing activity in TCS genes other than CovRS. Another example of a mechanism to acquire invasiveness is found in mutations in the negative regulator *rgg* (*ropB*) that also results in *S. pyogenes* strains acquiring invasiveness (Ikebe, et al., 2010). *S. pyogenes* genomes contain 13 TCSs, on average (Ribardo, Lambert, & McIver, 2004), and considering that the TCSs are important for regulation of expression of various genes, mutations in these TCS genes may be a key factor for the invasiveness of *S. pyogenes*. Further studies will be required, not only to understand the molecular interactions between regulated genes and uncharacterized TCS proteins, but also to investigate the invasive role of certain SNPs.

Conclusion and Perspectives

Comparative genomic studies provide an important avenue for understanding how particular lineages emerge, as well as the mechanisms involved in the overall evolution of disease involvement by an organism. *S. pyogenes* is a remarkable example of a pathogen that can invade and survive in host human cells (Nakagawa, et al., 2004). Though it may be captured by autophagosomes in autophagy (Nakagawa, et al., 2004), *S. pyogenes* can persist in host cells for a long time, and therefore, the machinery for intracellular survival by host protein degradation and propagation may also exist in *S. pyogenes*, as was previously shown in *Shigella flexineri* and *Listeria monocytogenes* (Ogawa, Yoshimori, Suzuki, Sagara, Mizushima, & Sasakawa, 2005; Py, Lipinski, & Yuan, 2007; Yoshikawa, et al., 2009). Asymptomatic carriage and antibiotic treatment failure suggest that *S. pyogenes* may be able to persist in a host after an initial infection. The asymptomatic carriage rates of 2.5% to 32% in school-age children and 1.3% to 4.9% in adult health care workers and military recruits reminds us that a considerable reservoir for future infection still remains (Wood, et al., 2009). Future research on pharyngeal tissue will be required, since it is an environment that appears to be more hydrodynamically and chemically stable for survival than an extracellular environment. Extensive comparative genomics will support these perspectives by investigating the differences between invasive and non-invasive *S. pyogenes* strains. In addition, multiple comparisons of various *emm* strains will provide insights into the role of the *emm* gene in *S. pyogenes* evolution and geographic differences.

In conclusion, this chapter has reviewed the current approach and practical application of comparative genomics to *S. pyogenes*, and has described the importance of next-generation sequencing in understanding the molecular events associated with *S. pyogenes* evolution. Comparative genomics research will continue in the future to provide significant clues for a further understanding of *S. pyogenes* itself.

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The Bacteriophages of *Streptococcus pyogenes*

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Abstract

Bacteriophages typically may be grouped into two categories by their life cycle: lytic phages and lysogenic (temperate) phages. The five decades following the discovery of phages saw numerous investigations on the lytic phages of *S. pyogenes*, which included studies on host range, basic biology, and their ability to mediate general transduction. In contrast to lysogenic phages, lytic phages do not alter the phenotype of the host streptococcal cell during a long-term genetic relationship, but can shape the host population by eliminating susceptible cells in a population, or by facilitating genetic exchange by transduction. This chapter also presents a summary of information on the lysogenic phages of group A streptococci, their distribution and attachment sites, morphology and genome organization, and associated virulence genes. Additional areas of overview include the diversity of lysogenic phages and the horizontal transfer of genes from other species. Finally, there is a discussion of prophages as vectors for virulence genes, phage-like elements that carry antimicrobial resistance genes, and regulation of host gene expression.

Lytic phages of group A streptococci

Biology and distribution of lytic phages

Bacteriophages may typically be grouped into two categories by their life cycle: lytic phages and lysogenic (temperate) phages. Lytic phages infect their host cell and begin the viral replicative cycle within a short time frame. At the end of replication and assembly, the host bacterial cell typically lyses and releases the newly formed bacteriophage particles. The five decades following the discovery of phages saw numerous investigations on the lytic phages of *S. pyogenes*, which included studies on host range, basic biology, and their ability to mediate general transduction. In contrast to lysogenic phages, lytic phages do not alter the phenotype of the host streptococcal cell during a long-term genetic relationship, but they can shape the host population by eliminating susceptible cells in a population, or by facilitating genetic exchange by transduction.

Bacteriophage A25

The best studied lytic phage of *S. pyogenes* is bacteriophage A25, which was originally isolated from Paris sewage in the early 1950s (Maxted, 1952; Maxted, 1955) and was found to mediate generalized transduction in *S. pyogenes* (Leonard, Colón, & Cole, 1968). The literature also refers to phage A25 as phage 12204, which is its designation by the American Type Culture Collection (ATCC 12204). To date, the genome sequence of A25 has not been determined, but one study estimated its linear dsDNA genome to be about 34.6 kb in length (Pomrenke & Ferretti, 1989). Electron microscopy shows that it belongs to the Siphoviridae with an isometric, octahedral head measuring 58-60 nm across and a long flexible tail that measures 180-190 nm in length and 10 nm in diameter (Malke, 1970; Zabriskie, Read, & Fischetti, 1972). The tail of this phage is composed of 8nm circular subunits and terminates in a transverse plate with a single projecting spike that is about 20nm long (Zabriskie, Read, & Fischetti, 1972; Read & Reed, 1972). In contrast to many lysogenic phages, it appears that A25 does not

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encode a hyaluronidase (hyaluronate lyase) as part of its tail fiber. However, it does encode a lysin that has activity against groups A, C, G, and H streptococci (Hill & Wannamaker, 1981).

One-step growth experiments have shown that phage A25 has an average burst size that may vary depending upon the host strain, with a reported average burst sizes of 30 PFU/cell when grown on strain K56 (Malke, 1969a) and 12 CFU/cell on strain $T25_3$ (Fischetti, Barron, & Zabriskie, 1968). Peptidoglycan is the cell receptor for A25, and treatment of the cells with the group C streptococcus phage C1 lysin (PlyC) destroyed the receptor binding (Cleary, Wannamaker, Fisher, & Laible, 1977). Phage A25 has a broad host range, being adaptable to group G streptococci after passage (Cleary, Wannamaker, Fisher, & Laible, 1977). Wannamaker and co-workers showed that phage A25 also could infect 48% of group C strains tested (Wannamaker, Almquist, & Skjold, 1973). Other *S. pyogenes* lytic phages in the same study could also infect group C strains at frequencies that range from 34% to 47%. The possibility that phage A25 and other lytic *S. pyogenes* phages can infect multiple species of streptococci may have contributed to the horizontal transfer of both host and prophage genes via transduction. At present, it is unknown whether the A25 phage packages its DNA through a terminase-mediated headful mechanism or by the recognition of *pac* sites. However, it is known that the efficiency of transduction by this phage is not highly stringent, as discussed below.

Transduction in *S. pyogenes*

Transformation, conjugation, and transduction are common means of genetic exchange in bacteria. In *S. pyogenes*, natural transformation may occur when the cells live in a biofilm (Marks, Mashburn-Warren, Federle, & Hakansson, 2014), but such an exchange has not been seen in the laboratory. Conjugative transposons are frequent elements in *S. pyogenes*, but they are not associated with the sort of transfer events seen with the F plasmid of *E. coli*. In contrast, generalized transduction occurs in *S. pyogenes* and is mediated by both lytic and lysogenic phages.

Transduction in *S. pyogenes* was first reported in 1968, detailing how five phages (three lytic and two lysogenic) were able to transduce streptomycin resistance (Leonard, Colón, & Cole, 1968). Of this group of phages, phage A25 (phage 12204) was able to transduce antibiotic resistance at the highest frequency (1×10^{-6} transductants per PFU). The transfer was DNase resistant but was sensitive to antiphage serum, which supports generalized transduction as the mechanism of genetic exchange. The capsule of *S. pyogenes* is composed of hyaluronic acid, which was found to be a barrier to A25 infection (Maxted, 1952). Lysogenic phages often encode a hyaluronidase, but phage A25 apparently lacks such a gene, since this enzymatic activity has not been associated with it. The state of *S. pyogenes* encapsulation can vary during the growth phase (Crater & van de Rijn, 1995), and some strains, such as the recently described M4 isolate from Australia (Henningham, et al., 2014), do not express a capsule at all. Therefore, the susceptibility of cells to phage-mediated transduction probably varies by growth state and genetic background, both of which could influence horizontal transfer.

A number of strategies have been used to improve transduction frequencies. Malke showed that transduction frequencies could be improved by using specific A25 antiserum to block unabsorbed or progeny phages from infecting transductants that result from the initial adsorption (Malke, 1972). Increased levels could also be obtained by the use of temperature-sensitive mutants of phage A25 (Malke, 1969a), as could UV irradiation of transducing lysates prior to adsorption to the host streptococci (Malke, 1972; Colón, Cole, & Leonard, 1970; Malke, 1969b).

Lysogenic bacteriophages of *S. pyogenes* are capable of mediating transfer of antibiotic resistance by transduction. Strains with bacteriophage T12-like prophages can produce transducing lysates that are capable of transferring resistance to tetracycline, chloramphenicol, macrolides, lincomycin, and clindamycin, following lysogen induction. Generalized transduction transfer of erythromycin and streptomycin resistance, following mitomycin C treatment of endogenous prophages, has also been observed (Hyder & Streitfeld, 1978).

Transduction may play a role in the dissemination of genes among related streptococcal species. Some bacteriophages isolated from groups A and G streptococci can infect serotype A, C, G, H, and L strains, and some were capable of infecting multiple serotypes (Colón, Cole, & Leonard, 1972). The same study showed that phage A25 could transduce streptomycin resistance to a group G strain. Wannamaker and co-workers further showed that streptomycin resistance could be transferred to *S. pyogenes* strains by a temperate transducing phage isolated from group C streptococcus (Wannamaker, Almquist, & Skjold, 1973). The wealth of *S. pyogenes* genome data supports the idea that horizontal gene transfer has been important in the evolution of this pathogen (Bessen, et al., 2015), and transduction is assumed to play an important role in this process. However, the molecular mechanisms that would drive this process are not well understood. The majority of studies on streptococcal transduction were performed before the advent of modern techniques of molecular biology and genomics, and as a result, this may be an opportune time to reexamine this phenomenon. A better understanding of streptococcal transduction may prove essential to understanding the flow of genetic information in natural populations of *S. pyogenes* and the horizontal transfer of information from other genera.

Lysogenic phages of group A streptococci

Genome prophages, their distribution, and attachment sites

Lysogenic bacteriophages are defined by their ability to integrate their DNA into the host bacterium's chromosome via site-specific recombination, becoming a stable genetic element that can be passed to daughter cells after cell division. Studies from the pre-genomics era suggested that lysogeny was common in *S. pyogenes* (Kjems E. , 1960; Krause, 1957; Chaussee, Liu, Stevens, & Ferretti, 1996; Hynes, Hancock, & Ferretti, 1995; Wannamaker, Skjold, & Maxted, 1970; Yu & Ferretti, 1989), but it was genome sequencing (starting with the first one completed and confirmed by almost every subsequent one) that demonstrated that toxin-carrying prophages were not only common, but were prominent genetic features that shaped the fundamental biology of this bacterial pathogen (Table 1). The number of lambdoid prophages or phage-like chromosomal islands found in a given genome strain has ranged from a low of one (MGAS15252) to as many as eight (MGAS10394), with three to four elements being most common. These prophages are found to be integrated into multiple sites on the *S. pyogenes* genome, and can be found in each quadrant (Figure 1 and Table 2). The majority of the genome prophages (72%) are found to be integrated into genes encoded on the lagging strand (relative to *ori*C). No prophages have been found to target genes in the hypervariable regions, which include the M-protein (*emm)* or the streptococcal pilus. Some sites are frequent targets for prophage integration; the genes for DNA binding protein HU, tmRNA, and the DNA mismatch repair (MMR) protein MutL are very commonly occupied by a prophage or prophage-like chromosomal island.

The integration of prophages occurs via a homologous exchange between sequences shared between the phage and host chromosomes (*attP* and *attB*, respectively); this process is mediated by a phage-encoded integrase. These duplications between the phage and host DNA can be as few as a few nucleotides to over 100 bp, and can often include the coding regions of the bacterial genome (Campbell, 1992; Groth & Calos, 2004; Fouts, 2006). In *S. pyogenes*, the identifiable duplications between *attB* and *attP* range from 12 bp (MGAS10394.1) to 96 bp (T12). An extensive survey of bacterial genome prophages found that prophages usually integrate into a gene ORF (69% of identified prophages) while integration into an intragenic region, such as that seen in coliphage Lambda, is less common and accounts for only 31% of prophages (Fouts, 2006). Gene targets included tRNA genes (33%), tmRNA (8%), and various other genes (28%). Examples of each target site can be observed in the *S. pyogenes* genome prophages (Table 2). Most commonly, the duplication occurs between the phage and the 3' end of the host gene, and integration leaves the open reading frame intact via the duplicated sequence (Fouts, 2006; McShan & Ferretti, 2007). However, in *S. pyogenes*, the 5' end of genes are frequently targeted for integration, which could potentially lead to an altered expression of the host gene (Table 2). The best characterized system of an altered host gene expression is the control of MMR in strain SF370 by SpyCIM1 (*S. pyogenes* chromosomal island, serotype M1) where the expression of genes for MMR, multidrug efflux, Holliday junction resolution, and

base excision repair are controlled by this phage-like chromosomal island, in response to growth (McShan & Ferretti, 2007; Nguyen & McShan, 2014; Scott, Nguyen, Hendrickson, King, & McShan, 2012; Scott, Thompson-Mayberry, Lahmamsi, King, & McShan, 2008). Similarly, the phage-like transposon MGAS10394.4 (also known as Tn1207.3 (D'Ercole, et al., 2005)) separates the DNA translocation machinery channel protein ComEC operon proteins 2 and 3, which potentially creates a polar mutation that silences protein 3. A number of other streptococcal genes are targeted at their 5' ends by prophages, including genes that encode the recombination protein RecX, a HAD-like hydrolase, and DNA-binding protein HU (Table 2). Other prophages integrate into the promoter region that precedes the ORF in dipeptidase Spy0713, *yesN* and a gamma-glutamyl kinase. In the case of dipeptidase Spy0713 that is targeted by members of the SF370.1 family, integration separates the ORF from the predicted native promoter and may replace it with a phage-encoded promoter found immediately upstream of the coding region following integration (Figure 2). This phage-encoded promoter is preceded by a canonical CinA box (Claverys & Martin, 1998), which suggests that this putative alternate promoter may also change the transcriptional program of the gene. In another example, two serotype M3 prophages were found to be integrated into a CRISPR type II system direct repeat; remarkably, this event may have led to the loss of CRISPR function in these cells. In all of these examples, the integration of a phage or phage-like element into the 5' end of a gene has the potential to alter streptococcal gene expression by blocking transcription or providing an alternative promoter, and the frequency of such transcription-altering prophages may be an important regulatory strategy in *S. pyogenes*.

Not all *S. pyogenes* prophages inactivate host genes following integration. Those that integrate into the 3' end of genes typically preserve gene function through the shared DNA sequence between the DNA molecules, and a number of examples can be found in the genome prophages (Table 2). Bacteriophage T12 integrates by site-specific recombination into what was initially identified as a gene for a serine tRNA (McShan, Tang, & Ferretti, 1997), but was correctly identified as a tmRNA gene after the completion of genome sequencing. Genes that encode tmRNA are frequently used as bacterial attachment sites (*attB*) for prophages that infect a range of bacterial species, including *Escherichia coli*, *Vibrio cholerae*, and *Dichelobacter nodosus* (Fouts, 2006; Williams, 2002). Besides the tmRNA gene, other 3' gene targets used by genome prophages include the histone-like protein HU, dTDP-glucose-4,6-dehydratase, a putative SNF helicase, and recombination protein *rec*O.

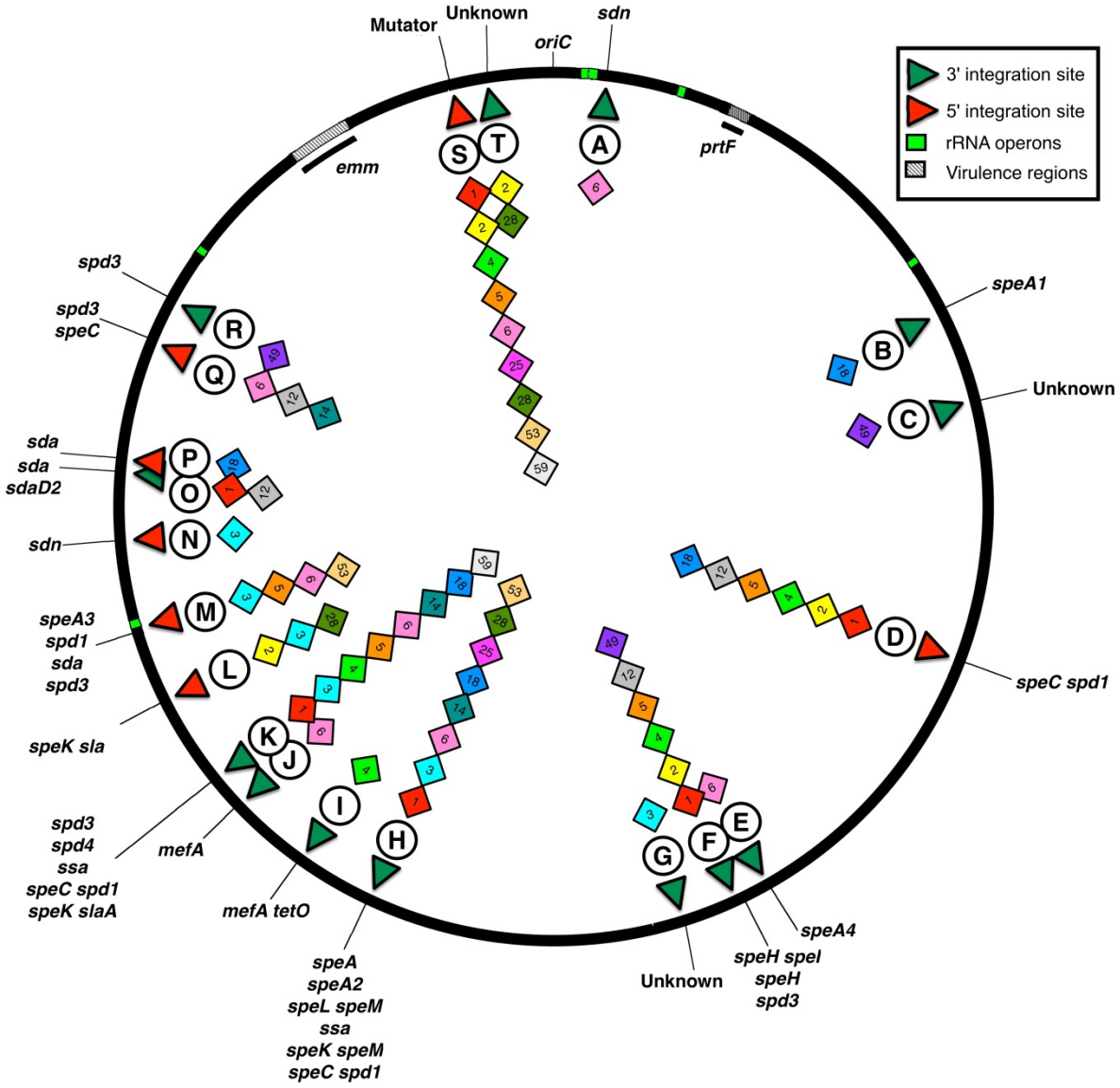

Figure 1. Prophage attachment sites in the *S. pyogenes* genome. The locations of the genome prophages are shown as a generalized chromosome backbone based upon the SF370 M1 genome; each diamond represents a genome prophage that was identified at that site. The M-type of the host for each prophage is indicated by the number within the diamond, and the circled letter is the identifier linked to **Table 2** for *attB* gene identification, integration target within that gene (5' or 3'), and associated prophage virulence genes. The rRNA operons are indicated as green blocks, while the hypervariable regions that contain virulence genes associated with *emm* or *prtF* are hatched. The origin of replication is indicated (OriC).

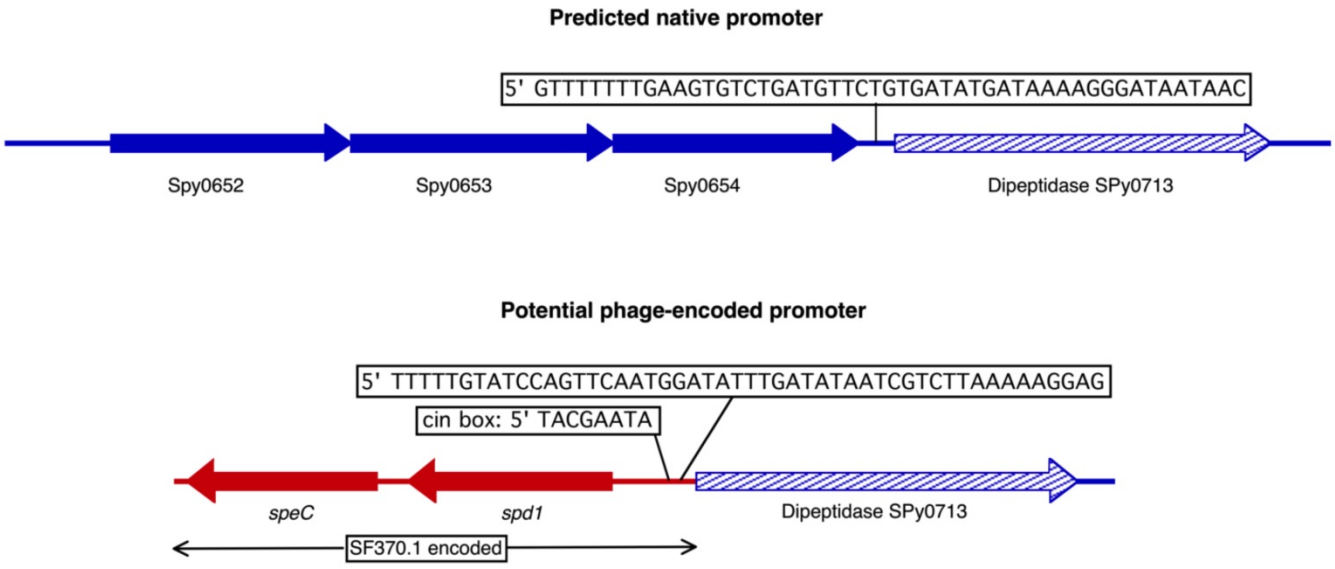

Figure 2. Integration of prophage SF370.1 may provide an alternate promoter for dipeptidase Spy0713. In strains that lack an integrated prophage at this site, the native promoter for dipeptidase Spy0713 is downstream from the uncharacterized gene Spy0654; the predicted sequence is shown above. Integration of phage SF370.1 into Spy0713 separates this gene from that native promoter, and a predicted promoter encoded by the prophage is now positioned in front of the dipeptidase ORF. This phage-encoded promoter is preceded also by a canonical CinA box (Claverys & Martin, 1998), which is not part of the native promoter. The transcription of prophage virulence genes *speC* and *spd1* is from the opposite strand and should not influence transcription of Spy0713. Promoter predictions were done using the online tool at http://www.fruitfly.org/seq_tools/promoter.html (Reese & Eeckman, 1995).

Table 1. Assembled and annotated genomes of *S. pyogenes* that are hosts to prophages.

Strain	M type	Prophages (CI)*	Origin	Disease**	Genome size (bp)	Accession no.	Reference
SF370	M1	3 (1)	USA	Wound	1852441	NC_002737	(Ferretti, et al., 2001)
MGAS5005	M1	3	Canada	Invasive (CSF)	1838554	NC_007297	(Sumby, et al., 2005)
M1 476	M1	3	Japan	STSS	1831128	NC_020540.2	(Miyoshi-Akiyama, Watanabe, & Kirikae, 2012)
A20	M1	3	Taiwan	Necrotizing fasciitis	1837281	NC_018936.1	(Zheng, et al., 2013)
MGAS10270	M2	4 (1)	USA	Pharyngitis	1928252	NC_008022	(Beres, et al., 2006)
MGAS315	M3	6	USA	STSS	1900521	NC_004070	(Beres, et al., 2002)
SSI-1	M3	6	Japan	STSS	1894275	NC_004606	(Nakagawa, et al., 2003)
MGAS10750	M4	3 (1)	USA	Pharyngitis	1937111	NC_008024	(Beres, et al., 2006)
Manfredo	M5	4 (1)	USA	ARF	1841271	NC_009332	(Holden, et al., 2007)
MGAS10394	M6	7 (1)	USA	Pharyngitis	1899877	NC_006086	(Beres, et al., 2002)
MGAS2096	M12	2	Trinidad	AGN	1860355	NC_008023	(Beres, et al., 2006)
MGAS9429	M12	3	USA	Pharyngitis	1836467	NC_008021	(Beres, et al., 2006)
HKU16	M12	3	Hong Kong	Scarlet fever	1908100	AFRY00000001	(Tse, et al., 2012)
HSC5	M14	3	USA	Not known	1818351	NC_021807.1	(Port, Paluscio, & Caparon, 2013)

Table 1. continued from previous page.

Strain	M type	Prophages (CI)*	Origin	Disease**	Genome size (bp)	Accession no.	Reference
MGAS8232	M18	5	USA	ARF	1895017	NC_003485	(Smoot, et al., 2002)
MGAS6180	M28	2 (2)	USA	Puerperal sepsis	1897573	NC_007296	(Green, et al., 2005)
NZ131	M49	3	New Zealand	AGN	1815785	NC_011375	(McShan, et al., 2008)
Alab49	M53	3 (1)	USA	Impetigo	1827308	NC_017596	(Bessen, et al., 2011)
MGAS15252	M59	0 (1)	Canada	SSTI	1750832	NC_017040	(Fittipaldi, et al., 2012)
MGAS1882	M59	1 (1)	USA	AGN, pyoderma	1781029	NC_017053	(Fittipaldi, et al., 2012)

* Number of lambdoid prophages (Number of phage-like chromosomal islands)
** AGN, acute glomerulonephritis; ARF, acute rheumatic fever; CSF, cerebrospinal fluid; SSTI, skin or soft tissue infection; STSS, streptococcal toxic shock syndrome

Table 2. Prophages of *S. pyogenes*, and their integration sites and associated virulence genes.

Target gene (attB)	Gene target for Integration (*attB*)	Associated Phages (Virulence Genes*)	Identifier**
ssDNA binding protein recO	3’	MGAS10394.1 (*sdn*)	A
RNA helicase snf	3’	MGAS8232.1 (*speA1*)	B
Promoter of Hypothetical Spy49_0371	5’	NZ131.1 (None identified)	C
Dipeptidase	5’	SF370.1 (*speC-spd1*) MGAS10270.1 (*speC-spd1*) MGAS10750.1 (*speC-spd1*) Man.4 (*speC-spd1*) MGAS2096.1 (*speC-spd1*) MGAS9429.1 (*speC-spd1*) MGAS8232.2 (*speC-spd1*)	D
tRNAarg	3’	MGAS10394.2 (speA4)	E
dTDP-glucose-4,6-dehydratase	3’	SF370.2 (speH-speI) MGAS10270.2 (spd3) MGAS10750.2 (spd3) Man.3 (speH-speI) MGAS9429.2 (speH-speI) HKU16.3 (speH-speI) NZ131.2 (speH)	F
CRISPR type II system direct repeat sequence	5’	MGAS315.1 (None identified) SPsP6 (None identified)	G
tmRNA	3’	T12 (*speA*) MGAS5005.1 (*speA2*) A20.1 (*speA2*) M1 476.1 (*speA2*) HSC5.1 (*speL-speM*) MGAS315.2 (*ssa*) SpsP5 (*ssa*) MGAS10394.3 (*speK-slaA*) MGAS8232.3 (*speL-speM*) MGAS6180.1 (*speC-spd1*) Alab49.1 (*speL-speM*)	H
trmA	3’	m46.1 (mefA)	I

Table 2. continued from previous page.

Target gene (attB)	Gene target for Integration (*attB*)	Associated Phages (Virulence Genes*)	Identifier**
comE	ND	MGAS10394.4 (mefA)	J
DNA-binding protein HU	5’	SF370.3 (spd3) MGAS5005.2 (spd3) A20.2 (spd3) M1 476.2 (spd3) MGAS315.3 (spd4) SPsP4 (ssa) MGAS10750.3 (ssa) Man.2 (spd4) MGAS10394.5 (speC-spd1) MGAS8232.4 (spd3) HSC5.2 (spd3) Alab49.2 (speC-spd1) MGAS1882.1 (speK-slaA)	K
Promoter of *yesN*	5’	MGAS10270.3 (speK-sla) MGAS315.4 (speK-sla) SPsP3 (speK-sla) MGAS6180.2 (speK-slaA)	L
Regulatory protein *recX*	5’	MGAS315.5 (speA3) SPsP2 (speA3) Man.1 (spd1) MGAS10394.6 (sda) Alab49.3 (spd3)	M
Putative gamma-glutamyl kinase	5’	MGAS315.6 (sdn) SPsP1 (sdn)	N
tRNAser	3’	MGAS5005.3 (sda) A20.3 (sdaD2) M1 476.3 (sdaD2) HKU16.2 (sdaD2) MGAS2096.2 (sdaD2) MGAS9429.3 (sda)	O
HAD-like hydrolase	5’	MGAS8232.5 (sda)	P
Excinuclease subunit *uvrA*	5’	HSC5.3 (spd3) HKU16.1 (ssa speC) MGAS10394.7 (spd3)	Q
Conserved hypothetical protein Spy49_1532	5’	NZ131.3 (spd3)	R
DNA mismatch repair protein *mutL*	5’	SpyCIM1 (SF370.4) SpyCIM2 (MGAS10270.4) SpyCIM4 (MGAS10750.4) SpyCIM5 (man.5) SpyCIM6 (MGAS10394.8) SpyCIM25 SpyCIM28 (MGAS6180.3) SpyCIM53 (Alab49.4) SpyCIM59 (MGAS15252.1) SpyCIM59.1 (MGAS1882.1)	S

Table 2. continued from previous page.

Target gene (attB)	Gene target for Integration (*attB*)	Associated Phages (Virulence Genes*)	Identifier**
SSU ribosomal protein S4P	3'	MGAS10270.5 (None identified) MGAS6180.4 (None identified) MGAS15252.2 (None identified	T

* Prophage associated genes: Superantigens: *speA*, *speC*, *speH*, *speI*, *speK*, *speL*, *speM*, *ssa,* and their alleles; DNases (streptodornases): *sda*, *spn*, *spd,* and their alleles; phospholipase: *sla* and alleles; macrolide efflux pump: *mefA*.
** Identifier for the phylogenetic tree in Figure 5
ND – not determined

Morphology and genome organization

Tailed phages with dsDNA genomes (*Caudovirales*) are abundant in the biosphere, and are perhaps the most frequently found form of life on Earth (Brüssow & Hendrix, 2002). Of the *Caudovirales*, the phage subset *Siphoviridae* (icosahedral heads with long, non-contractile tails) comprise about 60% of the total (Ackermann, 2005). A bacteriophage survey from the pre-genomics era found that 92% of phages of the genus *Streptococcus* were *Siphoviridae* by current classification (Ackermann & DuBow, 1987), and the few early electron micrographs published reflect this prevalence (Kjems, 1958; Malke, 1970; Zabriskie, Read, & Fischetti, 1972; Malke, 1972). Figure 3 shows the typical *Siphoviridae* morphology of two well-studied *S. pyogenes* phages, SF370.1 and T12. The tail fibers of SF370.1, which contain the hyaluronidase (hyaluronate lyase) used for capsule penetration during phage infection (Smith, et al., 2005), can be seen in the micrograph. The lytic transducing phage A25 also has typical *Siphoviridae* morphology (Malke, 1970; Malke, 1972). While lysogenic phages may be found to be mostly members of the *Siphoviridae*, given their probable common pool of genetic modules (see below), other phage morphotypes may be found in the lytic phages, such as the *Podoviridae* C1 phage of the related group C streptococci (Nelson, Schuch, Zhu, Tscherne, & Fischetti, 2003). The coliphage Lambda has been the prototype for lysogenic prophages, and the genetic organization of most group A streptococcal genome prophages follows a similar general plan (Desiere, McShan, van Sinderen, Ferretti, & Brüssow, 2001; Canchaya, et al., 2002), as they have identifiable genetic modules for integration and lysogeny, replication, regulation, head morphogenesis, head-tail joining, tail and tail fiber genes, lysis, and virulence (Figure 4).

Lysogeny module

Temperate phages are defined by their carriage of genes that establish and maintain a stable condition within a host cell, usually via site-specific integration. Minimally, lysogeny requires genes that encode an integrase (recombinase) and excisionase to mediate prophage DNA integration and excision, as well as genes that encode repressor and antirepressor proteins to direct and control this process, following the pattern seen in coliphage Lambda (Ptashne, 2004). Phage integrases typically mediate a recombination event between an identical sequence shared between the circular form of the prophage genome (*attP*) and the bacterial chromosome (*attB*), and the recognition of these DNA sequences is inherent in a given integrase protein (Groth & Calos, 2004; Campbell, del-Campillo-Campbell, & Ginsberg, 2002; Argos, et al., 1986). Most lambdoid phages usually have integrases that belong to the tyrosine integrase family, and the integrases of *S. pyogenes* prophages belong to this group. The excisionase gene in Lambda and many other Gram-negative host phages is positioned upstream of integrase; however, in the lactic acid bacteria and other Gram-positives, its genome location is variable (Bruttin, Desiere, Lucchini, Foley, & Brüssow, 1997; Breüner, Brøndsted, & Hammer, 1999). Indeed, since excisionase proteins often show little conservation (Lewis & Hatfull, 2001), it is often difficult to identify the correct ORF in a given prophage. Some excisionase genes may be provisionally identified in the *S. pyogenes* genome prophages by their homology to other phages (Desiere, McShan, van Sinderen, Ferretti, & Brüssow, 2001); however, to date, none have been experimentally confirmed.

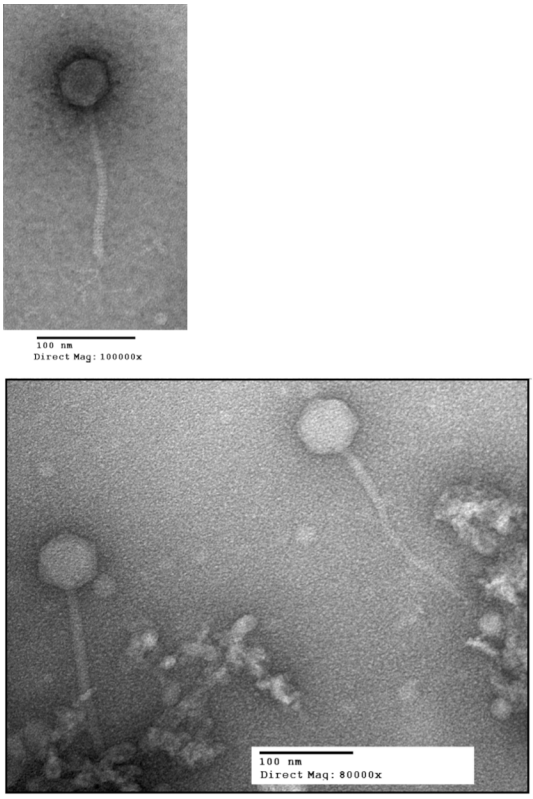

Figure 3. Morphology of streptococcal lysogenic phages. Prophages SF370.1 (A) and T12 (B) release typical *Siphoviridae* virions, following induction. In this micrograph, the SF370.1 head is about 55nm across and the tail is 168nm in length. In this image, the tail fibers that contain hyaluronate lyase (hyaluronidase) are visible. The T12 capsid has similar dimensions with the head being about 66nm and the tail length about 196nm. Electron micrographs have been provided by W. M. McShan and S. V. Nguyen.

DNA replication and modification

A region that shows considerable diversity between individual prophages, and which encodes genes involving DNA replication and modification, follows the lysogeny module. Homologs of DNA polymerases, replisome organizer, restriction-modification systems, and primase genes are present in these regions, as well as potential sequences that may function as the origins of phage DNA replication (Desiere, McShan, van Sinderen, Ferretti, & Brüssow, 2001; Canchaya, et al., 2002). Inspection of the genome annotations of these regions also shows that while many genes are unique to *S. pyogenes* phages, others have close homologs to phages from other streptococcal species such as *Streptococcus thermophilus* or *Streptococcus equi*, which suggests that a pool of genetic material is shared by a diverse group of phages (discussed below).

DNA packaging, capsid structural genes and host lysis genes

The next region of prophage genomes is dedicated to the genes that encode the proteins for the assembly of phage heads and tails, as well as the proteins needed to package the phage DNA into the heads and join this complex to the tails. The function of many of these genes has been inferred by homology to known phage proteins or sequences, or by presumption of function due to their relative order in the chromosome. However, with the exception of the hyaluronate lyase (hyaluronidase) gene found in some *S. pyogenes* phage tail fibers (Hynes, Hancock, & Ferretti, 1995; Smith, et al., 2005; Hynes & Ferretti, 1989), most of these genes have not been experimentally characterized, and as a result, these function assignments remain provisional. The typical holin-lysin genes that are employed at the end of the lytic phase to lyse the infected bacterial cell and release the newly formed phage particles follow the capsid genes.

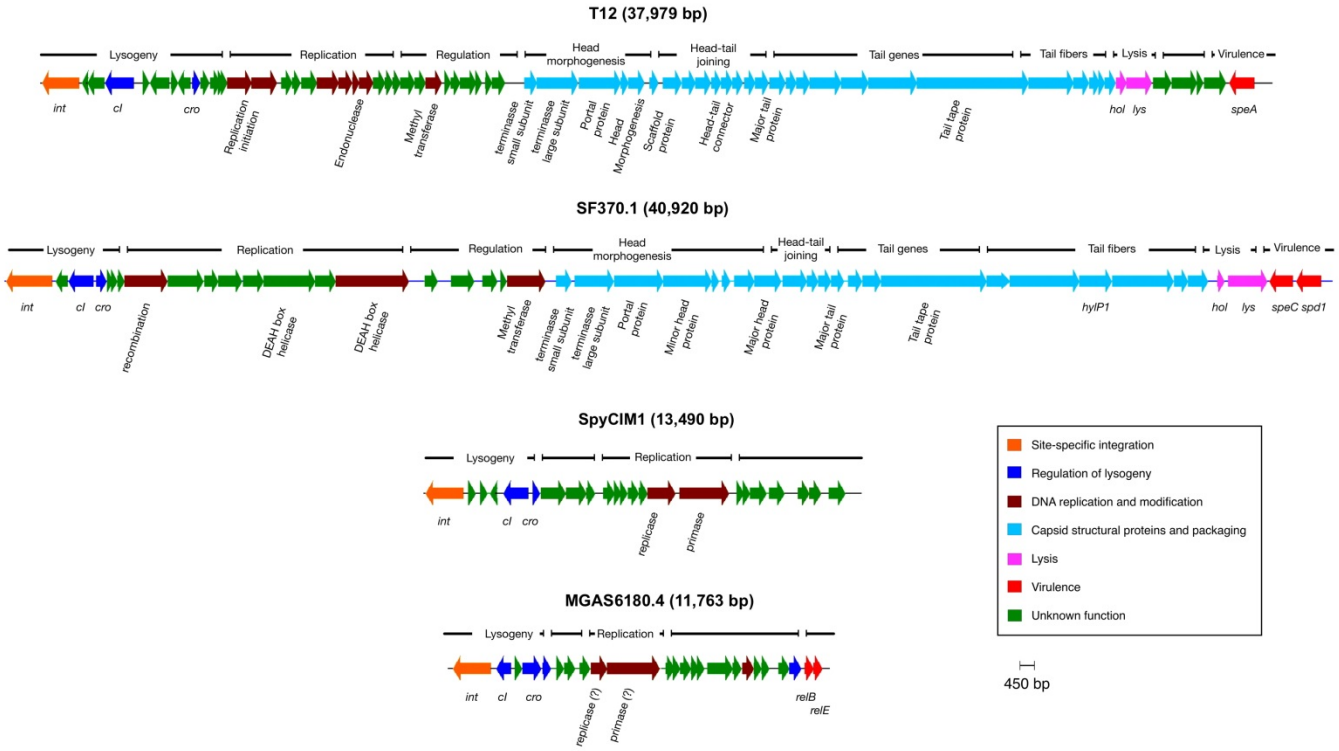

Figure 4. The genetic structure of streptococcal prophages and phage-like chromosomal islands. The prophages found in the genomes of *S. pyogenes* follow a typical lambdoid pattern in their organization with genetic modules for lysogeny, DNA replication, regulation, head morphogenesis, head-tail joining, tails and tail fibers, lysis, and virulence.

Virulence genes

Finally, at the distal end of the phage chromosome are the genes for host conversion that encode a range of virulence factors; exotoxins that are often superantigens, as well as DNases like streptodornase, are prominent (Table 2). The biology of these virulence factors is covered in a separate chapter in this book. The origin of phage-encoded toxins remains unclear—but since these toxin genes play no known role in the replication of the phage, it suggests that such genes were acquired at some point late in the phage's evolutionary history. It has been proposed that virulence factors may be acquired by phages by imprecise excision events (Barksdale & Arden, 1974), but independently finding known phage-associated virulence genes on the bacterial chromosome has not been observed. Some superantigen genes are not associated with prophages (Proft, Sriskandan, Yang, & Fraser, 2003; Proft, Moffatt, Berkahn, & Fraser, 1999), but it remains unclear whether these genes are a genetic source of prophage superantigens. The lateral gene capture of virulence genes has undoubtedly been important in their dissemination (Ochman, Lawrence, & Groisman, 2000), and indeed, such exotoxins may have evolved *de novo* as elements to increase bacterial host cell fitness (Brüssow, Canchaya, & Hardt, 2004). Decayed prophage remnants with superantigen domains may be seen in the *S. pyogenes* genome (Canchaya, et al., 2002), and these regions may serve as a genetic reservoir for virulence genes. Similarly, host-range variants of phages from different bacterial species (or even genera) are another potential reservoir for toxin genes; for example, the *speA* gene of *S. pyogenes* and the enterotoxins B and C1 of *Staphylococcus aureus* show a significant degree of homology, and thus may share a common origin (Weeks & Ferretti, 1986).

Diversity of lysogenic phages

Structural genes dominate phylogenic relations

In a seminal 1980 paper, Botstein proposed that the product of bacteriophage evolution is not the individual virus but a pool of interchangeable genetic modules, each of which carries out a biological function in the phage lifecycle; therefore, natural selection acts at the level of these individual modules (functional units) (Botstein, 1980). Phylogenetic analysis of the Lambdoid *S. pyogenes* prophages predicts that several prominent groups exist (Figure 5). Within each branch, considerable group diversity may exist in terms of targeted bacterial attachment sites and encoded virulence factors (Table 2). Inspection of each group shows that phylogeny is driven by large shared blocks of genes encoding proteins for DNA packaging, heads and tails, and host lysis (Table 3); for example, see the analysis of the group of prophages that contain phage T12, where all members of the group minimally share these structural genes (Figure 6). However, other ones, such as the SF370.1 group, are much more clonal, with all prophages sharing the same virulence and lysogeny modules. Therefore, the phylogeny of individual functional modules (such as toxin genes) may be unrelated to other regions of the genome (such as the lysogeny module or capsid genes). The apparent shuffling of the individual modules may be driven by both homologous and non-homologous recombination (Desiere, McShan, van Sinderen, Ferretti, & Brüssow, 2001; Ford, Sarkis, Belanger, Hendrix, & Hatfull, 1998; Monod, Repoila, Kutateladze, Tétart, & Krisch, 1997; Juhala, et al., 2000). The actual driving force behind this engine of prophage diversity in *S. pyogenes* remains poorly understood, but some clues have emerged. In a survey of 21 toxin-carrying *S. pyogenes* strains, 18 were found to carry a highly conserved ORF adjacent to the toxin genes (Aziz, et al., 2005). This ORF, which was named paratox, may help promote homologous recombination between phages so that toxin genes may be exchanged, which would lead to phage diversity. Some genome phages or phage-like elements do not readily fall into any group: MGAS10394.2, HKU16.1, NZ131.1, m46.1, and MGAS10394.4 are phylogenetic outliers that have little commonality with other prophages. When the prophages are clustered by the M-type of their host cell, some patterns do emerge (Figure 7). There appears to be a pool of phages shared by M1 and M12 strains; for example, M3 strains have some phages that appear more frequently within this serotype, but the current sample size is too small to draw any definite conclusions. More genomic data will be required to obtain a clearer picture of prophage distribution by serotype.

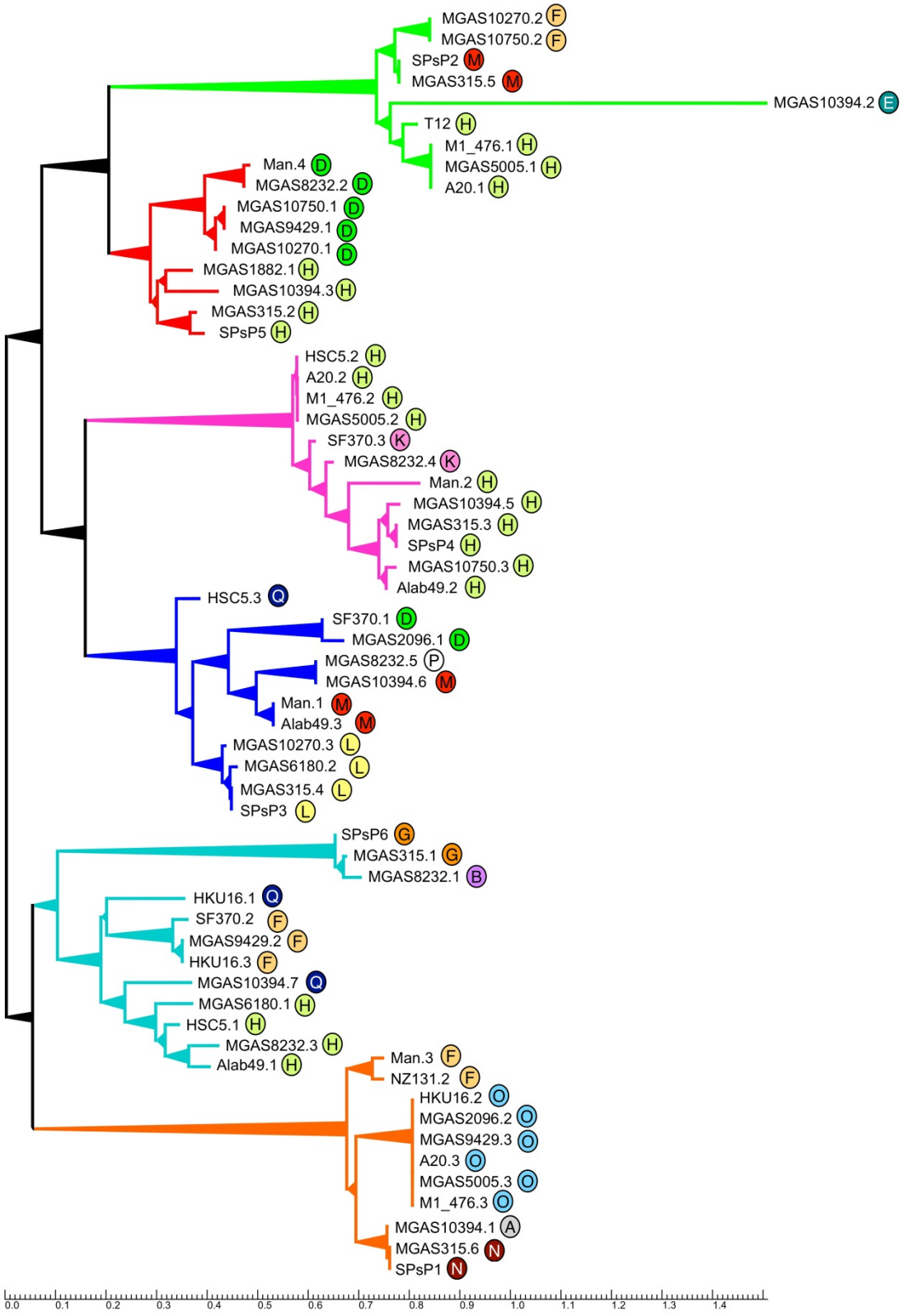

Figure 5. Phylogenetic relationships of *S. pyogenes* prophages. An unrooted phylogenetic tree was created by DNA alignment of the genome prophages. Prophages MGAS10394.2, HKU16.1, NZ131.1, m46.1, and MGAS10394.4 were so dissimilar from the other prophages that each occupied an independent branch; consequently, they are not shown on the tree for clarity. The alignment organized the remaining prophages into six major branches, and the encircled letter identifier by each prophage refers to its associated attachment site (*attB*) described in Table 2; each identifier is colored to facilitate viewing. The groups are defined by shared modules for structural genes (Table 3). The tree was created using the software packages Clustal-omega and TreeGraph 2(Stöver & Müller, 2010; Sievers, et al., 2011).

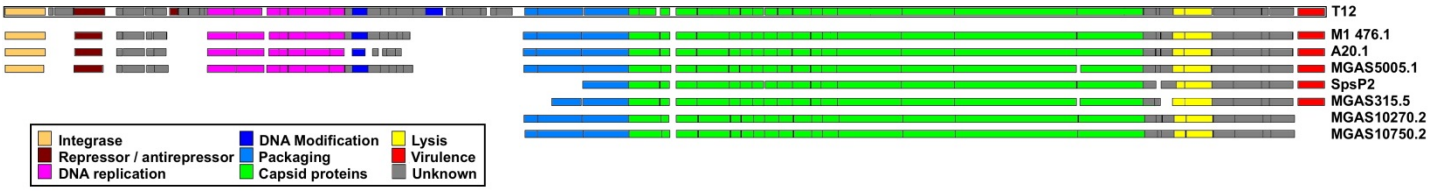

Figure 6. Shared genetic modules of the T12-related prophage family. The top line is the simplified genetic map of bacteriophage T12, colored by gene or genetic module for the integrase, repressor-antirepressor, DNA replication, DNA modification, DNA packaging, capsid proteins, lysis, and virulence (*speA*). Regions of unknown or uncertain function are colored gray. Beneath T12, the genetic maps of the other genome prophages that share the extended region dedicated to packaging, capsid proteins, and lysis are shown. DNA regions that are divergent from T12 are not shown. The figure illustrates that a structural gene module can be associated with divergent attachment sites or virulence genes. The alignment was derived from the phylogenetic tree presented in Figure 5.

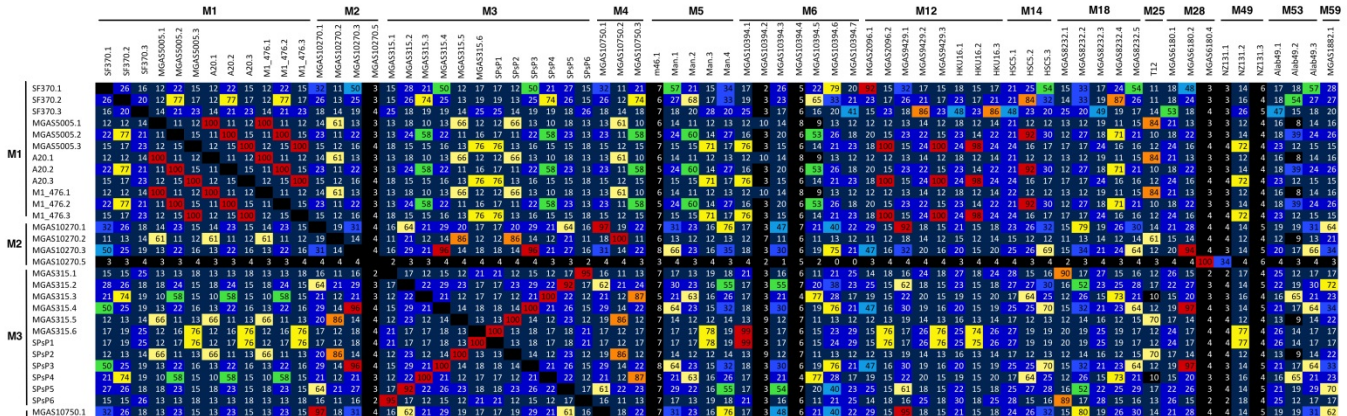

Figure 7. Identity matrix of genome prophages grouped by M-type. The identity matrix presents the Clustal-omega DNA alignment from Figure 5 as percentage identity between genome prophages, which are grouped by the M-type of their host streptococcus. The numbers within each cell represent the identity rounded to the nearest whole number, and the cell colors show the range into which each identity falls, by increasing percentages of 10.

Table 3. *S. pyogenes* prophages, grouped by shared structural modules.

T12 Group	SF370.1 group	SF370.2 group	SF370.3 group	MGAS315.1 group	MGAS315.2 group	MGAS315.6 group	MGAS6180.4 group
MGAS5005.1	SF370.1*	SF370.2*	SF370.3*	MGAS315.1*	MGAS10270.1*	MGAS5005.3	MGAS10270.5
M1 476.1	MGAS10270.3*	MGAS10394.7*	MGAS5005.2	SPsP5*	MGAS315.2*	M1 476.3	MGAS6180.4

Table 3. continued from previous page.

T12 Group	SF370.1 group	SF370.2 group	SF370.3 group	MGAS315.1 group	MGAS315.2 group	MGAS315.6 group	MGAS6180.4 group
A20.1	MGAS315.4*	MGAS9429.2*	M1 476.2	MGAS8232.1*	SpsP6*	A20.3	MGAS15252.2
MGAS10270.2	SPsP3*	HKU16.3	A20.2		MGAS10750.1*	MGAS315.6*	
MGAS315.5	Man.1*	HSC5.1*	MGAS315.3*		Man.4*	SPsP1*	
SPsP2	MGAS10394.6*	MGAS8232.3*	SPsP4*		MGAS10394.3*	Man.3*	
MGAS10750.2	MGAS2096.1*	MGAS6180.1	MGAS10750.3*		MGAS9429.1*	MGAS10394.1*	
T12	HSC5.3*	Alab49.1*	Man.2*		MGAS8232.2*	MGAS2096.2*	
	MGAS8232.5*		MGAS10394.5*		MGAS1882.1*	MGAS9429.3*	
	MGAS6180.2*		HKU16.1*			HKU16.2	
	Alab49.3*		HSC5.2*			NZ131.2*	
			MGAS8232.4*				
			NZ131.3*				
			Alab49.2*				

* Contain a hyaluronate lyase (hyaluronidase) as a component of the tail fiber

Prophage hyaluronidase (hyaluronate lyase)

A subset of genome prophages encode a hyaluronidase (hyaluronate lyase, hyaluronoglucosaminidase) gene (Table 3). Two alleles (*hylP* and *hylP2*) of this gene were originally identified (Hynes, Hancock, & Ferretti, 1995; Hynes & Ferretti, 1989), and subsequent studies show that this gene exists in multiple alleles that are mainly distinguished by SNPs and by collagen-like domain indels in some variants (Marciel, Kapur, & Musser, 1997; Mylvaganam, Bjorvatn, Hofstad, & Osland, 2000). The phage hyaluronidase gene found in the genome sequences also show considerable diversity that may have resulted from recombination (Hynes, Hancock, & Ferretti, 1995; Hynes & Ferretti, 1989; Marciel, Kapur, & Musser, 1997; Mylvaganam, Bjorvatn, Hofstad, & Osland, 2000). It had been suggested that phage hyaluronidase was a potential virulence factor; however, the crystallization and structural analysis of HylP1 from phage SF370.1 suggested that the function of this enzyme is to introduce widely spaced cuts in the bacterial hyaluronic acid to cause a local reduction in capsule viscosity and aid phage invasion during infection (Smith, et al., 2005). Similar structural properties were observed in the hyaluronidase proteins encoded by prophages SF370.2 and SF370.3 (Martinez-Fleites, Smith, Turkenburg, Black, & Taylor, 2009).

Horizontal transfer of genes from other species

One observation that comes from extensive genome sequencing is that the lysogenic phages of *S. pyogenes* share a gene pool with other streptococcal species, including those that are closely related (such as *Streptococcus equi*), as well as those that are more distant (*S. pneumoniae*). These shared genes include those that are essential to the basic phage life cycle, such as capsid proteins, as well as virulence genes, like exotoxins and superantigens. For example, *Streptococcus equi* prophages share many superantigens or virulence genes with *S. pyogenes* phages, including *slaA*, *speL*, *speM*, *speH*, and *speI* (Holden, et al., 2009). This study found that phage Seq.4 from *S. equi* strain Se4047 was very closely related at the DNA level to *S. pyogenes* Manfredo phage Man.3, including the two phage-encoded exotoxins. Similarly, *Streptococcus agalactiae* phage JX01, isolated in milk from cattle with mastitis, shares an extensive homology with *S. pyogenes* prophage MGAS315.2 in the modules that control DNA replication, tail, head-tail connector, head capsid, and DNA packaging, with over 97% amino acid identity between their terminase subunits; however, no virulence associated genes were identified in this phage (Bai, et

al., 2013). The temperate phage MM1 of *S. pneumoniae* also draws from a common pool of structural genes, sharing the DNA packaging, head-to-tail joining, and tail genes with phage SF370.1 (Obregón, García, García, López, & García, 2003). Additionally, this phage and MGAS315.4 share tail and tape measure genes. Some of the oral streptococci also have phages that contribute to this common pool. The portal, terminase, major capsid protein, major tail protein, and tape measure protein of *Streptococcus mitis* phage SM1 share homology with prophage SF370.3 and a number of other phages of low-GC Gram-positive hosts (Siboo, Bensing, & Sullam, 2003). While acquisition of these phages structural genes from other streptococcal species may not directly impact virulence in the same way that a novel exotoxin would, these capsid genes may help to expand the host range within the group A organisms. The dairy species *Lactococcus lactis* also is included in this pool of shared phage genes, since prophage SF370.3 closely resembles the cos-site temperate phage r1t of *L. lactis* (Desiere, McShan, van Sinderen, Ferretti, & Brüssow, 2001; van Sinderen, et al., 1996).

Group A streptococcal prophages and the host phenotype

Prophages as vectors for virulence genes

As described above, the link between bacteriophages and virulence in *S. pyogenes* may be traced to the earliest days of bacteriophage research. A considerable range of virulence-associated genes are carried by these prophages and prophage-like elements, including superantigens (*speA*, *speC*, *speG*, *speH*, *speI*, *speJ*, *speK*, *speL*, *SSA*, and variants), DNases (*spd1*, MF2, MF3, and MF4), phospholipase A2 (*sla*), and macrolide resistance (*mefA*). It is quite common for a given prophage to carry more than one virulence gene, such as in the case of phage SF370.1, which contains both *speC* and *spd1*. The number and diversity of prophage-associated virulence genes in *S. pyogenes* argues that these frequently play an important role in pathogenesis.

The expression of phage-encoded virulence genes, rather than an autonomous event, may be linked to the host streptococcal cell genetic background (Venturini, et al., 2013) or physiological state (Anbalagan & Chaussee, 2013). For example, the reception of signals from co-cultured human cells influences *S. pyogenes* prophage virulence gene expression. Human pharyngeal cells release a soluble factor that stimulates expression of pyrogenic exotoxin C (SpeC) and phage DNase Spd1, as well as the induction and release of phages by *S. pyogenes* grown in co-culture (Broudy, Pancholi, & Fischetti, 2001; Broudy, Pancholi, & Fischetti, 2002). Similar results were independently observed where the expression of prophage-encoded toxins SpeK and Sla were enhanced by their co-culture with pharyngeal cells (Banks, Lei, & Musser, 2003). Eukaryotic cells also provide an environment that promotes the transfer of toxin-producing phages from a lysogen to a new host; this phenomenon can occur in either *in vitro* culture or in a mouse model (Broudy & Fischetti, 2003). However, the genetic background of the streptococcus influences whether or not the acquisition of a new prophage-expressing DNase results in enhanced pathogenic potential of the resulting lysogen (Venturini, et al., 2013). Some of these bacteria-phage interactions appear to be linked to the cellular regulatory networks; for example, the *S. pyogenes* global regulator Rgg can control the expression of the phage-encoded DNase Spd3 by interacting with phage promoters of that gene (Anbalagan & Chaussee, 2013). The alteration of streptococcal gene expression may also impact the levels of phage toxins released during an infection. By employing a murine subcutaneous chamber model, Aziz and co-workers showed that the expression of *S. pyogenes* protease SpeB diminished after extended colonization in the mouse; this loss of protease activity occurred with the simultaneous enhancement of phage-encoded exotoxin SpeA and streptodornase expression (Aziz, et al., 2004). Notably, these altered expression patterns were independent events.

Phage-like elements that carry antimicrobial resistance genes

S. pyogenes is known to harbor a number of genetic elements that appear to have phage sequences combined with sequences from transposons or plasmids, and that are vectors for antibiotic resistance. One example is Φm46.1, the main *S. pyogenes* element that carries the *mefA* and *tetO* genes (Brenciani, et al., 2010). The

chromosome integration site of Φm46.1 is a 23S rRNA uracil methyltransferase gene, and this phage has high levels of amino acid sequence similarity to Φ10394.4 of *S. pyogenes* strain MGAS10394.4 and λSa04 of *S. agalactiae* A909 (Banks, et al., 2004; Tettelin, et al., 2005). The antibiotic resistance cassette of the PhiM46.1 family may be a recent acquisition: the lysogeny module appears to be split, due to the insertion of a segment that contains *tet*O and *mef*A into the phage's DNA (Brenciani, et al., 2010). Besides being found frequently in *S. pyogenes*, phage Φm46.1 has a broad host range that allows it to transduce antibiotic resistance to strains of *S. agalactiae*, *S. gordonii*, and *S. suis* (Giovanetti, et al., 2014). All of these species share a highly conserved *attB* site, which undoubtedly facilitates the dissemination of this phage. Further, within *S. pyogenes*, Φm46.1 appears to be able to infect a wide range of M-types (Di Luca, et al., 2010). The ability of this phage family to mediate the transfer of antibiotic resistance again shows how frequently *S. pyogenes* prophages modify the phenotypes of their hosts to improve fitness.

Phage-like element and regulation of host gene expression

A frequent mobile element found in *S. pyogenes* genomes are the phage-like SpyCI (*S*treptococcal *p*yogenes *c*hromosomal *i*slands) that integrate into the 5' end of the MMR gene *mutL* (Nguyen & McShan, 2014; Scott, Nguyen, Hendrickson, King, & McShan, 2012; Scott, Thompson-Mayberry, Lahmamsi, King, & McShan, 2008). The SpyCI share integrase modules with related phage-like chromosomal islands from *Streptococcus anginosus*; *Streptococcus canis*; *Streptococcus dysgalactiae*, subsp. *equisimilis*; *Streptococcus intermedius*; and *Streptococcus parauberis* (Nguyen & McShan, 2014). The DNA replication module is even more widespread among other species, and in addition to the ones named above, is found in related chromosomal islands found in *S. agalactiae*, *S. mitis*, *S. pneumoniae*, *Streptococcus pseudopneumoniae*, *Streptococcus suis*, and *Streptococcus thermophilus* (Nguyen & McShan, 2014). The remarkable defining characteristic of SpyCI is how they regulate MMR and the other genes in the operon (major facilitator family efflux pump *lmrP*, Holliday junction resolvase *ruvA*, and base excision repair glycosylase *tag*). The best studied member of this family, SpyCIM1 from strain SF370, is a dynamic element that excises from the bacterial chromosome during early logarithmic growth and replicates as a circular episome (Scott, Thompson-Mayberry, Lahmamsi, King, & McShan, 2008). As the bacterial population reaches the end of the logarithmic phase and enters the stationary phase, SpyCIM1 re-integrates into the unique attachment site at the beginning of *mutL* ORF (Figure 8). The result of this cycle is that SpyCIM1 acts as a growth-dependent molecular switch to control the expression of MMR, which causes SF370 to alternate between a mutator and wild-type phenotype in response to growth. During rapid cell division and DNA replication, the integrity of the genome is maintained by an active MMR system; during periods of infrequent cell division, mutations may accumulate at a higher rate (Scott, Nguyen, Hendrickson, King, & McShan, 2012; Scott, Thompson-Mayberry, Lahmamsi, King, & McShan, 2008). Preliminary studies suggest that the SpyCI, which lacks identifiable structural genes, may employ a helper prophage for packaging and dissemination, in a fashion similar to the well-characterized *Staphylococcus aureus* pathogenicity islands (SaPI) (Novick, Christie, & Penadés, 2010). Further, the impact of this genetic regulatory switch upon *S. pyogenes* gene expression beyond the MMR operon needs to be characterized.

Conclusions

The association of *S. pyogenes* with its bacteriophages is a major factor in the biology of this human pathogen, which influences the distribution of virulence genes, the spread of antibiotic resistance, the horizontal transfer of host genes, and the population distribution of cells. These relationships can range from simple predator-prey models to complex symbiotic associations that promote the evolutionary success of both cell and phage. Furthermore, in prophages, the choice of integration site into the bacterial chromosome may alter the streptococcal genotype through either gene inactivation or the replacement of normal promoter elements with phage-encoded ones. The similarity that prophages have to pathogenicity islands can hardly be overlooked, and the range of prophage-mediated characteristics that add to host survival or virulence can be easily predicted to increase as new research is conducted. Genome sequencing has greatly contributed to our understanding of

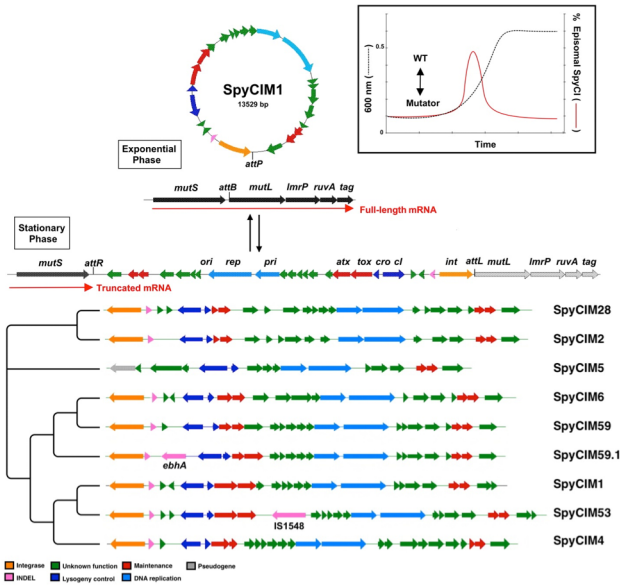

Figure 8.

A. SpyCIM1 regulation of the MMR operon through dynamic site-specific excision and integration. The MMR operon of *S. pyogenes* groups the genes that encode DNA mismatch repair (*mutS* and *mutL*), multidrug efflux (*lmrP*), Holliday-junction resolvase (*ruvA*), and base excision repair glycosylase (*tag*). The orientation of this chromosomal region is shown from the lagging strand to emphasize the MMR operon transcription. During the exponential phase, SpyCIM1 excises from the chromosome, circularizes, and replicates as an episome, which restores the transcription of the entire DNA mismatch repair operon (WT). Excision and mobilization occurs early in logarithmic growth in response to as-yet unknown cellular signals (Insert; adapted from Scott et al., 2014 (Scott, Nguyen, Hendrickson, King, & McShan, 2012)). As logarithmic growth continues, SpyCIM1 re-integrates into *mutL* at *attB*, and by the time the culture reaches stationary phase, the integration process has completed, again blocking the transcription of the MMR operon. WT: Wild-type phenotype associated with unimpeded expression of the MMR operon. Reproduced from Frontiers in Microbiology (Nguyen & McShan, 2014) under the Creative Commons Attribution License (CC-BY 4.0).

B. Phylogenetic tree of the SpyCI. The phylogenetic tree of the SpyCI DNA sequences is presented. The tree was created with TreeGraph 2(Stöver & Müller, 2010) using previously analyzed data (Nguyen & McShan, 2014).

prophage distribution and genetic composition, and this bank of knowledge has been and will be an important foundation for future biological studies on the interactions of *S. pyogenes* and its phages that will be certain to reveal many novel—and perhaps surprising—relationships.

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The CRISPR-Cas system of *Streptococcus pyogenes*: function and applications

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Abstract

Many *Streptococcus pyogenes* strains harbor CRISPR-Cas loci that encode for the RNA-guided nuclease Cas9. Due to the use of this nuclease in genome editing, there has been a great deal of focus on the study of CRISPR-Cas immunity in these organisms, and as a result the *S. pyogenes* CRISPR-Cas9 system is one of the most studied and better understood of these systems. In this chapter, we review how the Cas9 nuclease mediates anti-phage immunity and how it can be repurposed for the genetic engineering of human cells and other eukaryotic organisms.

Introduction

Streptococcus pyogenes is the source of the most significant genetic tool of the twenty first century: the RNA-guided Cas9 nuclease (Pennisi, 2013). This nuclease is widely used to introduce genetic modifications in a variety of cells and organisms, from bacteria (Jiang, Bikard, Cox, Zhang, & Marraffini, 2013) and yeast (DiCarlo, et al., 2013) to monkeys (Niu, et al., 2014) and human cell lines (Cong, et al., 2013; Mali, et al., 2013a). The high efficiency and simplicity of the Cas9 genome editing technique has accelerated the possibilities of human gene therapy.

What is the function of Cas9 in *Streptococcus pyogenes*? The nuclease is a central player of the adaptive immunity that is provided by clustered regularly interspaced short palindromic repeats (CRISPR) loci (Marraffini, 2015). These loci consist of short repetitive sequences (30-40 bp) that are intercalated by equally short sequences of viral (bacteriophage) and plasmid origin (Bolotin, Quinguis, Sorokin, & Ehrlich, 2005; Mojica, Díez-Villaseñor, García-Martínez, & Soria, 2005; Pourcel, Salvignol, & Vergnaud, 2005) called "spacers." The presence of a spacer sequence that matches the genome of a bacteriophage or conjugative plasmid prevents the host from becoming infected by these genetic invaders (Barrangou, et al., 2007; Marraffini & Sontheimer, 2008). Therefore, spacers provide sequence-specific immunity against bacteriophage and plasmid infection. Importantly, in a process known as adaptation, new spacers can be introduced into the CRISPR locus during infection (Barrangou, et al., 2007); in this way, the CRISPR system creates a memory of the infection that is used to provide immunity in subsequent encounters with the same invader or a related invader (for example, one that harbors the same spacer sequence in its genome) (Fig. 1A).

How do spacer sequences provide immunity? The CRISPR locus is usually transcribed into a long precursor RNA that contains both repeats and spacers. This precursor is subsequently cleaved at the repeat sequences to liberate small CRISPR RNAs (crRNAs) that contain the intervening spacer sequence (Tang, et al., 2002) (Fig. 1B). Cleavage is usually carried out by CRISPR-associated (Cas), repeat-specific endoribonucleases (Brouns, et al., 2008; Carte, Wang, Li, Terns, & Terns, 2008). The crRNA forms a ribonucleoprotein complex with an effector Cas nuclease, which finds its target (also known as the protospacer) through base-pairing of the spacer sequence in the crRNA and the genome of the invader, and proceeds to cleave it (Gasiunas, Barrangou, Horvath, & Siksnys, 2012; Hale, et al., 2009; Jinek, et al., 2012; Jore, et al., 2011; Samai, et al., 2015; Westra, et al., 2012) (Fig.

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1C). The destruction of the viral or plasmid DNA stops the infection and confers immunity to the host (Garneau, et al., 2010). As expected from any host-pathogen interaction, this event is just a first step in the arms race between the host CRISPR-Cas systems and the extrachromosomal invaders, since bacteriophages and plasmids can evolve by generating mutations in the target site that prevent a perfect base-pairing with the spacer sequence of the crRNA (Deveau, et al., 2008). These mutations allow the invader to escape CRISPR-Cas immunity and re-establish infection. The cycle restarts when the CRISPR-Cas system acquires a new spacer sequence that matches perfectly with the genome of the invader (Levin, Moineau, Bushman, & Barrangou, 2013).

Streptococcus pyogenes CRISPR loci

Depending on the *cas* gene content, CRISPR-Cas systems can be classified into five types (Makarova, et al., 2015). Bioinformatic analysis revealed that seven of 13 available *S. pyogenes* genomes contain two CRISPR-Cas loci belonging to the types II (CRISPR-2) and 1 (CRISPR-1) (Nozawa, et al., 2011). Interestingly, the strains that lack CRISPR sequences have the highest number of prophages (Table 1, highlighted in green). More importantly, analysis of the CRISPR targets shows a mutually exclusive relationship between CRISPR spacer sequences and their prophage targets (Nozawa, et al., 2011; Marraffini L. A., 2010; Marraffini & Sontheimer, 2010). For example, strain SF370 has a total of 9 spacers, of which 6 match perfectly sequences found in 22 prophages present in other strains, and none matches any of the endogenous Φ370.1-4 prophages; the same is true for the other CRISPR-containing *S. pyogenes* strains (Table 1, highlighted in red). In addition, many spacers display 1-4 mismatches with prophage targets (within a 30-35 bp long spacer). Furthermore, in the M3 serotype MGAS315 and SSI-1 strains, the type II CRISPR-Cas locus is interrupted and inactivated by the prophages Φ315.1 and ΦSPsP5, respectively. The data suggests (i) that CRISPR immunity can prevent prophage acquisition in *S. pyogenes*; (ii) that phages have acquired mutations to evade CRISPR immunity and lysogenize; and (iii) that there is a dynamic relationship between *S. pyogenes* and its phages that results in the selection of strains with increased pathogenic adaptations. However, none of these hypotheses have been rigorously tested empirically, and the relationship between the CRISPR, prophages, and virulence properties of *S. pyogenes* strains still remains to be established. It also remains unclear whether CRISPR immunity prevents conjugation of the many different ICE elements present in *S. pyogenes* strains (Beres & Musser, 2007). Although CRISPR-Cas systems have been shown to prevent conjugation in other organisms, such as staphylococci (Marraffini & Sontheimer, 2008), no spacers have been found that match the conjugative elements of *S. pyogenes*.

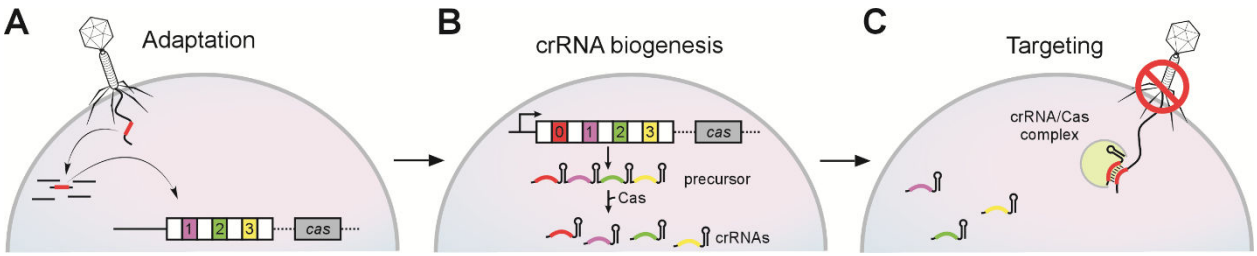

Figure 1. The CRISPR-Cas immunity pathway. CRISPR-Cas loci contain clusters of repeats (white boxes) and spacers (colored boxes) that are flanked by CRISPR-associated (*cas*) genes. (**A**) During adaptation, new spacers derived from the invading DNA are incorporated into the CRISPR array through an unknown mechanism. (**B**) During crRNA biogenesis, a CRISPR precursor transcript is processed by Cas endoribonucleases within repeat sequences to generate small crRNAs. (**C**) During targeting, the match between the crRNA spacer and target sequences specifies the nucleolytic cleavage of the invading nucleic acid.

Table 1. CRISPR and prophage content of current *S. pyogenes* genomes.

Strain[a]	Serotype	CRISPR 2A[b]	CRISPR 1C[b]	Prophages in the genome[c]	Prophages targeted by CRISPR		Ref.
SF370	M1	6	3	370.1 – 4	10270.1, 2 315.2, 3, 4, 5 SPsP2, 3, 4, 5 10750.1, 2, 3 10394.3, 4, 5	9429.2 8232.2, 3, 5 6180.2 NZ131.3	(Ferretti, et al., 2001)
MGAS9429	M12	2	7	9429.1 – 3	5005.1, 2 315.1, 2 SPsP5, 6 Man.1, 4	8232.1, 2, 5 6180.1 NZ131.3	(Beres, et al., 2006)
NZ131	M49	4	5	NZ131.1 - 3	370.1, 3 5005.2 10270.3 315.3, 4 SPsP3, 4 10750.3	Man.1, 2 10394.4, 5, 6 2096.1 8232.2, 4, 5 6180.2	(McShan, et al., 2008)
MGAS2096	M12	2	6	2096.1 – 2	5005.1 315.1, 2 SPsP5 Man.1, 4	8232.1, 2, 5 6180.1 NZ131.3	(Beres, et al., 2006)
MGAS5005	M1	3	4	5005.1 – 3	370.1 10270.1, 2, 3 315.2, 4, 5 SPsP2, 4, 5 10750.1, 2	Man.1, 3, 4 10394.6 9429.1 8232.2, 5 NZ131.3	(Sumby, et al., 2005)
MGAS10270	M2	3	3	10270.1 – 5	370.1 315.1, 2, 3 SPsP3, 4, 5 10750.3	Man.2, 3, 4 10394.3, 5 8232.1, 2, 3 6180.1	(Beres, et al., 2006)
MGAS6180	M28	4	1	6180.1 – 4	370.2, 3 5005.2 315.3, 5 SPsP2, 4, 5	10750.3 10394.4, 5 9424.2 8232.3, 4, 5	(Green , et al., 2005)

Table 1. continued from previous page.

Strain[a]	Serotype	CRISPR 2A[b]	CRISPR 1C[b]	Prophages in the genome[c]	Prophages targeted by CRISPR		Ref.
MGAS10750	M4	-	5	10750.1 – 4	370.3 315.5 SPsP2	Man.1 8232.4	(Beres, et al., 2006)
MGAS315	M3	-	-	315.1 – 6	-		(Beres, et al., 2002)
SSI-1	M3	-	-	SPsP1 – 6	-		(Nakagawa, et al., 2003)
Manfredo	M5	-	-	Man.1 – 5	-		(Holden, et al., 2007)
MGAS10394	M6	-	-	10394-1 – 8	-		(Banks, et al., 2004)
MGAS8232	M18	-	-	8232.1 – 5	-		(Smoot, et al., 2002)

(a) Strains are ordered from highest to lowest total number of CRISPR spacers.
(b) Number of spacers are indicated.
(c) Prophages and prophage remnants are indicated.

Cas9-mediated defense

Although two CRISPR-Cas loci are present in many *S. pyogenes* strains (Nozawa, et al., 2011), it is not known whether the type I system is actually functional and/or if its *cas* genes and CRISPR sequences are expressed. In contrast, the molecular mechanism of *S. pyogenes* type II CRISPR-Cas immunity has been studied in detail. *S. pyogenes* SF370 contains a type IIA CRISPR-Cas system (Fig. 2A) that harbors four *cas* genes (*cas9*, *cas1*, *cas2,* and *csn2*), six 30-nt spacers flanked at each side by 36-nt repeats, and an additional gene encoding for a trans-encoded crRNA (tracrRNA) (Deltcheva, et al., 2011). Cas9 is central to the defense provided by the type II CRISPR Cas system, since it has been shown to be essential for all three stages of immunity (Fig. 1): adaptation (Heler, et al., 2015), crRNA biogenesis, (Deltcheva, et al., 2011) and interference (Sapranauskas, et al., 2011).

During the biogenesis of the crRNA guides, the CRISPR array of repeats and spacers is transcribed into a long precursor RNA. As opposed to the type I and III crRNA biogenesis pathways, the *S. pyogenes* type II systems do not require a repeat-specific endoribonuclease to process the crRNA precursor (Brouns, et al., 2008; Carte, Wang, Li, Terns, & Terns, 2008). Instead, the precursor is then processed by the combined action of the tracrRNA, RNase III, and Cas9 (Deltcheva, et al., 2011) (Fig. 2B). The tracrRNA contains an extensive secondary structure that is recognized and bound by Cas9. The tracrRNA also harbors a sequence that is complementary to the repeat sequence of the crRNA precursor. The annealing of these complementary sequences leads to the formation of a dsRNA that is cleaved at one end by RNase III. This cleavage liberates the small crRNAs from the precursor, which remain bound to Cas9 via their association with the tracrRNA. In this way, the processing of the type II crRNA precursor generates Cas9 molecules that are loaded with crRNA guides and ready to search invading DNA molecules for its targets. Although CRISPR immunity against phage infection has not been experimentally demonstrated in *S. pyogenes*, the *S. pyogenes* type II CRISPR system has been studied using vectors that were engineered to harbor prophage sequences that match the spacers contained in the CRISPR array (Deltcheva, et al., 2011). These experiments showed that the absence of the tracrRNA, RNase III, or Cas9 all prevent the destruction of the target-containing vector, a finding that is consistent with the essential role that these three elements play in the generation of crRNA guides.

Work performed in *S. thermophilus* determined that type II CRISPR-Cas immunity results in the introduction of double-strand DNA breaks (DSBs) on the genome of the invading phage or plasmid at the target site specified by spacer sequences (Garneau, et al., 2010). In addition, genetic analysis of the *S. thermophilus* CRISPR system demonstrated that *cas9* is the only *cas* gene necessary for the interference phase of CRISPR immunity (Sapranauskas, et al., 2011). This mechanism presents the potential for an “autoimmune” nucleolytic reaction

against the CRISPR locus itself; that is, how can Cas9 loaded with tracRNA and a crRNA avoid cleaving the spacer sequence from which the crRNA was transcribed? Early work in *S. thermophilus* revealed that the phage targets of the type II CRISPR-Cas system of this organism display a strong conservation of the sequence downstream of the target (Deveau, et al., 2008). The conserved nucleotides are referred to as the protospacer-adjacent motif or PAM (Mojica, Díez-Villaseñor, García-Martínez, & Almendros, 2009). The PAM is only present downstream of the target DNA, but is absent from the repeat sequences; that is, downstream of the spacer sequence in the CRISPR locus. Studies of the *S. pyogenes* type II CRISPR system confirmed the findings obtained for the *S. thermophilus* system: that *cas9* is the only *cas* gene required for immunity (Heler, et al., 2015) and determined that the PAM motif sequence is NGG (Jiang, Bikard, Cox, Zhang, & Marraffini, 2013; Deltcheva, et al., 2011).

Biochemical and structural studies have meticulously characterized the crRNA-guide nucleolytic activity of Cas9 that is essential to provide immunity to the host (Fig. 2C). *S. pyogenes* Cas9 contains six domains: an HNH nuclease domain, a RuvC nuclease domain, an α-helical lobe, an arginine-rich region, a Topo-homology domain, and a PAM-recognition C-terminal domain (Jinek, et al., 2014; Nishimasu, et al., 2014). Single-molecule fluorescent experiments demonstrated that Cas9 searches for GG dinucleotide PAM sequences on the target dsDNA (Sternberg, Redding, Jinek, Greene, & Doudna, 2014). Transient binding to the PAM provides the necessary energy to unwind the dsDNA that is immediately upstream of the GG dinucleotide. Unwinding is followed by base-pairing of the crRNA with the seed sequence of the target. An inability to anneal leads to the quick release of Cas9, which continues sampling other DNA sequences. In contrast, if base pairing is productive, the rest of the crRNA sequence pairs with the target, forming an R-loop structure consisting of an RNA:DNA hybrid that is formed by the crRNA spacer sequence and its complementary DNA sequence (the target strand) and a displaced ssDNA (the PAM-containing, non-target strand) (Jiang, et al., 2016) (Fig. 2C). The formation of the R-loop triggers the cleavage of both DNA strands, with the HNH domain cleaving the target strand and the RuvC domain cleaving the PAM-containing strand (Jinek, et al., 2012; Jiang, et al., 2016).

Generation of a memory of infection

In contrast to crRNA biogenesis and Cas9 nuclease activity, the acquisition of new spacers following phage infection by the type IIA CRISPR-Cas system of *S. pyogenes* is less well understood. The original observation of spacer acquisition was done in *Streptococcus thermophilus*, where lytic phages were added to liquid cultures and phage-resistant bacteria were isolated (Barrangou, et al., 2007). The study showed that many of the bacteriophage-insensitive mutants (BIMs) expanded the CRISPR array by the incorporation of a new spacer with a sequence matching the genome of the infecting phage. However, studies like this are difficult to carry out in *S. pyogenes*, due to the lack of potent lytic phages that enable the isolation of BIMs. To overcome this limitation, a recent study transplanted the type IIA CRISPR-Cas system from *S. pyogenes* SF370 to *Staphylococcus aureus* (Heler, et al., 2015). Using staphylococcal lytic phages, the authors were able to observe and study the acquisition of new spacers in this CRISPR-Cas system. This arrangement was used to investigate the sampling of spacer sequences with functional flanking PAM sequences. Two scenarios are possible: (i) only spacers with the required NGG flanking nucleotide sequence are acquired; (ii) any phage sequence can become a new spacer, but only those flanked by NGG PAMs can provide immunity, and therefore are selected during phage infection. Genetic and biochemical analysis demonstrated the first scenario, with the PAM-binding domain of Cas9 being required during spacer acquisition to determine phage sequences that are flanked by a correct PAM (Heler, et al., 2015). This is a simple mechanism in which the same enzyme that is responsible for the recognition of the PAM during the implementation of immunity, Cas9, is also used to ensure that new spacers are flanked by such sequences.

The next step after the selection of a 30-bp phage sequence flanked by NGG is to integrate this sequence into the CRISPR array as a new spacer. This has not been investigated in *S. pyogenes*, but experiments with *Escherichia coli* demonstrated that Cas1 and Cas2 form a complex that is necessary and sufficient to integrate new spacers

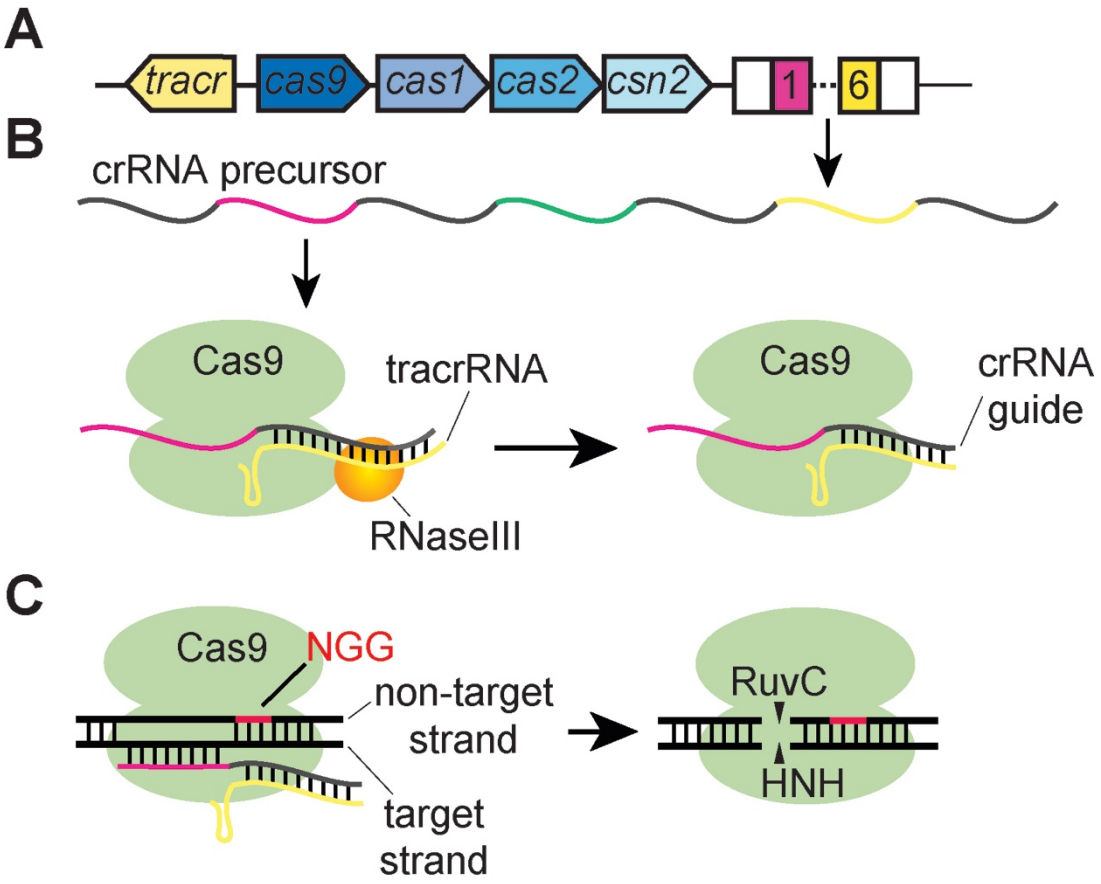

Figure 2. Cas9-mediated CRISPR immunity in *S. pyogenes*. (**A**) Organization of the *S. pyogenes* SF370 CRISPR-Cas locus. It contains four protein-coding *cas* genes, the *tracrRNA* gene, and seven repeats (white boxes), intercalated with six spacer sequences (the numbered, colored boxes). (**B**) CrRNA biogenesis begins with the transcription of the long crRNA precursor whose repeats form a dsRNA with complementary sequences present in the tracrRNA, which is bound by Cas9. RNase III cleavage of this dsRNA separates each spacer sequence from the precursor to generate a Cas9-tracrRNA-crRNA complex ready for surveillance and attack of foreign genetic elements that harbor a sequence complementary to the crRNA spacer. (**C**) Cas9 cleavage of target DNA. The Cas9 ribonucleoprotein complex scans dsDNA molecules for the presence of a PAM sequence (NGG). Binding of the PAM is followed by an attempt to pair the crRNA with the target DNA strand. If there is a productive annealing, an R-loop structure forms and Cas9 proceeds to the cleavage of each DNA strand using different nuclease domains: RuvC cleaves the PAM-containing, displaced, or non-target strand, and HNH cleaves the target strand.

(Arslan, Hermanns, Wurm, Wagner, & Pul, 2014; Nuñez, et al., 2014; Nuñez, Lee, Engelman, & Doudna, 2015). Genetic analysis demonstrated that all four *cas* genes (*cas9*, *cas1*, *cas2*, and *csn2*) of the *S. pyogenes* system are necessary for spacer acquisition (Heler, et al., 2015). Altogether, these results suggest that Cas1 and Cas2 also perform spacer integration in *S. pyogenes*—possibly through a more elaborate mechanism than that of the type I *E. coli* CRISPR-Cas system, since Csn2 and Cas9 (which are present in the type II CRISPR-Cas system from *S. pyogenes*, but not in the type I system of *E. coli*) also seem to form a complex with Cas1 and Cas2 (Heler, et al., 2015). As explained above, the function of Cas9 in this complex is presumably to sample PAM sequences, but the function of Csn2 is unknown (Arslan, et al., 2013).

Cas9-based genetic applications

Since the examination of the crRNA-guided DNA targeting mechanism (Garneau, et al., 2010; Marraffini, 2010), Cas nucleases have been proposed as useful biotechnological tools that require the sequence-specific cleavage of DNA (Marraffini & Sontheimer, 2008; Sontheimer & Marraffini, 2010). One such application is the genetic engineering of eukaryotic cells. Early work with the yeast *Saccharomyces cerevisiae* demonstrated that the

introduction of DSBs results in the generation of indels at the cleavage site after repair of the break via non-homologous end joining (NHEJ) (Plessis, Perrin, Haber, & Dujon, 1992; Rudin, Sugarman, & Haber, 1989). In addition, if the appropriate DNA template is provided after chromosomal cleavage, it could be used by the homology-directed repair (HDR) mechanism. Through this method, specific point mutations contained in the repair template can be introduced into the genome (Choulika, Perrin, Dujon, & Nicolas, 1995; Rouet, Smih, & Jasin, 1994). Therefore, a central aspect of this technique is the generation of a DSB at the desired sequence; however, the tools to achieve this have been difficult to engineer. Sequence-specific nucleases, such as zinc-finger (Bibikova, et al., 2001) and TALE nucleases (Christian, et al., 2010) were developed for this purpose, but programming them to achieve sequence-specific cleavage is difficult. In contrast, *S. pyogenes* Cas9 provides a simple and robust tool for the generation of precise DSBs, since the specificity can be easily programmed with the crRNA guide (Fig. 3). To make the system more amenable to genome editing, the tracrRNA and crRNA can be fused into a single-guide RNA (sgRNA) (Jinek, et al., 2012), thus reducing the number of components that need to be transferred from *S. pyogenes* to the target organism from three (tracrRNA, crRNA, and Cas9) to two (sgRNA and Cas9).

Genome editing of human cells mediated by *S. pyogenes* Cas9 was first achieved by transfecting a vector that harbored the *sgRNA* and the *cas9* genes into HK293 cells to generate indels in the *EMX1* chromosomal gene (Cong, et al., 2013) or in a *gfp* reporter gene (Mali, et al., 2013b) through the NHEJ pathway. In addition, it was shown that using an appropriate template for HDR-specific point mutations can be introduced into the *EMX1* gene (Cong, et al., 2013). For this, the expression of *cas9* was driven by the elongation factor 1α (EF1 α) promoter, its codons were changed for optimal translation in human cells, and nuclear localization signals were added to direct the nuclease to the cell nucleus. Gene knock-out through NHEJ-mediated indel generation is highly efficient and therefore can also be performed using libraries of sgRNAs that target the whole human genome to facilitate forward genetic experiments (Shalem, et al., 2014; Wang, Wei, Sabatini, & Lander, 2014). In this approach, a library of lentiviral vectors carrying *cas9* and multiple sgRNAs is used to infect cells. Upon integration of the lentiviral vector into the human genome, Cas9 introduces a sgRNA-specific indel, with each cell expressing a different sgRNA and therefore having a different gene knock-out. This genetically heterogeneous cell population can be subjected to different selection pressures that favor or disfavor different genotypes. Next-generation sequencing of the lentiviral *sgRNA* locus of the cells under selection allows the identification of the gene knock-outs that are enriched or depleted, thus assigning the responsibility for particular phenotypes to specific genes. Other versions of the Cas9 technology were developed by the direct injection of *cas9* mRNA and sgRNA molecules (Wang, et al., 2013), or of the Cas9 nuclease loaded with a sgRNA (Sung, et al., 2014). These techniques have been implemented to mutate multiple organisms, including mice (Wang, et al., 2013), flies (Gratz, et al., 2013), worms (Friedland, et al., 2013), livestock (Tan, et al., 2013), monkeys (Niu, et al., 2014), and many more. Moreover, the potential exists to use the technology to mutate the human germline: an outcome with important ethical consequences that requires serious study (Baltimore, et al., 2015).

The simplicity of the Cas9 DNA recognition mechanism has been successfully exploited to develop other applications besides genome editing. The cleavage of bacterial chromosomal sequences by *S. pyogenes* Cas9 is lethal (Bikard, Hatoum-Aslan, Mucida, & Marraffini, 2012), presumably because most bacteria lack the ability for NHEJ repair (Shuman & Glickman, 2007), and the nuclease repeatedly cleaves the target every time it is repaired by HDR. This lethality has been exploited to select for bacteria that carry mutations to prevent Cas9 cleavage (such as in the PAM or seed sequences) and thus enhance bacterial mutagenesis protocols (Jiang, Bikard, Cox, Zhang, & Marraffini, 2013), as well as to develop sequence-specific antimicrobials (Bikard, et al., 2014; Citorik, Mimee, & Lu, 2014). In addition, *S. pyogenes* Cas9 can be converted into an RNA-guided dsDNA binding protein if key residues are mutated in each of the nucleolytic active sites (D10A in the RuvC domain; H840A in the HNH domain). This "dead" protein, or dCas9, can be fused to different functional domains to bring them to specific sequences of the human genome. For example, the binding to promoter sequences of dCas9 fused to transcription activators or repressors can be used to modulate gene expression in human cells

(Qi, et al., 2013). Similarly, the binding of fusion proteins that consist of dCas9 and chromatin modification enzymes can lead to the modification of nucleosomal histones for the silencing or activation of particular chromosomal regions (Hilton, et al., 2015; Kearns, et al., 2015). In addition, dCas9-Gfp fusions can be used to fluorescently mark different loci (Chen, et al., 2013).

Conclusions

Although the diseases caused by *S. pyogenes* produce a great deal of human suffering, this organism is also the source of Cas9, a nuclease that holds an enormous promise for both human genome editing and gene therapy. Ironically, an organism responsible for some of the most prevalent infectious diseases in the world could harbor a cure for a number of genetic diseases. This irony highlights the importance of the research being performed with *S. pyogenes* and other bacterial pathogens, which may not only provide new therapies to combat disease, but also could lead to new genetic tools that can revolutionize medicine. Because of the revolution in human genetics caused by Cas9, much of the recent research on the CRISPR-Cas system of *S. pyogenes* has been centered on the biochemistry and structure of this nuclease. Future work will address the function of the CRISPR-Cas locus in the ecology, evolution and pathogenesis of *S. pyogenes*.

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Abbreviations

BIMs, bacteriophage-insensitive mutants

Cas, CRISPR-associated protein

CRISPR, clustered regularly interspaced short palindromic repeats

crRNA, CRISPR RNA

DSBs, double-strand DNA breaks

HDR, homology directed repair

ICE, integrated chromosomal element

NGG, PAM sequence for *S. pyogenes* Cas9

NHEJ, non-homologous end joining

PAM, protospacer-adjacent motif

sgRNA, single-guide RNA

TALE, transcription activator-like effector

tracrRNA, trans-encoded crRNA

ZFN, Zinc-finger nuclease

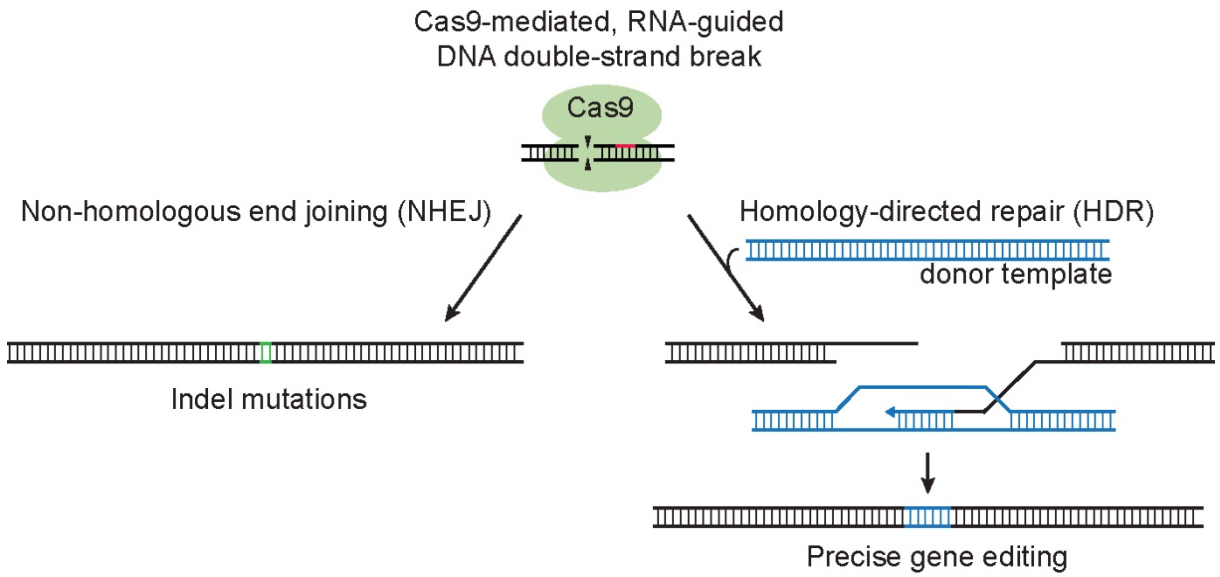

Figure 3. Cas9-mediated genome editing. Engineered Cas9 containing nuclear localization signals is expressed in human cells, along with a single-guide RNA (sgRNA) to introduce a sequence-specific double strand DNA break (DSB) in the gene to be mutated. In the absence of a repair template, the break is repaired by non-homologous end joining (NHEJ), leading to the introduction of a small insertion or deletion that usually creates an out of frame mutation that prevents the expression of the protein encoded by the gene. Therefore, this option is chosen when a gene knock-out is desired. In contrast, when a donor template that harbors a single-nucleotide mutation is added to repair the break by homologous recombination (homology-directed repair, HDR), the mutations will be incorporated into the desired gene. This approach is chosen to achieve site-directed mutagenesis; for example, to change a particular amino acid.

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The Streptococcal Proteome

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Introduction

The nucleotide sequence of the *Streptococcus pyogenes* genome was first determined in 2001, which inferred a proteome of 1,752 proteins (Ferretti, et al., 2001). The genomic sequencing of additional isolates showed that all streptococcal chromosomes are poly-lysogenized with bacteriophages (Sumby, Whitney, Graviss, DeLeo, & Musser, 2006), which account for approximately 10% of their genome content (Ferretti, et al., 2001). The number and types of prophages that are present in the genomes of different clinical isolates varies significantly, which suggests a stochastic process of co-evolution (Canchaya, Fournous, & Brüssow, 2004). Horizontally transmitted integrative conjugative elements, transposons, and insertion sequences also contribute to genomic variation within the species. Due to the extent of horizontal DNA transmission, *S. pyogenes* is thought to have a theoretically infinite *pan*-genome and—by extension—a theoretically infinite proteome (Desiere, McShan, van Sinderen, Ferretti, & Brüssow, 2001). Moreover, multiple protein isoforms can be derived from a single open reading frame (ORF) following post-translational modifications, such as truncation or phosphorylation, and each isoform may have a unique function. As a result, the complexity of an organism's proteome is estimated to be at least two to three orders of magnitude greater than that of the genome (Figure 1) (Matthiesen & Jensen, 2008; Cain, Solis, & Cordwell, 2014).

The availability of the genome sequence of *S. pyogenes* and advances in mass spectrometry (MS) have greatly enhanced our ability to characterize proteins on a genome-wide scale. Proteomic studies of *S. pyogenes* have been motivated by the pursuit of answers to fundamental questions, such as: How are proteins trafficked to specific sub-cellular locations? What is the functional significance of protein localization? How does the proteome transition in response to changing conditions encountered in the host? What are the differences between streptococci associated with a localized infection, as compared to those associated with life-threatening invasive infections? What are the functional significances of interactions between streptococcal proteins and human host proteins? What physiologic changes in the proteome occur in response to the presence of antimicrobials? What are correlates of protective immunity? What Streptococcal proteins can be used to vaccinate against disease? What proteins elicit pathogenic antibodies? Answering these questions is important in developing new approaches to mitigate the morbidity and mortality associated with *S. pyogenes*.

In this chapter, we focus on the advances in our understanding of the *S. pyogenes* proteome from the perspective shaped by results obtained using proteomics, or studies that have simultaneously characterized a set of proteins. We start by describing the methods that have been used to study the proteome and then discuss how these approaches have led to insights into the pathogen's response to changing conditions, circumvention of the immune response, and the organization and regulation of extracellular proteins. Finally, we will review advances in identifying proteins that evoke auto- and protective immunity. Given the rapid pace at which various aspects of proteomic investigation are progressing, including instrumentation, workflow strategies, and bioinformatics, the characterization of the *S. pyogenes* proteome is, in many ways, just beginning.

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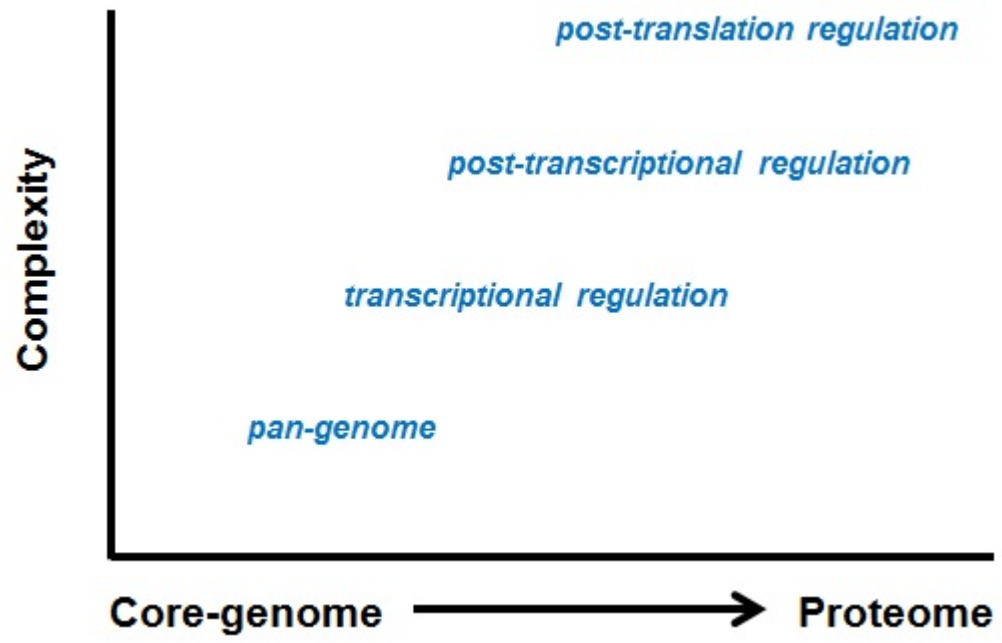

Figure 1: The complexity of the Streptococcal proteome is significantly greater than the corresponding core genome due to the acquisition of horizontally transmitted DNA contributing to the *pan* genome, the regulation of gene expression and post-translational modification of proteins.

Sub-cellular proteomes

The term *proteome* refers to the entire set of proteins that are expressed by an organism at a certain time under specific conditions. Unlike genomics and transcriptomics, proteomics examines compartmentalized sets of proteins. Therefore, protein localization is an important feature for characterizing the proteome. There are four spatially defined sub-proteomes of *S. pyogenes*: cytoplasmic proteins, cell membrane proteins, cell wall-associated proteins, and soluble exoproteins or culture supernatant proteins (CSPs).

Protein Trafficking

S. pyogenes lacks a twin-arginine translocation pathway (Dilks, Rose, Hartmann, & Pohlschröder, 2003) and most proteins are translocated from the cytoplasm by the general secretion pathway, which is often referred to as the Sec pathway (Schneewind & Missiakas, 2014). These proteins possess an amino-terminal signal peptide that consists of an "N region" of hydrophilic amino acids, an "H region" of approximately 17 hydrophobic amino acids, and a slightly hydrophilic "C region." During translation, the signal peptide is recognized by the signal recognition particle (SRP), which is composed of the Ffh protein and a small cytoplasmic RNA (Miller, Bernstein, & Walter, 1994). SRP targets the nascent polypeptide to the membrane receptor FtsY and the SecYEG translocon (Schneewind & Missiakas, 2014; Halic, et al., 2006). In *S. pyogenes*, SecA and other accessory proteins, such as the HtrA chaperone, have been reported to be localized to a single area of the cytoplasmic membrane, or microdomain, which is known as the ExPortal (Rosch & Caparon, 2004), although a separate study has observed SecA throughout the membrane (Carlsson, et al., 2006). Similar to *S. mutans* (Crowley, Svensäter, Snoep, Bleiweis, & Brady, 2004), the SRP protein Ffh is not required for the viability of *S. pyogenes* (Rosch, Vega, Beyer, Lin, & Caparon, 2008).

Proteins secreted by the Sec pathway are destined for the cytoplasmic membrane, the cell wall, or for release into the extracellular environment. These proteins are often present in more than one compartment. Proteins covalently attached to the cell wall possess a carboxyl terminal motif LPXTG or a similar motif (Fischetti,

Pancholi, & Schneewind, 1990). During export from the cytoplasm, the motif is recognized by the membrane-localized sortase, which cleaves the polypeptide and covalently anchors the protein to peptidoglycan (Mazmanian, Liu, Ton-That, & Schneewind, 1999; Perry, Ton-That, Mazmanian, & Schneewind, 2002). Several proteins that are important to the virulence of *S. pyogenes* contain the LPXTG motif, including the antiphagocytic M protein, which is often considered to be the single most important virulence factor of *S. pyogenes* (Cunningham, 2000).

Three sortases have been identified in *S. pyogenes*. SrtA is a housekeeping sortase that catalyzes the attachment of most cell wall-associated proteins in a two-step reaction. First, the LPXTG motif is cleaved, which leaves the carboxyl group of threonine to react with a free amine present in lipid II and results in the attachment of the protein to the cell wall (Mazmanian, Liu, Ton-That, & Schneewind, 1999; Perry, Ton-That, Mazmanian, & Schneewind, 2002). SrtA anchors the M protein, C5a peptidase (ScpA) protein, G-related α 2M-binding protein (GRAB), protein F (Barnett & Scott, 2002), and probably several more proteins to the cell wall. SrtA is localized to specific foci of the cytoplasmic membrane, mostly the septum (Raz & Fischetti, 2008). A second sortase, SrtB, is encoded in the FCT chromosomal region (so named because it encodes fibronectin, collagen, and T-antigen proteins) and catalyzes the attachment of the T antigen (pilin) to the cell wall (Barnett & Scott, 2002). SrtB recognizes an LPSTG motif. A third sortase, SrtC, is also encoded in the FCT region and anchors the protein encoded by the adjacent gene via a QVPTGV motif (Barnett, Patel, & Scott, 2004).

Recent studies suggest that additional information is contained within the signal peptide than was previously known. For example, M6 and the fibronectin binding protein PrtF.2 both possess a signal peptide and LPXTG motif. M6 is secreted at the septum and is localized throughout the cell wall, while PrtF.2 is concentrated at the poles. Swapping the signal sequences between the proteins reverses the pattern of localization, which indicates that the signal peptides determine the final location of the proteins (Carlsson, et al., 2006). In a related study, the localization of four proteins (M protein; SPN, an NAD(+)-glycohydrolase; SLO, a secreted cytolysin; and SpeB, a secreted cysteine protease) that possess signal peptides was analyzed in an Ffh mutant (lacking a functional SRP) (Rosch, Vega, Beyer, Lin, & Caparon, 2008). M protein localization to the cell wall was not affected by the absence of SRP (the Ffh mutant). In contrast, both SPN and SLO were not secreted in the mutant strain, which indicates that their export requires SRP. Notably, SpeB was not secreted by the mutant, unless glucose was added to the media. The studies indicate that more remains to be learned about the sorting and localization of proteins to the cell wall and extracellular milieu, as well as the functional consequences of the arrangement of proteins within these compartments.

Many studies have shown the importance of secreted proteins to virulence. In support of those findings, an SRP-deficient mutant of *S. pyogenes* is less virulent in animal models of infection (Rosch, Vega, Beyer, Lin, & Caparon, 2008), which suggests that therapeutic strategies designed to inhibit SRP-mediated protein localization could be effective in mitigating disease.

Fractionation techniques

The streptococcal proteome is partitioned during analyses and is often discussed in terms of these fractions, or sub-proteomes, which include CSPs, cell wall-associated proteins, cytoplasmic membrane proteins, and cytoplasmic proteins. Figure 2 summarizes the typical workflow used for fractionating these proteins. The use of media devoid of peptides or proteins, such as chemically defined media or rich media that has been filtered to remove proteins and peptides, can simplify the characterization of the CSPs, because all the proteins and peptides present are derived from *S. pyogenes*. Following the centrifugation of bacterial cultures and the collection of CSPs, the cell pellet is suspended in a buffer, and the proteins are further fractionated by separating the cell wall-associated proteins from membrane and cytoplasmic proteins. This is done by treating the cells with enzymes that degrade peptidoglycan, thereby releasing proteins attached to the cell wall into solution. Mutanolysin and lysozyme have often been used; however, a proportion of the resulting protoplasts typically lyse during enzymatic treatment. As a result, the cell wall fraction is contaminated with cytoplasmic proteins; this

occurs even when using an osmoprotective buffer. One method to improve these results is through the use of a bacteriophage derived N-acetylmuramoyl-L-alanine amidase to hydrolyse the N-acetylmuramic acid, L-alanine bond of the cell wall (Nelson, Loomis, & Fischetti, 2001). The enzyme, known as PlyC, was validated for this purpose and was found to decrease contamination with cytoplasmic proteins (Köller, et al., 2008). Following amidase treatment, centrifugation will separate the cell wall-associated proteins present in the supernatant fraction from the protoplasts that contain the cytoplasmic and membrane localized proteins. These fractions are separated by lysing the protoplasts and separating the membrane proteins (the pellet) and cytoplasmic proteins (supernatant) by centrifugation. The comparative complexity of the cytoplasmic protein fraction and the technical challenges of working with cell membrane proteins have hindered the characterization of these fractions. Nonetheless, cell membrane proteins are present at the host-pathogen interface and are essential to cellular homeostasis, including communication between the host environment and the cytoplasm. As a result, further characterization of this sub-proteome is an important area for future investigation.

Separating proteins by gel electrophoresis and liquid chromatography

Streptococcal proteins are usually separated by either gel electrophoresis or liquid chromatography (LC), and proteins of interest are identified with mass spectrometry. Much of the investigation into the *S. pyogenes* proteome has used two-dimensional gel electrophoresis (2-DE) to resolve complex protein mixtures (O'Farrell, 1975). While it is remarkably powerful in its ability to separate very similar proteins, 2-DE has several limitations. First, to visualize the proteins, they must be stained, and the sensitivity and the dynamic range of protein detection are both dependent on the staining process. Although progress has been made in the past decade to develop more sensitive stains that are compatible with mass spectrometry, protein detection is still limited to relatively abundant proteins. This means that less abundant proteins that are present and that may be of interest, such as regulatory proteins, are below the limits of detection. In addition, not all proteins can be simultaneously analyzed by 2-DE, due to differences in their solubility, size, and other biochemical characteristics. Finally, the 2-DE process is relatively laborious and time consuming. Because of these limitations, the use of gel-free methods, such as multi-dimensional liquid chromatography, has attracted considerable attention, and is being used in many studies on the *S. pyogenes* proteome. This approach is also referred to as "shotgun" proteomics, due to its similarity to shotgun DNA sequencing. A key feature of this strategy is that a mixture of proteins is first digested with trypsin (or another protease with a specific recognition site) to facilitate identification by mass spectrometry. The peptides are then resolved by liquid chromatography. While protein digestion increases the number of molecules present in the sample, since multiple peptides are derived from each protein, the biochemical homogeneity of peptides simplifies their characterization, as compared to proteins. Nonetheless, because so many peptides are contained in samples, multi-dimensional LC is necessary to resolve the peptides. MuDPIT is one example of such multi-dimensional protein identification technology. In this case, peptides are initially separated by cation exchange chromatography, with the eluting peptides further separated by reverse phase liquid chromatography. The chromatography steps can either be done separately, or more commonly, by sequential analysis. This analysis is achieved by connecting two resolving columns so that peptides separated with the first column (1^{st} dimension) elute directly into a second column (2^{nd} dimension). Advances in the chemistries used for sequential separation and the development of LC pumps that can produce reliable gradients at nanoliters per minute flow rates have greatly contributed to the ability to use multi-dimensional liquid chromatography (Zhang, Fonslow, Shan, Baek, & Yates, 2013). Typically, the peptides from the 2^{nd} dimension column elute directly into a mass spectrometer for characterization and protein identification.

Identifying proteins with peptide mass fingerprinting

Proteins can be identified with mass spectrometry by a process known as peptide mass fingerprinting (PMF). To accomplish this process, the protein of interest (which is either present in solution or within an excised portion of a polyacrylamide gel) is digested with trypsin, which cleaves the peptide bonds adjacent to each lysine and

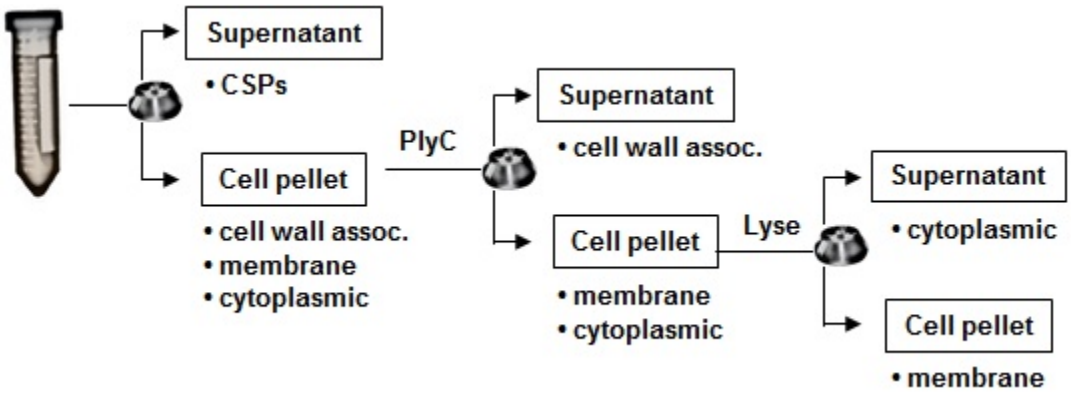

Figure 2: Fractionation of the *S. pyogenes* proteome. CSPs are obtained following centrifugation of the bacterial culture. The bacterial pellet is suspended in buffer and treated with PlcY (or mutanolysin) to release cell-wall–associated proteins covalently linked to peptidoglycan. Following centrifugation, the supernatant contains the cell-wall–associated proteins. Protoplasts are then lysed, and after centrifugation, the cytoplasmic membranes will be in the supernatant and the membrane proteins in the pellet.

arginine residue. The masses of the resulting peptides (tryptic peptides) are then measured with a mass spectrometer. Note that the mass measurement is highly accurate; it is well within 1 atomic mass unit, which means that there are a limited number of peptides encoded by the streptococcal genome with a nearly identical mass, as measured by the mass spectrometer. As the name indicates, PMF identifies proteins by matching experimentally determined peptide masses with theoretical masses inferred from the corresponding genome (Matthiesen & Carvalho, 2010).

Accurate protein identification with PMF requires matching several (usually a minimum of 3) measured peptide masses to inferred peptide masses. This is because a peptide from one protein could be modified so that it has nearly the same mass as an inferred peptide from a different protein; or in some cases, a highly conserved motif may be present in more than one protein. A routine solution to this limitation is to incorporate post source decay (PSD) or collision-induced dissociation (CID) into the analysis. Both techniques first measure the mass of a specific peptide. The peptide is then fragmented by breaking peptide bonds, and the masses of the smaller peptides and amino acids are measured. Contemporary mass spectrometers accomplish this process by isolating a peptide ion of interest from other peptides in the sample through a process known as ion gating. The mass of the isolated peptide is then measured and is referred to as the mass of the parent peptide (MS1). The parent peptide is then fragmented into a series of sequentially smaller peptides and amino acids, and their masses are determined (MS2). The entire process is known as tandem mass spectrometry (Figure 3), or MS/MS. The result of using MS/MS is that multiple fragment masses can be matched to those that are inferred from the genome sequence. In addition, the peptide can be sequenced *de novo* based on the masses of individually fragmented ions, since all the common amino acids (except leucine and isoleucine) can be distinguished by their unique masses. When using MS/MS, the detection and fragmentation of a single parent peptide can be sufficient to confidently identify the corresponding protein. Other methods of fragmenting peptides include collision-assisted dissociation (CAD), electron transfer dissociation (ETD), and electron capture dissociation (ECD) (Zubarev, Zubarev, & Savitski, 2008); however, these have not yet been applied to studies involving *S. pyogenes*.

Protein quantification

The quantification of proteins separated by either 1 or 2-dimensional gel electrophoresis is most often (and most easily) accomplished by staining the proteins and then scanning the stained gel. The sum of the number of pixels comprising the protein band, or spot, and the intensity of each pixel is proportional to the amount of protein within the band or spot. The majority of studies have used R-250 or G-250 Coomassie Blue, modified silver staining protocols compatible with mass spectrometry; or one of the many fluorescent stains available, such as

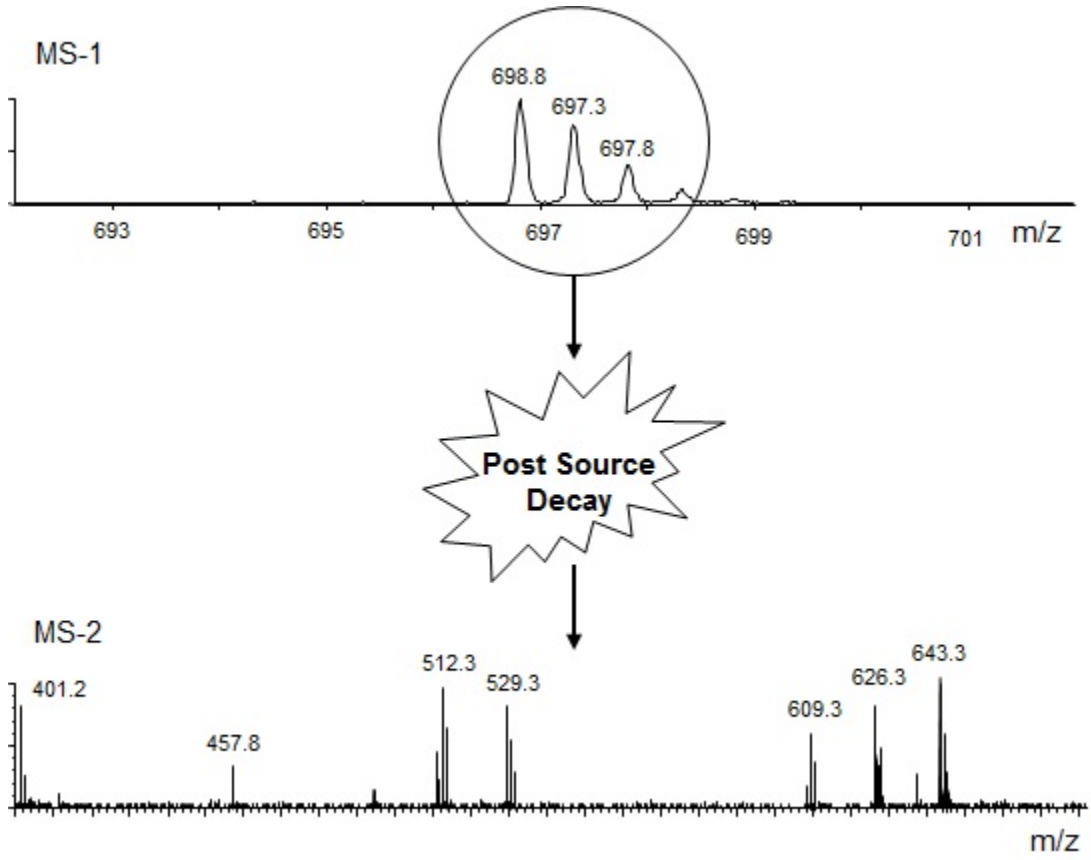

Figure 3: Protein identification by tandem mass spectrometry. Tryptic peptides are separated by liquid chromatography and elute into the mass spectrometer. The mass of the parent tryptic peptide is measured in MS-1. The peptide is then fragmented and the masses are measured. In the example above, the mass of the tryptic peptide is 1,392 (it has a charge of $^{+}2$). The fragments and masses are shown.

SYPRO Ruby, Deep Purple, or LavaPurple. The fluorescent stains are generally more sensitive and have a greater dynamic range, as compared to silver staining methods that are compatible with mass spectrometry. Because of limitations in sensitivity and other drawbacks associated with 2-DE, more studies using gel-free methods to separate proteins have been developed to quantitate proteins using the mass spectrometer.

Difference in-gel electrophoresis to compare protein abundance

Protocols have been developed to quantitatively compare proteins separated by 2-DE between two or more samples without using separate gels, which helps to avoid problems associated with trying match protein spots among gels. Difference in-gel electrophoresis (DIGE) uses a combination of fluorescent stains or probes with different excitation and emission wavelengths to quantitatively compare proteins from more than one sample (Unlü, Morgan, & Minden, 1997). For example, proteins isolated from a wild-type *S. pyogenes* isolate can be labeled via lysine residues with succinimidyl esters of propyl-Cy3, and those from a mutant derivative can be similarly labeled with a methyl-Cy5 ester. The two cyanine family fluorophores have the same masses and charges, but have different emission wavelengths. The labeled proteins from each sample are mixed prior to their separation by electrophoresis. Because the samples are separated simultaneously in a single gel, the differentially labeled proteins will migrate to exactly the same place within the gel. The gel is then scanned to measure the intensity of the individual flours. One of the limitations of this technique is that the fluorescent labels are less sensitive than SYPRO Ruby and silver staining (Matthiesen & Amorim, 2010).

Label-based protein quantification with mass spectrometry

Label-based strategies for quantitating proteins with mass spectrometry use either stable isotopes or the mass tag labeling of proteins or peptides. The advantage of these techniques as compared to quantifying stained proteins is that quantitation is done using the mass spectrometer, which is far more sensitive than the currently available protein dyes or flourophores . With these methods, proteins from two, or more, samples are covalently labeled with isotopically distinguishable elements, such as ^{14}N and ^{15}N, and are then analyzed with mass spectrometry. The labeling can be performed before or after protein digestion. After labeling, the samples are combined and the masses of tryptic peptides are determined. The relative difference in protein abundance between the two samples is determined by comparing the ratios of ion peak intensities from the differentially labeled samples (either peptides labeled directly or peptides derived from a labeled protein). For example, the intensity of a peptide from a wild-type sample can be directly compared to that from a mutant, because the specific peptide ions will differ by exactly one mass unit (when labeled with^{14}N and ^{15}N).

Proteins can also be labeled *in vivo* by growing streptococci in media containing isotopic precursors, most commonly amino acids. The process is known as stable isotope labeling by amino acids in culture (SILAC) (Ong, et al., 2002). For example, a wild-type strain can be cultured with media that contains ^{12}C-lysine and a mutant derivative similarly cultured with ^{13}C-lysine. When the peptides obtained from the two strains are simultaneously analyzed by mass spectrometry, they will differ by exactly one atomic mass unit, and the intensities of the isotopic ion peaks will be proportional to the abundance of the cognate protein (Figure 4).

In vitro protocols use a post-biosynthetic labeling strategy that can be applied to any set of protein samples obtained from streptococci. To do so, proteins are labeled with isotope-coded affinity tags (ICAT) before protein digestion, or with isobaric mass tags (isobaric tags for relative and absolute quantification, or iTRAQ) after protein digestion. ICAT uses biotinylated derivatives of iodoacetamide, which react with the cysteine residues of denatured proteins. The biotin labeling permits cysteine-containing peptides to be isolated from complex peptide mixtures, through their affinity for streptavidin. This strategy reduces the number of peptides introduced into the mass spectrometer, which can enhance both sensitivity and mass accuracy. To determine the relative amounts of proteins from two samples, such as from a wild-type and mutant strain, two isotopic biotin-containing tags are used (^{12}C and ^{13}C). The peptide ion peak intensities of each sample are then compared to determine the relative amounts of the corresponding protein in each sample. Some proteins may not be detected using this method if the cysteine residues are not present or reactive (Shiio & Aebersold, 2006).

The iTRAQ method involves labeling peptides from different sources, such as from a wild-type and mutant strain, after protein digestion with chemical groups that have the same mass. The label contains an amine-specific reactive group, a balancer group, and a reporter mass group. Because the mass of the tagged peptides is identical in both samples, potential differences in the efficiency of ionization associated with peptides that have different mass tags is eliminated. A decrease in ionization efficiency would decrease ion intensity, which is assumed to correlate with protein abundance. With this method, a single parental peptide mass from both samples is measured in MS1, because the peptides from the two samples have tags with identical masses; however, fragmentation of the tryptic peptides during MS2 analysis will release reporter ions with different masses that are unique to each sample (wild-type and mutant). Again, the intensities of the fragment ions correlate to the abundance of the parent protein. The advantage of this procedure is that it eliminates the possibility that a difference in the molecular weight of an isotopically labeled protein will change the efficiency at which tryptic peptides ionize. iTRAQ is the most widely used protocol for isobaric mass tag protein quantitation, and can be used to simultaneously compare up to eight samples (Ross, et al., 2004).

Label-free protein quantitation with mass spectrometry

Three different strategies can be used to determine the quantities of proteins without labeling them. One strategy is based on the assumption that the ion peak intensities of peptides correlate with the abundance of their parent

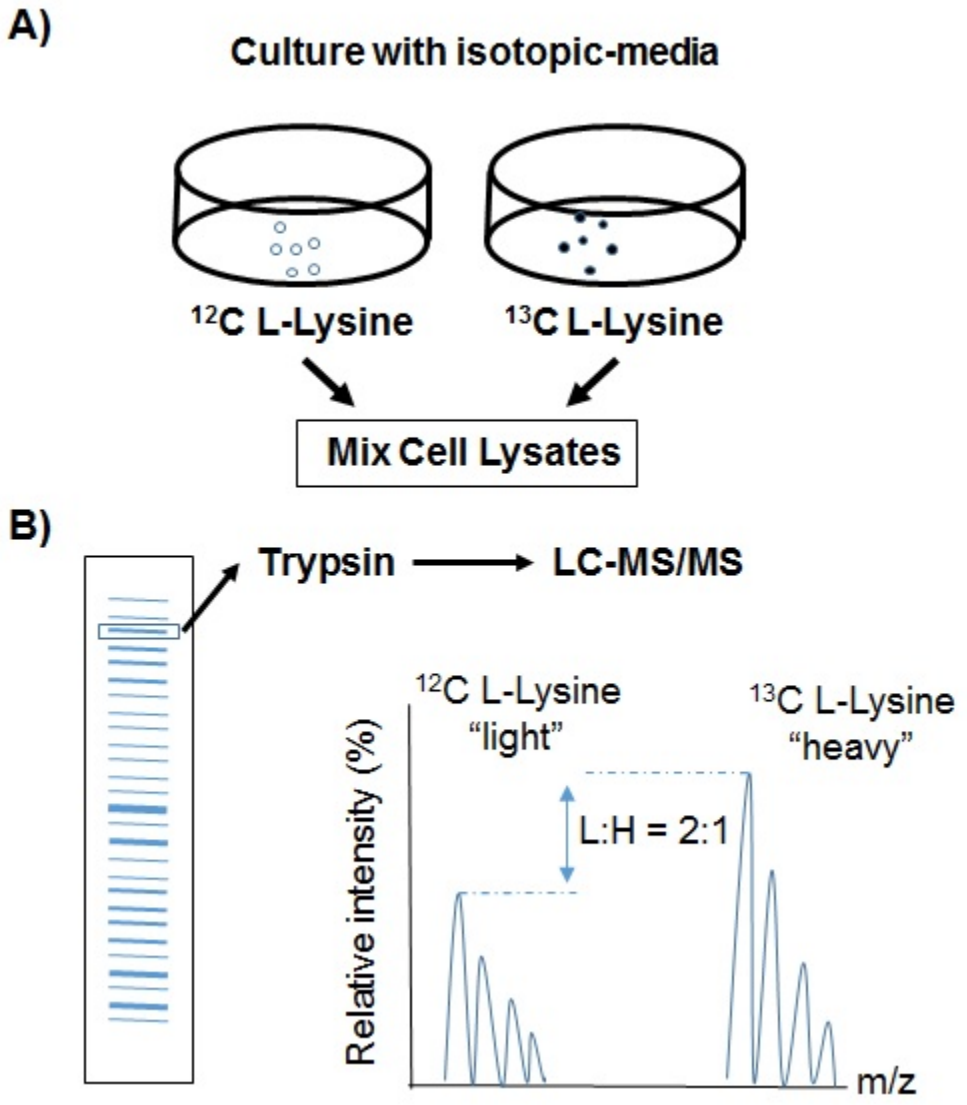

Figure 4: Stable isotope labeling by amino acids in culture (SILAC) to quantitatively compare the abundance of proteins. A) Two strains (wild type and mutant) are grown with either a ^{13}C or ^{12}C amino acid such as lysine. Following culture, cell lysates are obtained and the proteins combined and separated by gel electrophoresis. B) Protein bands are excised from the gel, digested with trypsin, and analyzed together with LC-MS/MS. The intensity of the peptide ions corresponds to the relative abundance of the parent proteins.

protein in complex samples, which is known as the protein abundance index method (PAI). The other two methods are targeted counting using either selected ion monitoring (SIM) or selected reaction monitoring (SRM).

The PAI method has two main features. First the number of tryptic peptide masses (assuming that trypsin is used in the digestion step) measured for a particular protein is compared to all the theoretical peptides that are predicted from *in silico* digestion of that particular protein. The assumption is that the number of peptides that are detected, as compared to the theoretical number of peptides, directly correlates to the abundance of the parent protein. When using this approach, it's important to validate the results by determining the normalized spectral abundance factor (NSAF). This validation is performed by calculating the ratio of the number of MS/MS spectra obtained for each protein (Wasinger, Zeng, & Yau, 2013) to the total number of spectra obtained from all of the proteins detected in the sample (Zhu, Smith, & Huang, 2010). Malmstrom et al. used this label-

free method to examine how the intracellular homeostasis of *S. pyogenes* is influenced by exposure to human plasma, as discussed in more detail below (Malmström, et al., 2012).

The SIM method can be used with scanning mass spectrometers (those instruments capable of detecting ions throughout a defined mass range, such as quadrupoles and Q-ToFs) by restricting the acquisition mass range to only the ion(s) of interest. In other words, a mass spectrometer is programmed to detect only the masses that correspond to peptides that are derived from the proteins of interest. The narrower the mass range, the more specific the SIM assay is to a particular protein. SIM is highly sensitive because by focusing on a particular mass, and ignoring all the others in the sample, more time is devoted by the instrument to measuring the mass of interest, which improves the overall signal intensity and accuracy of mass determination (Lange, et al., 2008).

Similarly, the SRM method uses a triple quadrupole (QQQ) mass spectrometer to detect one peptide derived from the protein of interest. Moreover, the mass of a specific fragment ion of the peptide of interest is also targeted for measurement. The peptide and fragment ion are referred to as a transition pair. Similar to SIM, the instrument dedicates its analysis to the measurement of only a few very specific masses while ignoring the other ions in the sample. A QQQ is particularly well suited to this application, because two quadrupoles (Q1 and Q3) are configured as mass filters that monitor ions to measure the masses of interest. The Q1 quadrupole creates a mass gate, in which only ions with approximately the mass of the tryptic peptide of interest are measured. After measuring the mass, the ions are then fragmented in Q2, and the fragmented mass of interest is measured in Q3. To determine the absolute quantity of the protein in a sample, a selected peptide derived from the protein of interest is biochemically synthesized in an isotopic form (^{13}C) and is added to samples at different concentrations. When the samples are analyzed, a standard curve can be created, based on the ion intensity of known amounts of the isotopic peptide. The intensity of the peptide of interest is then used with the standard curve to determine the absolute concentration of the protein of interest. When SRM is applied to multiple proteins, it is called multiple reaction monitoring (MRM) (Lange, et al., 2008). In MRM, the instrument is configured to detect several transition pairs of interest. Lange et al. have used MRM to quantitatively measure streptococcal virulence factors (Lange, et al., 2008). One of the challenges to performing SRM and MRM is in the selection of transition pairs, which has traditionally been a tedious, time-consuming process; however, the development of *in silico* tools has greatly simplified this process. Moreover, Karlsson et al. have validated over 10,000 transition pairs to quantitate streptococcal proteins by SRM (Karlsson, Malmström, Aebersold, & Malmström, 2012).

Characterization of the *S. pyogenes* proteome

In the following sections, we review studies that have used proteomic approaches to characterize the streptococcal proteome. Table 1 includes a summary of these studies, which includes the subproteome investigated, the methods used, and their major findings.

Table 1. Major studies characterizing the group A Streptococcus proteome.

Fraction studied	Technique	Experimental Condition	Major findings	Refs.
Cytoplasmic	2-DE	Exponential vs. post-exponential	Identified the most abundant cytoplasmic proteins, which were predominantly metabolic enzymes. In addition to growth phase associated changes in protein isoforms, quantitation of transcriptional changes with DNA microarray analysis showed that post-transcriptional regulation is an important aspect of pathogen adaptation to changes in the growth phase.	(Chaussee, Callegari, & Chaussee, 2004)

Table 1. continued from previous page.

Fraction studied	Technique	Experimental Condition	Major findings	Refs.
CSPs	2-DE	Influence of Fe, O_2, NaCl, CO_2, and temperature	The abundance of SpeB, Sic, SpeF, EndoS, Mf-3 and SKA varied in response to Fe, temperature, and NaCl.	(Nakamura, et al., 2004)
Cell wall-associated	2-DE	Addition of hyaluronic acid	Hyaluronic acid increased exoproteins involved in adherence including M protein, GAPDH, and a collagen like surface protein.	(Zhang, et al., 2007)
CSPs	2-DE	Growth in sub-inhibitory concentrations of clindamycin	The presence of clindamycin at various points in the growth curve differentially altered the abundance of CSPs, including SpeB, which was less abundant.	(Sawai, et al., 2007)
Cytoplasmic	2-DE	Exposure of a PenG susceptible and tolerant strain to PenG	The proteome of a PenG tolerant strain in the absence of PenG exposure was similar to that of the susceptible isolate after exposure to PenG. The results suggested the tolerant strain is pre-adapted for tolerance.	(Chaussee, McDowell, Rieck, Callegari, & Chaussee, 2006)
Cytoplasmic and CSPs	2-DE	Exposure to the phytochemical rhodomyrtron	The levels of several metabolic enzymes, including glyocolytic enzymes, were altered following rhodomyrtron exposure; however, the target of rhodomyrtron remains to be determined.	(Limsuwan, Hesseling-Meinders, Voravuthikunchai, van Dijl, & Kayser, 2011)
CSPs	2-DE	A *perR* mutant compared to the parental A20 isolate	Among 38 changes in the abundance of CSPs between the strains, there was less MF-3 produced by a *perR* mutant. The decrease was associated with decreased virulence of the mutant.	(Wen, et al., 2011)
CSPs and cell wall-associated	2-DE	An *nra* regulatory gene mutant compared to the parental isolate	The abundance of 67 proteins was altered in the mutant including the virulence associated proteins PrtF.2 and SclA.	(Kreikemeyer, et al., 2007)
CSPs	2-DE	An isolate with a naturally acquired *csrS*/*covS* mutation was compared to a derivative with a functional *csrS*/*covS* gene	Increased amounts of SclA and Gls24 were identified in the naturally acquired *csrS*/*covS* mutant. Both proteins were also discovered to be important for growth in human blood.	(Tsatsaronis, et al., 2013)
Surface proteins	SRM-MS	Compared the surface proteome after incubation with pooled human sera or saliva	*S. pyogenes* incubated with plasma bound IgG1, IgG3 and proteins associated with both the classical and alternative complement pathways. When incubated with saliva, the major proteins bound to the surface were IgG1, IgG2 and complement factor H, which inhibits complement activation.	(Nordenfelt, et al., 2012)
Surface proteins	SRM-MS	Compared the binding of human plasma proteins to the surfaces of wt AP1 and a *mga* mutant	The wt isolate bound 35 times more Fg, C4BP, and S protein compared to the mutant. The wt isolate also had fewer proteins associated with the complement membrane attack complex associated with its surface.	(Sjöholm, Karlsson, Linder, & Malmström, 2014)

Table 1. continued from previous page.

Fraction studied	Technique	Experimental Condition	Major findings	Refs.
Predicted surface localized proteins	Protein array	Used a protein chip consisting of 106 Streptococcal surface proteins to identify proteins that could bind human proteins including Fg, fibronectin, and C4BP	For the first time, two membrane proteins (Spy_1037 and Spy_1326) and Spy_0591 were discovered to be receptors for human C4BP and Fg proteins.	(Margarit, et al., 2009)
Surface proteins	Shotgun proteomics and SRM	Identified and quantified host proteins bound to surface when *S. pyogenes* was exposed to human plasma	The most abundant plasma proteins on the *S. pyogenes* surface were Fg, immunoglobulins, C4BP, albumin, and protein S.	(Sjöholm, Karlsson, Linder, & Malmström, 2014)
Cytoplasmic	Label free LC MS/MS	Changes in cytoplasmic proteins when cultured with various amounts of human plasma	Enzymes involved in fatty acid biosynthesis were less abundant when cultured with plasma. This was associated with the acquisition of fatty acids from plasma via the binding of albumin-fatty acid complexes to the pathogen's surface.	(Malmström, et al., 2012)
Secreted proteins with signal sequence, lipoprotein or LPXTG motifs	Protein array	Identify protein-protein interactions among extracellular proteins	A putative extracellular amidase, Mur1.2, bound several virulence associated proteins including HylA, SKA, SLO, and ScpA. Mur1.2 may play a role in the spatial organization of extracellular proteins.	(Zhu, Smith, & Huang, 2010)
CSPs	2-DE	Characterized CSPs from M1 and M3 isolates and identified those that reacted with sera from infected mice or humans	Nearly all the glycolytic enzymes were present in the CSP fraction and many were immunogenic, indicating they were also extracellular during infection.	(Lei, Mackie, Lukomski, & Musser, 2000)
Cell wall and CSPs	2-DE	Compared extracellular proteins obtained from wild-type HSC5 to an *htrA* mutant	HtrA in strain HSC5 promoted SpeB activation, which was associated with degradation of most extracellular proteins.	(Cole, et al., 2007)
CSPs	2-DE	Examined CSPs in M1T1 SpeB+ and SpeB- variants	Showed that active SpeB can degrade nearly all the CSPs.	(Aziz, et al., 2004)
Patient sera	*S. pyogenes* protein array	Examined patient sera for reactivity to Streptococcal proteins	Sera from patients with neurological symptoms had elevated antibody titers to multiple streptococcal proteins.	(Bombaci, et al., 2009)
Cell wall and membrane	SDS-PAGE gel sliced into multiple fractions	Identified proteins likely to be surface exposed as potential vaccine candidates	Detected canonical cytoplasmic proteins in the extracellular fractions.	(Sharma, et al., 2013)
CSPs	2-DE with Edman degradation	Compared CSPs from M1 and M3 isolates and identified proteins that elicited antibodies during human infection	Identified proteins in CSP that elicited antibodies during human infection. Also found that at least some protein isoforms detected by 2-DE are likely to be the result of truncations at the amino terminus.	(Lei, Mackie, Lukomski, & Musser, 2000)

Table 1. continued from previous page.

Fraction studied	Technique	Experimental Condition	Major findings	Refs.
Cell wall-associated	2-DE with MALDI TOF MS	Identified surface exposed proteins based on reactivity with human sera and accessibility to biotinylation	Nearly half of the proteins were immunogenic and about a third reacted with biotin, suggesting that many, but not all, of the proteins in the cell wall associated fractions are surface exposed.	(Cole, et al., 2005)
Intact cells	Surface shaving	Identified surface exposed proteins based on susceptibility to trypsin	Identified surface exposed proteins and tested their ability to protect against infection using a mouse model.	(Rodríguez-Ortega, et al., 2006)
Intact cells	Surface shaving	Identified surface exposed proteins based on susceptibility to trypsin	Identified 33 protein previously not recognized as surface exposed.	(Severin, et al., 2007)
Intact cells	Surface shaving	Used a systems approach to identify novel vaccine candidates	Identified six vaccine candidates including previously characterized SLO and ScpA, as well as SPy_0416, SPy_0269, SPy0019, and SPy_2010. The proteins were all highly expressed, surface exposed, and elicited antibodies during human infection.	(Bensi, et al., 2012)

Proteome changes in response to different environmental conditions

Identifying changes in gene expression is most conveniently done at the transcript level. This is partially due to the availability of highly sensitive methods to measure transcripts, including quantitative RT-PCR, and the biochemical homogeneity of RNA. Nonetheless, because proteins mediate the majority of cellular functions, and because post-transcriptional changes can greatly impact protein abundance and function, it is also important to characterize the changes that occur at the protein level in response to different environmental conditions.

The study of differences in protein abundance between the exponential and post-exponential phases of growth is an experimentally convenient way to characterize the microbes' response to changes in cell density, the accumulation of metabolites (quorum sensing), nutrient depletion, decreasing pH values, and other stresses. Many of these are relevant to the changing conditions that occur during infection. Cytoplasmic proteins from samples obtained during the exponential and post-exponential phases of growth were compared (Chaussee, Callegari, & Chaussee, 2004). The use of 2-DE resolved 527 proteins; 125 of these were identified, which corresponded to 78 genes, since multiple isomers of a single ORF were often identified. The most abundant cytoplasmic proteins were enolase and GAPDH. In addition to glycolytic enzymes, many other catabolic enzymes were detected, including those involved in pyruvate metabolism (AcoB, Spy_1028; AcoL, Spy_1031), which convert pyruvate to acetyl CoA and CO_2; L-lactate dehydrogenase; and the enzymes of the arginine deiminase pathway encoded by the ArcABC operon, which convert arginine to citrulline, NH_3, and ATP. The analysis was coupled with transcriptome characterization. Together, the results identified several growth phase associated changes mediated at the post-transcriptional level, including changes in the expression of lactate dehydrogenase and a cell division initiation protein (DivIAV). In addition, the abundance of several protein isoforms varied in a growth-phase–dependent manner, which indicates that regulation at the post-translational level is also an important (though poorly understood) means of adapting to changing environmental conditions.

Other conditions thought to be important to different stages of infection include the concentrations of hyaluronic acid and iron. Iron is essential for bacterial growth, and iron availability has a significant impact on the streptococcal proteome. Iron supplementation decreased the abundance of 17 exoproteins, including several established virulence factors (Sic, SpeF, and Ska (Nakamura, et al., 2004)). Hyaluronic acid, in addition to being the capsule produced by *S. pyogenes*, is a non-sulfated glycosaminoglycan that is widely distributed in human

tissues, including the skin. The addition of hyaluronic acid to cultures increased the abundance of several virulence factors, including the M protein, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a collagen-like surface protein, and two hypothetical proteins (Zhang, et al., 2007). One interpretation of these results is that hyaluronic acid promotes the production of exoproteins that facilitate adherence.

S. pyogenes is often exposed to antimicrobials during the course of infections. High doses of clindamycin and penicillin are recommended for the treatment of invasive diseases, including toxic shock syndrome; in part because clindamycin (an inhibitor of ribosome translocation) reduces the production of exoproteins that contribute to disease (Mascini, Jansze, Schouls, Verhoef, & Van Dijk, 2001; Coyle, Cha, & Rybak, 2003). An examination of the influence of sub-inhibitory levels of clindamycin on the secretome of *S. pyogenes* by using 2-DE showed that exposure decreased the abundance of the extracellular cysteine protease (SpeB) (Sawai, et al., 2007); however, the abundance of several other exoproteins increased when clindamycin was added to cultures at the beginning or early exponential phase of growth. Many of these proteins are known to be degraded by SpeB (Aziz, et al., 2004), which suggests that the increase in various exoproteins was due to decreased degradation by SpeB. Surprisingly, however, the increases in SLO, NAD-glycohydrolase, and the streptococcal inhibitor of the complement (SIC) were found to be due to increased transcription of the genes (Minami, et al., 2010). In contrast, the addition of clindamycin during the mid-exponential phase of growth decreased the abundance of exoproteins, which suggests that the effect of the antibiotic varies depending on the growth phase of the bacteria (Sawai, et al., 2007). It's noteworthy that sub-inhibitory concentrations were used in this study, which may occur in micro niches of the infected host—especially if the vasculature at the site of infection is compromised.

Penicillin is the antibiotic of choice for patients with pharyngitis who are not allergic to the drug. Since the 1950s, the incidence of the failure of penicillin to eradicate *S. pyogenes* has increased, even though *S. pyogenes* remains universally susceptible to penicillin *ex vivo* (Pichichero, 1991). Several explanations have been proposed to explain this observation, including the idea that some isolates can be tolerant, but not resistant, to penicillin. To explore changes in the proteome that are associated with phenotypic tolerance to penicillin, cytoplasmic proteins were compared between cultures of *S. pyogenes* exposed to penicillin, or not, by using 2-DE and tandem mass spectrometry (Chaussee, McDowell, Rieck, Callegari, & Chaussee, 2006). Changes in the abundance of proteins associated with fatty acid biosynthesis, glycolysis, and various stress responsive proteins were identified. In addition, the proteome of a penicillin-tolerant strain of *S. pyogenes* was characterized and compared to that of the non-tolerant parental strain in both the presence and absence of penicillin. Following penicillin exposure, the proteome of the parental strain was similar to that of the tolerant strain that had not been exposed to penicillin. This finding suggests that the wild-type bacteria respond to penicillin in a manner that reflects the pattern of gene expression in the tolerant strain. The implication is that tolerance is induced by penicillin exposure (at least in a fraction of the bacterial cells in the culture), which might be relevant to clinical treatment failure (Chaussee, McDowell, Rieck, Callegari, & Chaussee, 2006).

In addition to examining the response to antimicrobials that are currently in use, proteomics has been used to investigate the mechanism of action of compounds that are being developed as therapeutic agents. Rhodomyrtron is a phytochemical isolated from *Rhodomyrtus tomentosa* that inhibits the growth of *S. pyogenes* and other Gram-positive bacteria. Using a gel-based approach, cytoplasmic and CSPs were compared between cultures grown in the presence or absence of sub-inhibitory concentrations of rhodomyrtron in an attempt to determine the mechanism of inhibition. Changes in metabolic enzymes, as well as secreted virulence factors (CAMP factor and SpeC), were identified (Limsuwan, Hesseling-Meinders, Voravuthikunchai, van Dijl, & Kayser, 2011). While the results seem to indicate that the compound perturbs the proteome, additional study is needed to determine how exactly these changes influence the growth of *S. pyogenes*.

Transcriptional regulation of sub-proteomes

Proteins localized to the cell wall or the extracellular milieu directly influence the outcome of host-pathogen interactions. As a result, the characterization of these sub-proteomes can be useful in identifying the molecular

bases for differences in virulence. For example, proteomics have helped to identify at least part of the basis for the virulence attenuation associated with a *perR* mutant (Wen, et al., 2011). PerR is a Fur-like transcriptional regulator that contributes to iron homeostasis, the oxidative stress response, and virulence (Ricci, Janulczyk, & Björck, 2002). CSPs produced by wild-type strain A20 and a *perR* mutant were separated with 2-DE and compared. Thirty-eight differences in protein abundance were identified, including a difference in the production of MF-3, a secreted nuclease encoded by bacteriophage (Wen, et al., 2011). Follow-up studies confirmed that there was less MF-3 produced by the *perR* mutant; showed that PerR bound to the promoter region of the *mf-3* gene; and showed that decreased expression of *mf-3* in the parental A20 strain decreased virulence. Notably, the growth rate and yield of the *perR* mutant was less when DNA was the sole carbon source present in the media, which suggests that DNA was being utilized as a catabolic substrate; presumably following hydrolysis by extracellular nucleases, including MF-3 (Wen, et al., 2011).

Another study on using proteomics to characterize regulons focused on Nra, a transcriptional regulator in the RALP family that represses the expression of virulence factors localized primarily to the cell wall, including a fibronectin-binding protein (SfbX), serum opacity factor (SOF), C5a peptidase (ScpA), M protein, and others (Kreikemeyer, et al., 2007). Proteomics was used, in conjunction with DNA microarrays, to identify other members of the Nra regulon. Both culture supernatant and cell wall-associated proteins were obtained from wild-type and *nra* mutant strains and were separated with 2-DE. In this study, phage lysin C was used to release the cell wall-associated proteins. A total of 67 proteins were identified as being differentially expressed (Kreikemeyer, et al., 2007). The largest differences in protein abundance among CSPs and the cell wall-associated proteins were PrtF.2 (a fibronectin binding protein) and SclA (a collagen-like protein), respectively. With some exceptions, the changes identified with proteomics correlated to the results obtained by DNA microarrays.

A systems approach was used to investigate a serotype M89 isolate from an invasive infection (Tsatsaronis, et al., 2013). The isolate possessed a naturally acquired mutation in the *csrS*/*covS* gene, which is the histidine kinase component of the two component regulator *csrRS*/*covRS*. The secretomes of the clinical isolate and a derivative that possessed a functional CsrS/CovS protein were compared. SclA and Gls24 were identified in samples from the clinical isolate, but were absent from the secretome of the complemented isolate that possessed a functional *csrS*/*covS* gene. Gls24 is a stress-responsive protein that is required for virulence in *Entercoccus faecalis* (Teng, Nannini, & Murray, 2005). Subsequent investigation revealed that both SclA and Gls24 are required for the survival of *S. pyogenes* in human blood. As a result, proteomics has discovered a novel role for SclA and Gls24 in streptococcal pathogenesis.

Identifying the host-acquired proteome

S. pyogenes surface proteins bind human proteins, thereby acquiring a surface composition partially derived from humans; the so called "host-acquired proteome." Decorating the cell surface with human proteins confers several advantages to the microbe, including adherence, internalization, evasion of the host immune response, and pathogen dissemination.

Human proteins that regulate complement activation accumulate on the surfaces of pathogens and contribute to virulence. The cell wall-associated M protein is the best characterized protein involved in recruiting human proteins to the surface. M proteins typically bind to Factor H, fibrinogen (Fg), C4b-binding protein (C4BP), plasminogen, collagen, and albumin, although the binding specificity and affinity varies among the different serotypes (Smeesters, McMillan, & Sriprakash, 2010). Factor H and C4BP are large glycoproteins that are present in human plasma. They down-regulate complement activation on human cells. Fg binding to the surface of several *S. pyogenes* serotypes decreases complement deposition and activation (Smeesters, McMillan, & Sriprakash, 2010). SRM MS was used to measure the binding of IgG subclasses and complement proteins present in pooled human plasma and saliva to the surface of *S. pyogenes* (Nordenfelt, et al., 2012). The results showed a clear distinction between the fluids. After incubation with plasma, the host proteins bound to the bacterial surface were predominately IgG1 and IgG3, as well as proteins associated with both the classical and alternative

complement pathways. In contrast, the predominant host proteins identified on the bacterial surface following incubation with saliva were IgG1 and IgG2, as well as complement factor H, which inhibits complement activation (Nordenfelt, et al., 2012). The results highlight the dynamic nature of the bacterial surface protein composition during the colonization of different niches within the human host.

Mga is a global transcriptional regulator that is required for the expression of several extracellular proteins, including the M protein. SRM was used to compare the binding of human plasma proteins to the surfaces of both a wild-type M1 strain and an *mga* mutant derivative (Sjöholm, Karlsson, Linder, & Malmström, 2014). Plasma proteins partially mimic the environment to which the pathogen is exposed during both bacteremia and localized infections, where the inflammatory response causes vascular leakage and an increase in plasma proteins at the site of infection. The results showed differences in the binding of more than 28 plasma proteins. The *mga* mutant bound significantly less Fg, C4BP, and S proteins to the pathogen surface, which is as expected, due to the absence of M protein. The results also showed an increase in the binding of human complement proteins associated with the membrane attack complex to the bacterial surface of the *mga* mutant strain. The results re-emphasized the importance of the Mga regulon in regulating host-protein recruitment to the surface of the pathogen.

An alternate strategy to characterize a host-acquired proteome used protein chips to analyze multiple streptococcal proteins for their ability to bind various ligands, including host proteins. A chip consisting of 106 recombinant streptococcal proteins predicted to be surface localized was created by expressing the genes in *E. coli*, purifying the epitope tagged proteins, and arraying the proteins on nitrocellulose coated slides. Using the chip, Margarit et al. identified proteins with the capacity to bind Fg, fibronectin, and C4BP. In addition to confirming the previously described interactions, the study discovered some new interactions. Specifically, two streptococcal membrane proteins were identified that bind to C4BP (Spy_1037 and Spy_1326), and a novel Fg binding protein (Spy_0591) was discovered (Margarit, et al., 2009).

Using a combination of shotgun proteomics and SRM, Sjoholm (Sjöholm, Karlsson, Linder, & Malmström, 2014) examined several aspects of host protein binding to the bacterial surface. The most interesting results came from quantitating the abundance of plasma proteins bound to the streptococcal surface. The most abundant human proteins on the surface were Fg, immunoglobulins, C4BP, albumin, and protein S. Protein S is a glycoprotein that inhibits coagulation, among other functions. Much of the protein S in human plasma is associated with C4BP (Dahlbäck & Stenflo, 1981), which perhaps indicates that protein S-C4BP complexes bind to the bacterial surface, rather than through sequential binding of each protein, or the binding of each protein to distinct bacterial surface proteins.

Many of the human proteins recruited to the bacterial cell surface regulate different aspects of the immune response or mediate adherence and internalization; however, a unique function was discovered by comparing the abundance of streptococcal *cytoplasmic* proteins during culture with varying concentrations of human plasma. The presence of plasma altered the abundance of over 200 streptococcal proteins, as determined by using label free LC MS/MS. The most prominent changes included a decrease in the abundance of enzymes involved in fatty acid biosynthesis. Malmstrom et al. further investigated this finding and discovered that the plasma component responsible for repression was human serum albumin (HSA) bound to fatty acids (Malmström, et al., 2012). While it has been known for years that streptococcal M (and M-like) proteins bind HSA (Wagner, Schmidt, Wagner, & Köhler, 1986; Retnoningrum & Cleary, 1994), it was not known that binding resulted in the acquisition of fatty acids and a subsequent decrease in the expression of fatty acid biosynthetic enzymes. Thus, in addition to this new information, these results provide an example of how using an unbiased proteomics approach can lead to novel insights.

Additional human proteins are likely to be bound to the *S. pyogenes* surface, either in amounts below the current limits of detection or as a result of indirect binding through other human proteins. For example, several cell wall-associated streptococcal proteins bind human fibronectin (Schwarz-Linek, Höök, & Potts, 2006). By using

LC-MS/MS, over 30 different human plasma proteins were identified that bind to the amino terminal region of human fibronectin. As a result of fibronectin binding to the bacterial cell surface, dozens of other human proteins may also be recruited to the streptococcal surface (Moussavi-Harami, et al., 2013). Thus, the bacterial surface composition (surfome) is likely to be quite different in various micro-niches (including blood, pharynx, subcutaneous tissue, or lung, among others) occupied by *S. pyogenes*, depending on the expression of cell wall-associated proteins, posttranslational changes to cell surface proteins (discussed below), and the availability of different human proteins for recruitment to the surface.

Protein-protein interactions at the cell surface

While a great deal of progress has been made in identifying proteins associated with the bacterial cell surface, little is known about their spatial organization, which is likely to influence their functions. Proteomics can be used as an alternative to two-hybrid type experiments to identify protein-protein interactions. One such study explored the protein-protein interactions of *S. pyogenes* surface proteins using protein chips that consisted of 83 purified streptococcal proteins, which were selected based on their possession of a signal peptide, a lipoprotein motif, or the presence of an LPXTG motif. The capacity of each of these immobilized proteins to bind to other extracellular proteins was then assessed by incubating the array with soluble, biotinylated derivatives of each protein. Protein-protein interactions were further characterized by surface plasma resonance and confocal microscopy (Galeotti, et al., 2012). Among the highlights of the study was the finding that a surface associated protein annotated as Mur1.2 binds to several proteins that are known to be important to virulence, including hyaluronidase (HylA), SKA, SLO, a likely superantigen (SpeI), ScpA, and a fibronectin binding protein (SpyM3_0104). The primary structure of Mur1.2 is similar to N-acetyl muramidases, such as FlgJ. Among some Gram-negative species, FlgJ remodels peptidoglycan and binds to other extracellular proteins to facilitate the assembly of flagella. While speculative, the results suggest that Mur1.2 is involved in the spatial organization of streptococcal cell wall-associated proteins. The future use of proteomics, in combination with microscopy, is likely to aid in determining the architecture of the bacterial cell surface and contribute to our understanding of the ways in which the organization of extracellular proteins influences the function of these proteins.

Culture supernatant proteins

One might predict that CSPs would only include proteins that possess a type II signal sequence for transport across the cytoplasmic membrane and that lack a cell wall anchoring motif. Moreover, given the amino acid conservation of these motifs, one might also suspect the straightforward prediction of CSPs, based on inferences of the genome sequence. Experimental results obtained from proteomics have shown this is not the case. Initial reports that the glycolytic enzymes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Pancholi & Fischetti, 1992) and enolase (Pancholi & Fischetti, 1998) were localized to the cell wall and culture supernatant were confirmed by a proteomic analysis that showed the presence of nearly all the glycolytic enzymes (with the exception being glucose phosphate isomerase) in culture supernatant fractions (Lei, Mackie, Lukomski, & Musser, 2000). Moreover, the results showed that approximately half of all the CSPs have no discernable type II secretion signal, and that many are also associated with the bacterial cell surface.

The canonical cytoplasmic proteins (such as enolase) possess different functions when localized extracellularly, as compared to when they are cytoplasmic. As a result, they are often referred to as “moonlighting” proteins., Most of these proteins are involved in metabolism when localized to the cytoplasm and virulence when localized extracellularly (Henderson & Martin, 2011). For example, surface-localized GAPDH, which binds to human plasminogen (Pancholi & Fischetti, 1992), mediates adherence to pharyngeal cells and is antiphagocytic (Boël, Jin, & Pancholi, 2005). Similarly, surface-localized enolase binds to human plasminogen (Pancholi & Fischetti, 1998) with an even greater affinity than that of GAPDH. Plasminogen can subsequently be activated by SKA to plasmin, which degrades host tissue and promotes pathogen dissemination. The presence of moonlighting proteins on the surface of microbes has been described in several other bacterial species. Moreover, similar to *S.*

pyogenes, many of the moonlighting extracellular glycolytic enzymes contribute to virulence (Henderson & Martin, 2011). The paradigm even extends to other kingdoms, including mammalian cells, where normally cytosolic glycolytic enzymes (hexokinase, lactate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase) also localize to the nucleus and function in transcriptional regulation. Others (such as glucose-6-phosphate isomerase) influence motility and apoptosis (such as glucokinase, glyceraldehyde-3-phosphate dehydrogenase, and hexokinase) (Kim & Dang, 2005). As a result, protein localization is a critical component to the understanding of protein function.

The trafficking of moonlighting proteins has been, and continues to be, an enigma. In mammalian cells, the phosphorylation of lactate dehydrogenase is thought to be the signal for localization to the nucleus (Henderson & Martin, 2011). In *S. pyogenes*, one theory is that the extracellular localization of proteins that are usually considered to be cytoplasmic proteins is simply the result of autolysis and the coincidental affinity of the proteins for other proteins localized to the cell wall. This idea is attractive, because the most abundant extracellular moonlighting proteins are also the most abundant cytoplasmic proteins. However, the discovery that adding a twelve amino-acid peptide to the carboxyl terminus of GAPDH diminished extracellular localization indicates that autolysis may not be involved, and implies that an unidentified transport system specifically targets these proteins to the extracellular milieu (Boël, Jin, & Pancholi, 2005). While the mechanism of localization is not resolved, there is nonetheless a great deal of evidence that the glycolytic proteins are present on the cell surface and that they significantly influence host-pathogen interactions.

Post-translational regulation of the extracellular proteome

Proteins secreted to the extracellular milieu are important for the adaptation of *S. pyogenes* to various niches occupied by the pathogen during infection. The composition of proteins associated with the cell wall, and those that are freely soluble, is regulated both at the transcriptional level and post-translationally. In general, genes that encode proteins with the LPXTG motif are transcribed in the exponential phase of growth, while those with type II secretion peptides, but no LPXTG motif, are transcribed in the post-exponential phase of growth.

The secreted cysteine protease SpeB is the most important post-translational regulator of the extracellular proteome. The transcriptional regulation of *speB* expression is complex and was recently reviewed (Carroll & Musser, 2011). In general, *speB* is expressed in the post-exponential phase of growth following activation of expression by Rgg1 in response to a variety of signals, including glycolytic flux (Lyon, Gibson, & Caparon, 1998; Loughman & Caparon, 2006). In addition to transcriptional regulation, SpeB is secreted as zymogen, and specific environmental conditions are required for conversion of the zymogen to the enzymatically active protease. Proteins involved in the secretion and maturation of SpeB include RopA, PrsA, and HtrA (Lyon, Gibson, & Caparon, 1998; Lyon & Caparon, 2004). A proteomic study examined the effects of deleting the *htrA* gene on proteins localized to both the cell wall and the culture supernatant by using 2-DE, and indicated that HtrA indirectly enhances SpeB maturation, but is not essential for conversion of the zymogen to the active protease (Cole, et al., 2007). The diminished amounts of proteolytically active SpeB in the *htrA* mutant were associated with the presence of many proteins in cell wall and culture supernatant fractions that were likely degraded by SpeB in wild-type fractions. In addition, the results indicated that essentially all the proteolytic activity in culture supernatant fractions isolated from strain HSC5 could be attributed to SpeB (Cole, et al., 2007).

In 1945, Elliott reported that the extracellular SpeB protease (referred to then as streptococcal proteinase) degrades the cell wall-associated M protein (Elliott, 1945). This also results in the release of host proteins bound to M, such as Fg and immunoglobulin (Nelson, Garbe, & Collin, 2011). Subsequent studies showed that the protease can cleave, or degrade, a plethora of additional streptococcal and host proteins (Nelson, Garbe, & Collin, 2011). In fact, results obtained using proteomics showed that SpeB degrades nearly the entire secretome of an invasive serotype M1 isolate (Aziz, et al., 2004). As a result, SpeB can significantly alter the protein environment of the pathogen, which is comprised of both bacterial and host proteins.

SpeB-mediated remodeling of the streptococcal surface proteome significantly influences host-pathogen interactions and has been associated with pathogen dissemination. For example, when the bacterial cell density at the site of infection reaches a critical level, SpeB is expressed, secreted, and activated. Proteolysis results in both the enzymatic degradation of streptococcal proteins that mediate adherence and internalization (Chaussee, Cole, & van Putten, 2000), and the degradation of host extracellular matrix proteins (Kapur, et al., 1993). As a result, SpeB has been proposed to be a spreading factor that promotes pathogen *dissemination*.

In contrast, other results suggest that SpeB production is associated with *localized* infections. All clinical isolates possess the *speB* gene; however, not all of these isolates synthesize the protein. This can be the result of naturally selected mutations in either the *csrS*/*covS* gene or the *rgg1* gene. Mutations in these loci typically increase the expression of many virulence-associated exoproteins and decrease, or abrogate, the expression of SpeB (Chaussee, et al., 2002; Treviño, et al., 2009). Remarkably, isolates from patients with invasive diseases are more likely to have mutations in either *csrS*/*covS* or *rgg1*, as compared to isolates from localized pharyngeal infections (Sumby, Whitney, Graviss, DeLeo, & Musser, 2006; Ikebe, et al., 2010; Carroll, et al., 2011).

Two complementary mechanisms have been identified to explain the ways in which a loss of SpeB production (as occurs with *csrS*/*covS* and *rgg1* mutants) can increase pathogen dissemination. First, SpeB degrades an extracellular DNase (Sda1) that is important in mediating a pathogen's escape from neutrophil extracellular traps (NETs) (Walker, et al., 2007). Thus, when SpeB is active, there is insufficient Sda1 to mediate the escape from NETs and the infection tends to remain localized. On the other hand, in the absence of SpeB, Sda1 is active, and as a result, the pathogen is more likely to escape NETs and disseminate. Second, SpeB degrades SKA (an activator of human plasminogen) (Cole, et al., 2006). Therefore, in the absence of SpeB, SKA can convert human plasminogen to plasmin, which accumulates on the bacterial surface through binding to the M-like protein PAM, as well as the moonlighting proteins enolase and GAPDH. Because plasmin degrades many human proteins, including fibrin, fibronectin, thrombospondin, laminin, and others, the pathogen is able to invade tissues and disseminate.

From a practical standpoint, the results show the importance of accounting for SpeB protease activity when analyzing extracellular proteins. SpeB-mediated changes in the proteome can be experimentally controlled in several ways: first, by using *speB* mutants, which fail to produce either the protein (knock-out mutation) or site-specific mutants that lack the active site cysteine residue that is required for protease activity; second, by maintaining culture conditions that inhibit activation of the zymogen or by harvesting proteins prior to SpeB activation and subsequent protein degradation; and third, by including cysteine protease inhibitors in the media. In this instance, the epoxide cysteine protease inhibitor E64 is typically used.

Discovering immunogenic streptococcal proteins

Characterizing immunogenic streptococcal proteins is necessary to identify those that elicit cross-reactive antibodies involved in post-infection sequelae, as well as those that elicit protective antibodies, which may be used to vaccinate against *S. pyogenes*.

Streptococcal induced autoimmunity

Previous studies have identified antigens that contribute to the pathogenesis of post-infection sequelae. Cross-reactive antibodies directed at the M protein and the role they play in acute rheumatic fever (ARF) are of particular importance to this process. Neurological complications, including Syndeham's chorea, are well-known symptoms of ARF; however, in recent decades, a broader collection of neurological symptoms, including obsessive-compulsive disorders and tics, have been recognized as sequelae of *S. pyogenes* infections (Snider & Swedo, 2004). To identify streptococcal antigens that elicit neuropathogenic antibodies, Bombaci et al. used a protein chip that consists of over one hundred recombinant *S. pyogenes* proteins arrayed on glass slides to measure reactivity with sera obtained from 335 children, including 61 with neuropsychiatric symptoms

(Bombaci, et al., 2009). The results indicated that symptomatic patients had elevated antibody titers to multiple streptococcal proteins, as opposed to strong reactivity to just a few proteins (such as the M protein). The continued development and use of protein chips is likely to yield important new information into the pathogenesis of post-infection sequelae.

Vaccine development

Reverse vaccinology uses genomics (and often functional genomics) to identify proteins that are likely to be good candidates for vaccines. Protein localization to the cell wall or membrane is among the criteria often used to select candidate immunogens because these proteins are likely to be accessible to antibodies. Other criteria include proteins that are well conserved among isolates of *S. pyogenes*, those that are highly expressed, and those that are known to elicit antibodies during infection of either humans or mice. Proteins that meet these criteria are typically purified and tested in animal models to assess the ability of the protein to confer protective immunity.

Using bioinformatics, Sharma et al. identified between 199 and 237 proteins with a type II signal peptide among eight sequenced genomes. Next, the presence of the corresponding genes in the chromosome of isolates from India was determined (Sharma, et al., 2013). To identify these expressed genes, cell wall and membrane proteins were enriched from M1 and M49 strains and were separated by SDS-PAGE. The gels were then sliced into multiple pieces. The proteins were digested within the gel slices, the peptides were eluted, and the proteins were identified by MS. A total of 128 and 373 proteins were detected in the M1 and M49 strains, respectively; 116 proteins were identified in samples from both strains. As observed with other fractionation methods, many of the proteins identified are canonical cytoplasmic membranes, including a transcriptional regulator (Sharma, et al., 2013). Of all the proteins experimentally identified, 52 possessed a type II signal peptide.

Antibodies to surface proteins can be opsonic and can neutralize important functions related to virulence, such as adherence, which makes them attractive vaccine candidates. CSPs have also been investigated as vaccine targets for two major reasons. First, several CSPs are thought to be critical to virulence, and consequently, their neutralizing antibodies would likely decrease virulence. Second, proteomic studies indicate that many CSPs are also non-covalently associated with the cell wall. Thus, antibodies to CSPs may also be opsonic. The first study to use proteomics to identify CSP vaccine candidates analyzed the proteins collected from both exponential and stationary phase cultures of invasive isolates that represented serotypes M1 and M3. The proteins were separated with 2-DE, and the amino termini of selected proteins was determined through Edman degradation. Inter-strain differences in CSP composition were evident, including differences in the abundance of extracellular DNAse, NAD-glycohydrolase, SLO, and other proteins. CSPs that elicited an antibody response during the course of human infections were identified by 2-DE immunoblotting with patient sera (Lei, Mackie, Lukomski, & Musser, 2000). Many of the proteins that reacted with antibodies were moonlighting proteins that lacked a type II signal sequence. In addition, because Edman degradation (and not mass spectrometry) was used to identify protein spots excised from the gel, the results also showed that some of the protein isoforms separated by 2-DE were derived from truncation of the polypeptide at the amino terminus, presumably post-translationally. For example, two forms of mitogenic factor (a secreted DNase) were identified. The amino terminus of one corresponded to amino acid 43 of the inferred polypeptide, while the other corresponded to amino acid 42. While it is unclear if there are functional differences between the isoforms, the results illustrate the power of 2-DE to separate highly similar proteins and also provides insight into the types of differences that distinguish protein isoforms (Lei, Mackie, Lukomski, & Musser, 2000).

A similar study used human antisera to identify immunogenic cell wall-associated proteins produced by serotypes M69, M53, and M6. Proteins were isolated and separated with 2-DE. 155 protein spots were identified with MALDI-TOF MS, which corresponded to 74 proteins (Cole, et al., 2005). 45% of the identified proteins were antigenic, as determined by using pooled sera obtained from children living in a region of Australia where 70% of the children have impetigo. Cole et al. (Cole, et al., 2005) also exposed *S. pyogenes* to biotin prior to

isolating cell wall proteins, which were then separated with 2-DE. To identify proteins that were surface-accessible (based on their being biotinylated) immunoblotting was done with streptavidin and anti-streptavidin antibodies. About 30% of the proteins reacted with anti-streptavidin, which implies that they are surface exposed. The biotinylated proteins generally also reacted with pooled sera from the children. For proteins that were not biotinylated but that reacted with sera, it is difficult to know if the proteins were actually not surface exposed during biotinylation (or if a reactive amino group necessary for biotinylation was not surface exposed. As with other studies, several metabolic enzymes were identified in the cell wall-associated fraction.

Another strategy to identify surface-exposed proteins for potential use as immunogens involves treating washed intact streptococcal cells with a protease, such as trypsin (Rodríguez-Ortega, et al., 2006; Severin, et al., 2007). The assumption is that trypsin will only cleave those proteins that are surface exposed. Following centrifugation to pellet the bacterial cells, the tryptic peptides present in the supernatant were collected, separated with liquid chromatography, and analyzed with MS/MS to identify the corresponding proteins (Rodríguez-Ortega, et al., 2006; Severin, et al., 2007). This technique is often referred to as "surface shaving" (Figure 5). Bioinformatics predicted that 72 proteins encoded by the SF370 genome were surface exposed, and by using surface shaving, 95% of these proteins were identified (Rodríguez-Ortega, et al., 2006). The shaving approach should also identify membrane proteins with relatively large extracellular domains; however, only 37 of the estimated 524 membrane proteins encoded in the streptococcal genome were identified in the study (Rodríguez-Ortega, et al., 2006). It's possible that the cell wall and/or polysaccharide capsules may inhibit trypsin activity near the cytoplasmic membrane surface, thereby reducing the number of membrane proteins identified with this approach (Rodríguez-Ortega, et al., 2006). Rodriguez-Ortega tested 14 surface exposed proteins to determine if any could confer protection in a mouse model against subsequent challenges with S. *pyogenes*. The results of this work identified a new protective protein, Spy_0416, which is annotated as a peptidase that contains the LPXTG motif (Rodríguez-Ortega, et al., 2006).

Surface proteins of strain SF370 were similarly analyzed during the exponential and post-exponential phases of growth. 79 proteins were identified, including 14 which possessed the LPXTG motif (Severin, et al., 2007). Approximately one-quarter of the proteins were moonlighting proteins—a finding that is similar to results obtained by using cell wall hydrolases to extract surface associated proteins. When combined with results obtained by Rodriquez-Ortega, 118 surface exposed-proteins were identified. Remarkably, despite using similar approaches, only about 30% of the proteins identified were common to both studies.

Bensi et al. (Bensi, et al., 2012) used bioinformatics to identify proteins that are likely to be secreted to the surface. The corresponding genes were then expressed in *E. coli*, purified, and used to create a protein array. The purified proteins were also used to generate antibodies, which were used to identify highly expressed surface proteins by measuring protein production among a panel of clinical isolates. In addition, sera from patients with pharyngitis were used to identify proteins that were expressed during the course of human infection. Finally, the surface-shaving method was used to identify surface-exposed proteins. Six proteins were identified that were surface exposed, highly expressed, and elicited antibodies during human infection. Notably, two of these proteins, SLO and ScpA, have already been considered as vaccine candidates. The other four were SpyCEP protease (SPy_0416); PrgA (SPy_0269), which is annotated as a surface exclusion protein; SPy0019, which is a surface protein with an amidase motif; and internalin InlA (SPy_2010).

Conclusions and Perspective

Considering that the genome sequence of *S. pyogenes* was determined a little over a decade ago, significant achievements have been made in understanding streptococcal biology by using functional genomics, including proteomics. Major developments include a better understanding of the composition and quantity of human proteins recruited to the pathogen surface, and the role that the host-acquired proteome plays in both virulence and metabolism. We've also better characterized the composition of bacterial surface proteins, including

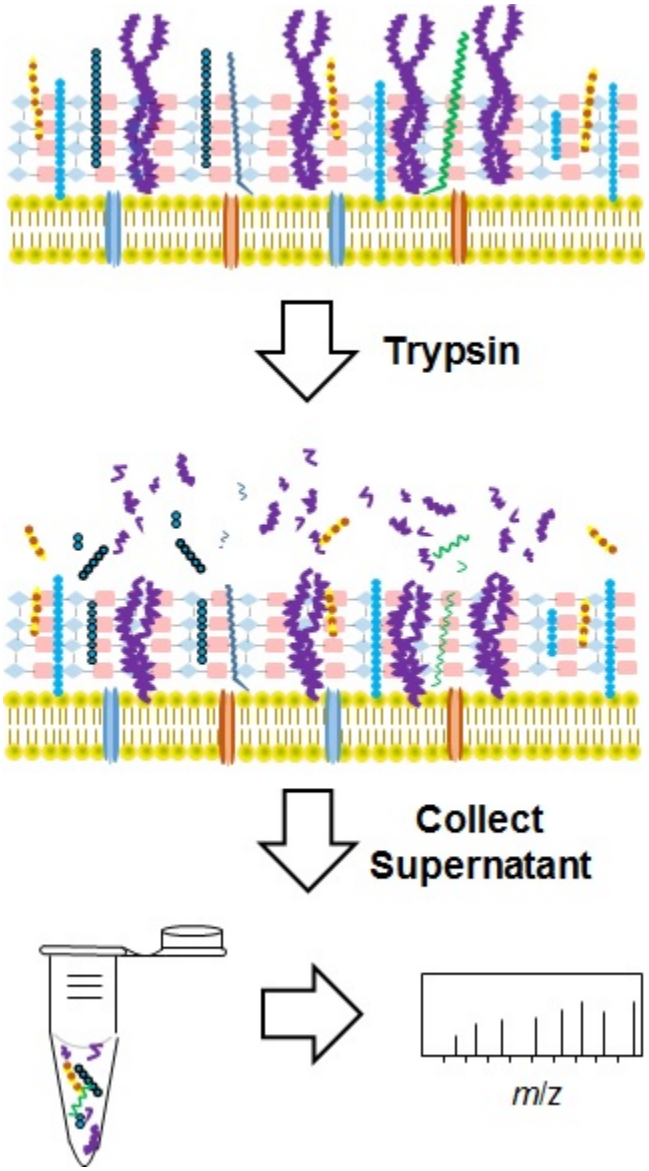

Figure 5: Proteolytic shaving to identify surface exposed proteins. Washed *S. pyogenes* cells are suspended in buffer and incubated with trypsin. Centrifugation is used to pellet the cells and tryptic peptides derived from surface exposed proteins are collected from the supernatant and analyzed with MS/MS to identify the cognate protein.

moonlighting proteins, although the trafficking of many of these proteins remains unclear. Finally, proteomics has enhanced the rate at which *S. pyogenes* proteins are qualified as vaccine candidates.

Several areas of investigation are poised for significant advancement in the upcoming decade. Peptide mediated signaling within the species (quorum sensing) between related species, and between the pathogen and host, promises to be an area of intense and fruitful investigation. Technical advances in peptidomics will hasten progress and lead to new insights into host-pathogen interactions. A more complete understanding of the spatiotemporal and protein-protein interactions that occur among proteins localized to the cytoplasmic membrane and cell wall is likely to enhance our understanding of the role that the proximal macromolecular environment plays in protein function. Additional information on the turnover of not only extracellular proteins, but also cytoplasmic proteins, is likely to complement the abundant amount of information on gene expression obtained at the transcript level, and to reveal insights into the pathogens' responses to changing environments.

Given the large number of exciting research questions that have yet to be addressed and the rapid development of instrumentation, techniques, and strategies to probe the streptococcal proteome, the upcoming decade promises to be marked with advances just as important as the last.

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Virulence-Related Transcriptional Regulators of *Streptococcus pyogenes*

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Abstract

Control of virulence gene expression in *S. pyogenes* is under the charge of 13 two-component regulatory systems and at least 30 transcriptional regulators known thus far. These virulence-related regulators are tasked with integrating environmental host cues with the pathogen's own metabolic state, as well as feedback signals from the expressed genome, into a coordinated response. This overview of the current understanding of *S. pyogenes* virulence-related regulators describes their roles in one or multiple of the following categories: *Master regulators*, such as CovR/S and RofA-like proteins (RALPs), that control the activity of multiple virulence-related regulators; *Metabolite-responsive regulators*, which sense and respond to changes in availability of sources of energy; carbohydrates (CcpA, Mga), amino acid/nitrogen supplies (Rsh, CodY), and other metabolites *S. pyogenes* must monitor in the host; *Metabolic-control regulators,* such as VicR/S, MtsR, CiaH/R, that influence expression of metabolism-related genes to maintain the necessary homeostasis to promote colonization; and *Environmental/host immunity-responsive regulators*, which control responses to a variety of cues, like oxidative stress (PerR), quorum sensing (Rgg2/3), saliva (SalK/R), and neutrophils (Ihk/Irr). Emphasis is made on the interdependence among regulators and the ways in which variation in their connectivity depends on their genotypic background, likely due to coevolution with its human host.

Under natural conditions, *Streptococcus pyogenes* (group A streptococci) is a strictly human-specific pathogen, implying that this fastidious species (i) lacks an independent environmental reservoir, and (ii) in order to succeed as a pathogen, needs to spread before extinguishing its host. *S. pyogenes* has demonstrated a high capacity to colonize multiple tissue sites and cause a wide variety of different diseases. These range from mild superficial infections of the skin (such as impetigo) and mucosal membranes (such as pharyngitis) to severe invasive infections (such as streptococcal toxic-shock-like syndrome or necrotizing fasciitis) to autoimmune sequelae (such as rheumatic fever). Infection of mammals other than humans requires experimental conditions that are characterized by unnaturally high bacterial titers and specific genotypes that are capable of breaking species barriers. Ideally, on the scale of population biology, the pathogen and its specific host should coevolve and exhibit an evolutionary equilibrium, with coevolution rates balancing each other (Rosenzweig, Brown, & Vincent, 1987), as suggested by the "Red Queen" argument ("It takes all the running you can do, to keep in the same place" (Van Valen, 1973)). In mammalian streptococcal pathogens, the well-explored example of host plasminogen activation mediated by streptokinase illustrates the workings of coevolution and renders streptokinase responsible for the host's specificity of infection (Gladysheva, Turner, Sazonova, Liu, & Reed, 2003; Sun, et al., 2004). The ample and varied repertoire of virulence factors that *S. pyogenes* expresses in the course of infection (as detailed elsewhere) and their highly coordinated regulation results from the coevolution described by the Red Queen principle. Control of virulence factor expression in *S. pyogenes* is under the charge of 13 two-component regulatory systems and at least 30 transcriptional regulators known thus far (Kreikemeyer, McIver, & Podbielski, 2003). These virulence-related regulators are tasked with integrating environmental cues (such as

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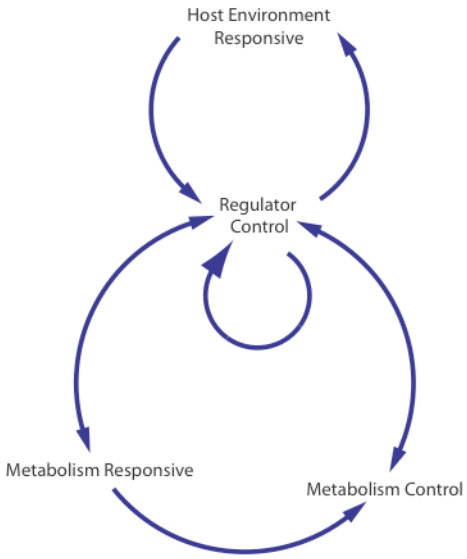

Figure 1. Categories of regulation in *S. pyogenes*. Gene regulators can be involved in regulator control, and can influence the expression of other regulators and themselves (for example, CovR, RALPs). Such regulators can control the activities of metabolite responsive (such as Mga) and metabolism controlling (such as CcpA) genes and be influenced by these themselves. Regulators that respond to changing conditions in the host environment (such as Ihk/Irr) can act on metabolic control and virulence gene control through master regulators.

nutrient availability, chemical stressors, host immune components, and temperature) with information on the pathogen's own metabolic state, as well as feedback signals from the expressed genome, into a coordinated response. As a result, the virulence-related regulators of *S. pyogenes* constitute a highly interconnected network that is in constant flux, which makes it quite difficult to elucidate and represent. Most of what is known about virulence factor regulation in *S. pyogenes* has stemmed from the examination of the pathogen's responses to different environmental and internal inputs. Moreover, emerging literature reveals that some of these regulators are capable of translating metabolic inputs unrelated to disease (like carbohydrate levels) into changes in the expression of factors for the evasion of host immunity. As a result, we have organized this overview of the current understanding of *S. pyogenes* virulence-related regulators by describing their roles in one, multiple or each (in some cases) of the following categories: metabolite sensing, metabolism control, coordination among regulators, and environmental/host immunity responsiveness (Figure 1). An emphasis is made on the interdependence among regulators and the ways in which variation in their connectivity depends on their serotypic/genotypic background, most likely as a result from coevolution with their primary host niches. Several regulators will be described in more than one of these categories, given their activity, especially in the case of those involved in coordinating other regulators, which are referred to here as "master" regulators.

Mechanisms of regulation

In terms of mechanisms of action, the virulence-related regulators of *S. pyogenes* have been found to belong to three types: two-component signal transduction systems (TCSs) (Supplemental Table 1, 2); so-called "stand alone" transcriptional activators/repressors; and non-coding RNAs (Supplemental Table 3, 4). TCSs are a common mechanism among bacteria to integrate the detection of extracellular signals with intracellular adaptive responses. These consist of a transmembrane sensor histidine kinase component, which recognizes an extracellular signal; and an associated cytoplasmic response regulator, which transduces the input sensed by the kinase into activation/repression of virulence-related gene expression. Upon interaction with the signaling

stimulus, two sensor kinase molecules often form a dimer, and are activated and autophosphorylated at one or both conserved histidine residues in the cytoplasmic transducer domain. The phosphorylated transducer signals to the cytoplasmic response regulator by transferring its phosphoryl group to an aspartyl residue of the regulator, which in turn acts as a transcription factor to control target gene expression. The genome sequences of *S. pyogenes* serotypes M1, M3 and M18 (Ferretti, et al., 2001; Beres, et al., 2002; Smoot, et al., 2002) have revealed the presence of an average of 13 TCSs, of which 11 are shared in serotypes M1 and M18 (Beres, et al., 2002). All systems have been annotated and assigned a putative function on the basis of sequence homology towards similar systems from other species (Graham, et al., 2002) or defined functions, as will be discussed later.

In contrast to TCSs, stand-alone regulators activate and/or repress the transcription of virulence genes without inputs from cognate TCS sensor kinases. This is not to say that all virulence regulators of this kind function without inputs from other cell signaling components; rather, that associated sensory kinases or other sensory elements have not been identified for them. The best-characterized stand-alone virulence regulators are Mga and the RofA-like protein type regulators (RALPs); we will use existing knowledge of these regulators to illustrate what is understood about this type of virulence regulator. For the most part, these regulators have been identified as containing DNA-binding domains that can interact directly with sequences in the promoter regions of the genes they control, but for many specific binding sequences, either they have not been characterized or they are hypothesized to indirectly affect the expression of their targets (Kreikemeyer, McIver, & Podbielski, 2003).

Alternatively, small non-coding RNAs of *S. pyogenes*, like in other bacteria, regulate gene expression primarily at the translational level in response to environmental signals (Wassarman, Zhang, & Storz, 1999). Most non-coding RNAs participate in post-transcriptional regulation by base pairing with target mRNAs, which results in either inhibition/activation of translation or degradation of the mRNAs (Gottesman, 2004). A small group of non-coding RNAs function by interacting with RNA-binding proteins to modify their activities (Majdalani, Vanderpool, & Gottesman, 2005). Only a relatively small number of non-coding RNAs have been identified and characterized in *S. pyogenes* so far, as compared to those observed in other bacteria (Tesorero, et al., 2013).

Master regulators/Virulence regulator control

In the first category, we examine the role that some regulators play in the control of other virulence-related regulators, which causes them to be referred to as master regulators. This is the case for TCSs like CovR/S, as well as RofA and other RALPs.

CovR/S

The best-characterized TCS of *S. pyogenes* is control of virulence (CovR/S) or capsule synthesis regulator (CsrR/S). CovS senses external signals, including environmental Mg^{2+} and host antimicrobial peptides (Gryllos, Levin, & Wessels, 2003; Gryllos, et al., 2008a) and transduces them to CovR, which in turn regulates the expression of several virulence gene regulons (Supplemental Table 2). Phosphorylation of CovR enhances oligomerization of the activated protein and subsequent DNA binding to long AT-rich sequences within the target promoter regions (Miller, Engleberg, & DiRita, 2001; Gusa, Gao, Stringer, Churchward, & Scott, 2006). CovR mechanisms of binding to DNA appear to vary across promoters and display a high affinity for single DNA binding sites (Churchward, Bates, Gusa, Stringer, & Scott, 2009) or cooperative binding along long stretches of promoter region DNA (Gao, Gusa, Scott, & Churchward, 2005). A consensus CovR-binding element (ATTARA) has been proposed on the basis of binding studies of the *hasA* promoter (Federle & Scott, 2002), but no single conserved binding box has been identified, even among the 18 most strongly CovR influenced genes (Graham, et al., 2002). For a thorough review of the molecular mechanisms of CovR transcriptional regulation, see (Churchward, 2007).

CovR/S TCS is considered to be a master regulator, given that microarray and quantitative RT–PCR-based analysis of *S. pyogenes* serotype M1 revealed that CovR influences the transcription of up to 15% (271 genes) of all chromosomal genes (Graham, et al., 2002), with its activity exerted during late exponential and stationary growth phases. CovR primarily acts as a transcriptional repressor, as it was observed that in a CovR mutant array analysis, only 26 and 4 of the 271 differentially expressed transcripts examined were down regulated during exponential and stationary phase growth, respectively (Graham, et al., 2002; Federle, McIver, & Scott, 1999). CovR both directly and indirectly regulates multiple virulence factors that repress the hyaluronic acid capsule operon (*hasABC*), and modulate the expression of streptolysin S (SagA), a cysteine protease (SpeB), an NAD glycohydrolase (Nga), streptolysin O (Slo), an IL-8 degrading protease (SpyCEP), streptokinase (Ska), streptodornase (Sda), an immunoglobulin modifying protein (EndoS), and a fibronectin binding protein (Fba) (Graham, et al., 2002; Heath, DiRita, Barg, & Engleberg, 1999; Darmstadt, Mentele, Podbielski, & Rubens, 2000; Levin & Wessels, 1998; Bernish & van de Rijn, 1999; Shelburne, et al., 2010). As a master regulator, CovR/S also controls the expression of other proven and putative transcriptional regulators, as observed in the up-regulation of 8 transcriptional regulators in the exponential growth phase, and that of 18 transcriptional regulators in the stationary phase of growth in a CovR-deficient mutant (Graham, et al., 2002). One directly CovR-repressed regulator is RivR (Ralp4), a member of the RofA-like protein type (RALP) family of stand-alone virulence-related regulators that repress the expression of capsule (*hasABC*) and G-related α_2-macroglobulin-binding proteins (Grab) involved in the inactivation of proteolytic blood plasma proteins (Roberts, Churchward, & Scott, 2007; Treviño, Liu, Cao, Ramirez-Peña, & Sumby, 2013). Significantly, CovR can regulate its target genes independently of CovS, as *S. pyogenes* strains with isogenic CovR or CovS mutations have different phenotypes (Treviño, et al., 2009) and the most studied invasive clinical isolate, the MGAS5005 (M1) strain, encodes a truncated and functionally inactive CovS protein (Treviño, et al., 2009; Sumby, Whitney, Graviss, DeLeo, & Musser, 2006; Shelburne, et al., 2008). The regulatory role of CovR/S in responding to the host environment and in metabolic control affecting virulence, whether directly or through the modulation of other virulence-related regulators (such as RALPs, Ihk/Irr, and LytR), is detailed in later sections.

RofA and the RofA-like protein type regulators (RALPs)

Four RALPs have been identified in *S. pyogenes*: RofA, Nra, Ralp3, and RivR (Ralp4). Among sequenced strains, they are 52% similar and 29% identical to each other on average (Granok, Parsonage, Ross, & Caparon, 2000), and are involved in the control of *S. pyogenes*–host-cell interactions, avoidance of host cell damage, and balanced virulence factor expression during stationary phase growth (Beckert, Kreikemeyer, & Podbielski, 2001; Podbielski, Woischnik, Leonard, & Schmidt, 1999; Molinari, et al., 2001). The most studied of the RALPs is the namesake of this family: RofA, a DNA-binding protein that was first identified in a serotype M6 strain as a transcriptional inducer under anaerobic growth conditions of *prtF*, the gene encoding fibronectin-binding protein F (*sfbI*) (Fogg & Caparon, 1997). RofA transcription occurs adjacent to and divergently from *prtF* and is positively autoregulated by RofA interaction with specific 17-bp sites within the intergenic region shared by the two genes (Granok, Parsonage, Ross, & Caparon, 2000; Fogg & Caparon, 1997). RofA directly modulates multiple virulence genes, up-regulating transcription of the FCT region genes (SfbI, the collagen binding protein Cpa, and T antigen) (Kreikemeyer, Beckert, Braun-Kiewnick, & Podbielski, 2002) and repressing transcription of the streptolysin S hemolysin (encoded by *sagA*), the SpeB protease, and the SpeA superantigen (Beckert, Kreikemeyer, & Podbielski, 2001). As a regulator of other virulence-related regulators, RofA down-regulates Mga in serotype M6 and thus can modulate Mga regulon virulence genes.

Nra is another well-studied RALP, which is only present in M serotypes that carry the FCT type 3 gene region (M3, 18 and 49), as all other M serotypes possess FCT gene regions that encode for RofA instead of Nra (Kratovac, Manoharan, Luo, Liziano, & Bessen, 2007). In the M49 strain, Nra was characterized as a repressor for the adjacent *cpa*, as well as for an unlinked gene that encodes a second fibronectin-binding protein, *prtF2* (Podbielski, Woischnik, Leonard, & Schmidt, 1999). Transcriptome analysis of a serotype M49 strain and its isogenic *nra* mutant strain showed that Nra is active in the exponential, transition, and stationary growth phases

(Kreikemeyer, et al., 2007). Maximum expression of Nra occurs in early stationary phase growth (Podbielski, Woischnik, Leonard, & Schmidt, 1999), and the highest number of differentially transcribed genes was observed in the transition phase of growth, with an Nra-knockout strain displaying 112 and 84 genes with increased and decreased transcript abundance, respectively (Kreikemeyer, et al., 2007). However, unlike some RofA-expressing strains, Nra does not appear to respond to changing atmospheric conditions (Podbielski, Woischnik, Leonard, & Schmidt, 1999). Nra has been shown to repress the transcription of FCT region genes, pilus protein-encoding genes, and the capsule biosynthesis operon (*hasABC*) (Kreikemeyer, et al., 2007). As a master regulator, Nra also represses virulence factors encoded by the Mga core regulon, and in the M49 serotype background, Nra has been observed to directly or indirectly repress expression of the global regulator Rgg, the Ihk/Irr TCS, additional RALPs (Ralp3 and RivR), and Mga itself (Podbielski, Woischnik, Leonard, & Schmidt, 1999; Kreikemeyer, et al., 2007). For other virulence genes, a negative regulatory effect of Nra has only been shown during one or two growth phases. These genes encode *prtF2*, a putative hemolysin (*hylX*), two hyaluronidases (*hylP2* and *hylP3*), an extracellular matrix-binding protein (*epf*), the laminin-binding protein (*lmb*), *speA*, and *ska*.

Ralp3 is encoded in the so-called *eno-ralp3-epf-sagA* (ERES) pathogenicity region, which is identified in selected M serotypes (M1, 4, 12, 28 and 49) where it controls the expression of Epf and Streptolysin S (Kreikemeyer, et al., 2007). Inactivation of Ralp3 in an M49 strain resulted in a diminished binding capacity to human plasminogen, and SpeB activity was significantly decreased (Siemens, et al., 2012). Transcriptome analysis identified 16 genes as up regulated, and 43 genes as down regulated in the *ralp3* mutant. Among those down regulated were the *lac* operon and the *fru* operon (encoding proteins involved in metabolism), the putative salivaricin operon (*sal*), and the whole Mga core regulon. The importance of the RALPs in metabolic control will be further discussed in the corresponding section. Every *S. pyogenes* strain tested to date encodes at least RofA or Nra, and some strains can express several RALP family members, which results in a widely variable combination of RALP-regulated genes and patterns of gene expression between different serotypes (Beckert, Kreikemeyer, & Podbielski, 2001; Podbielski, Woischnik, Leonard, & Schmidt, 1999; Kreikemeyer, Beckert, Braun-Kiewnick, & Podbielski, 2002; Kreikemeyer, et al., 2007).

Ralp4, or RivR (which stands for RALP, roman numeral "iv") was identified and characterized as a RALP, based on its 29% identity with RofA and 32% identity with Nra at the amino acid level (Roberts, Churchward, & Scott, 2007). Since phosphorylated CovR represses expression of RivR (Roberts, Churchward, & Scott, 2007), analysis of the RivR-regulated transcriptome in a MGAS5005 (M1T1) strain required its deletion and complementation in a Δ*covR* background (Roberts & Scott, 2007). This study showed that the overexpression of RivR resulted in the altered transcription of 31 genes, among which there were multiple virulence related genes that were either induced, in the case of *endoS* and several genes of the Mga regulon (*emm*, *scpA*, *fba*, *mga*, *sic*, *scl* and *grm*), or repressed by RivR activity. Further characterization revealed that RivR enhances transcriptional activation by Mga *in vitro*, increases expression of the Mga regulon *in vivo*, and is necessary for virulence in a CovR-deficient strain (Roberts & Scott, 2007). Additionally, as previously indicated, RivR also acts as a repressor of *hasABC* and *grab* expression in a CovR-dependent manner in two distinct M1T1 serotype strains (MGAS2221 and MGAS5005) (Treviño, Liu, Cao, Ramirez-Peña, & Sumby, 2013). In contrast to the earlier report (Roberts & Scott, 2007), the Mga regulon was not activated by RivR in these M1T1 serotype strains (Treviño, Liu, Cao, Ramirez-Peña, & Sumby, 2013).

Metabolite responsive regulators

Sensing and responding to changes in metabolite availability within a host is an integral part of virulence gene regulation in pathogenic bacteria, as virulence gene expression enables these organisms to infect and colonize the host. As a result, carbohydrate sources of energy, amino acid/nitrogen supplies necessary for protein and nucleic acid synthesis, as well as metal ions required for biochemical processes, are the principal metabolites that a pathogen like *S. pyogenes* must monitor throughout the course of host colonization. Here we summarize the

current understanding of the principal virulence gene regulators in *S. pyogenes* whose activity is directly determined by the availability of these metabolites.

Carbohydrate catabolite regulation (CcpA, LacD.1, Mga)

Bacteria of multiple species have been observed to coordinately alter the expression of carbohydrate utilization genes and virulence factor production in response to changes in carbohydrate availability (Moreno, Schneider, Maile, Weyler, & Saier, Jr., 2001; Deutscher, Francke, & Postma, 2006). Glucose is the primary carbohydrate source of energy for many bacteria, including *S. pyogenes*, which generates ATP from its conversion to pyruvate through the Embden-Meyerhof pathway. However, the utilization of alternative carbohydrates requires energy input to convert them to glucose in order to be metabolized. As a result, bacteria ensure that genes involved in alternative carbohydrate utilization are not expressed when glucose is available, through a process known as carbon catabolite repression (CCR) (Deutscher, Francke, & Postma, 2006). In *S. pyogenes*, CCR is not restricted to the regulation of carbohydrate catabolic enzymes and can affect the expression of virulence determinants via carbohydrate-sensitive regulators, such as CcpA, LacD.1, and Mga (Supplemental Table 3, 4).

CcpA and LacD.1

The catabolite control protein (CcpA) is a member of the LacI/GalR family of transcription factors that is common among Gram-positive pathogens, and a thorough review of its characterization can be found in (Deutscher, Francke, & Postma, 2006). Briefly, CcpA function centers on the activity of the phosphoenolpyruvate phosphotransferase system (PTS), which regulates carbohydrate uptake via a phosphorylation relay pathway that involves cytosolic (EI, HPr) and membrane bound (EII) enzymes (Deutscher, Francke, & Postma, 2006). Of these, HPr is tasked with determining the energy level of the cell by undergoing differential phosphorylation by the PTS and an HPr kinase at conserved histidine and serine residues, respectively. Phosphorylation of the serine residue enables the interaction of HPr with CcpA in order for CcpA to act as a carbohydrate-dependent transcriptional repressor by binding DNA at a palindromic consensus sequence in the promoter or coding regions of CCR-transcripts, known as a catabolite-responsive element or *cre* site (Deutscher, Francke, & Postma, 2006; Fujita, Miwa, Galinier, & Deutscher, 1995). Though primarily a repressor, CcpA is also known to function as an activator of transcripts that are required for glucose-dependent growth in other Gram-positive bacteria (Grundy, Waters, Allen, & Henkin, 1993). Transcriptome analysis of a MGAS5005 (M1T1) strain and its isogenic *ccpA* deletion mutant revealed that the transcription of 124 genes (~6% of the *S. pyogenes* genome) is affected by CcpA (Kinkel & McIver, 2008). In addition to the expected repression of genes relating to non-glucose sugar utilization, CcpA has influenced expression of virulence related genes, including the *sag* operon (repressing) and *rivR* (inducing). Under glucose-starved conditions, the expression of additional virulence related genes (*speB, mac, spd3*) were affected in the same CcpA-deficient strain (Shelburne, et al., 2008). Analysis of CcpA interaction with a *cre* site in the promoter region of *mga* in the JRS4 (M6) strain revealed that CcpA is necessary for full expression of the virulence regulator Mga in the late logarithmic phase of growth (Almengor, Kinkel, Day, & McIver, 2007). CcpA regulatory activity appears to be exerted both directly and indirectly and varies among strains, as CcpA was observed to bind to the *cre* site of the *mga* promoter in JRS4 (Almengor, Kinkel, Day, & McIver, 2007) and *speB* in the HSC5 (M14) strain, but not *sagA* in the latter, despite still repressing the expression of the *sag* operon (Kietzman & Caparon, 2010).

The CcpA regulon shares virulence gene targets with the CovR regulon, as demonstrated by the characterization of CcpA-deficient (*2221ΔccpA*), CovR-deficient (*2221ΔcovR*), and doubly-deficient (*2221ΔccpAΔcovR*) mutant strains in an M1T1 background with a functional CovS (MGAS2221), as well as by comparison with similarly constructed mutations in a CovS-deficient strain (MGAS5005) (Shelburne, et al., 2010). According to this study, CcpA and CovR co-regulate transcription of the virulence factor encoding genes *nga*, *slo*, *spyCEP*, *sagA*, *sdaD2, endoS*, *fba*, and *speB*. CcpA directly regulates expression of these genes in the strains examined, as CcpA and its complex with phosphorylated HPr protein, CcpA-(HPr-Ser46-P), bind with high affinity to *cre* sites within their

promoters. However, this shared regulation is dependent upon the status of CovS functionality; CcpA influences *speB*, *slo,* and *spyCEP* expression during *in vitro* growth only when CovS is functionally active (Shelburne, et al., 2010). Notably, CovR and CcpA do not regulate each other's expression, at least not directly in the strains and conditions examined—despite the fact that all of the virulence genes affected by CcpA inactivation are also affected by CovR inactivation (Shelburne, et al., 2010).

The CCR of virulence-related genes by CcpA occurs in conjunction with the activity of another carbohydrate-dependent regulator, called LacD.1. Identified as a glucose responsive regulator of the SpeB protease (Loughman & Caparon, 2006a), LacD.1 is a member of the Lac.1 operon and has the characteristics of a tagatose 1,6-bisphosphate aldolase involved in lactose and galactose metabolism. Though LacD.1 possesses enzymatic activity, it does not function to metabolize galactose (Loughman & Caparon, 2006a; Loughman & Caparon, 2007). LacD.1 regulatory function is independent of its catalytic activity, but the protein must bind to its substrate to exert its regulation of SpeB, a function that appears to have evolved by conferring increased fitness to *S. pyogenes*, as observed using competitive assays under *in vitro* growth conditions that mimic deep tissue environments (Cusumano & Caparon, 2013). An *in vivo* analysis of the carbon catabolite-responsive regulatory pathways constituted by CcpA and LacD.1 revealed that they can regulate up to 15% of the genome in response to glucose, and that together, they contribute to regulation of 60% of this subset in various combinations, including CcpA and LacD.1-specific and independent regulation, co-regulation, and even regulation independent of glucose (Kietzman & Caparon, 2011). Among the affected genes were several secreted factors, putative virulence genes, and previously characterized genes that affect virulence, such as *sagA* and *lctO* (Kietzman & Caparon, 2010). The most important virulence gene affected by CcpA and LacD.1 co-regulation is *speB*, which was induced by CcpA and repressed by LacD.1 both *in vitro* and *in vivo*. In the latter condition, CcpA and LacD.1 mutants evinced mis-regulation of SpeB at separate and distinct phases of infection, which highlights the spatial and temporal role that carbon catabolite sensing appears to play in virulence-related gene regulation during the course of infection (Kietzman & Caparon, 2011). Another important finding of this study was that multiple transcripts are responsive to glucose independent of CcpA and LacD.1 activity, which indicates that other carbohydrate responsive regulators act in *S. pyogenes*. One such regulator appears to be the virulence gene-related transcription factor Mga.

Mga

Mga is a large 500 amino acid DNA-binding with an approximate molecular size of 62 kDa. Orthologous proteins can be found in many pathogenic streptococci, including S*treptococcus dysgalactiae*, *S. pneumoniae*, *S. equi*, *S. gordonii*, *S. mitis*, *S. sanguinis*, and *S. uberis* (Geyer & Schmidt, 2000; Vasi, Frykberg, Carlsson, Lindberg, & Guss, 2000; Hava & Camilli, 2002; Vahling & McIver, 2006). Mga was first identified in *S. pyogenes* through spontaneous small deletions at a locus just upstream of the *emm* gene that resulted in M protein-negative strains (Spanier, Jones, & Cleary, 1984), and was later confirmed as a positive regulator of *emm* transcription and M protein production (Caparon & Scott, 1987). The identified loci immediately upstream of *emm* was predicted to produce a protein with homology to a DNA-binding transcriptional regulator (Perez-Casal, Caparon, & Scott, 1991; Chen, Bormann, & Cleary, 1993) and was eventually designated as *mga* for multiple gene regulator of group A streptococcus (Scott, et al., 1995). Mga is expressed in all *S. pyogenes* strains and its encoding gene is found in all strains examined to date (Podbielski, 1992; Bessen, Manoharan, Luo, Wertz, & Robinson, 2005). Its sequence varies up to 21% among *S. pyogenes* strains, and two divergent alleles of *mga* (*mga-1, mga-2*) have been identified (Haanes & Cleary, 1989; Hollingshead, Readdy, Yung, & Bessen, 1993), although the expressed Mga proteins maintain greater than 97% amino acid sequence identity. The *mga-1* allele is linked to serum opacity factor negative (SOF^-) class I strains isolated from throat infections, while the *mga-2* allele is almost exclusively observed in SOF^+ class II strains associated either with skin infections or with "generalist" strains (Bessen, Manoharan, Luo, Wertz, & Robinson, 2005), which suggests that the divergent Mga regulons likely evolved to adapt to distinct tissue environments (Bessen, Manoharan, Luo, Wertz, & Robinson, 2005).

Mga induces transcription of its encoding gene (*mga*) (Geist, Okada, & Caparon, 1993; Podbielski, Flosdorff, & Weber-Heynemann, 1995; McIver, Thurman, & Scott, 1999) and a core set of virulence genes during exponential growth *in vitro*. These include cell wall-associated surface molecules in addition to M protein involved in host tissue adherence, internalization into non-phagocytic cells and avoidance of the host innate and adaptive immune responses. The regulatory role of Mga during the course of infection in different tissue environments will be covered later in this chapter. Mga induces the transcription of multiple genes located downstream of *mga* and *emm* in a discrete region of the chromosome called the *mga* locus, identified in all *S. pyogenes* genomes thus far examined. This region includes, in varying organization and combinations depending on serotype, a cell wall-associated C5a peptidase (*scpA*), the M-like surface proteins Mrp (*fcrA*) and Enn (*enn*), secreted inhibitor of complement (*sic*), and a fibronectin-binding surface adhesin (*fba*) (Haanes, Heath, & Cleary, 1992; Podbielski, 1993; Hollingshead, Arnold, Readdy, & Bessen, 1994). The *scpA* gene product is involved in cleavage of the C5a chemotaxin, which inhibits recruitment of PMNs to infectious sites (Simpson, LaPenta, Chen, & Cleary, 1990). Mrp (*fcrA*) binds IgG and fibrinogen, and can provide resistance to phagocytosis; while Enn generally binds IgA, though antiphagocytic properties for it have not been shown (Bessen & Fischetti, 1992; Courtney & Li, 2013). The product of *sic* has the ability to block the membrane attack complex (MAC) of the complement, as well as inhibit the activity of innate immune effectors, such as lysozyme and host antimicrobial peptides (Akesson, Sjöholm, & Björck , 1996; Fernie-King, et al., 2001; Frick, Akesson, Rasmussen, Schmidtchen, & Björck, 2003). Fba constitutes a fibronectin-binding surface adhesin that is able to bind the complement regulators factor H and factor H-like protein 1 to block opsonophagocytosis (Terao, et al., 2001; Pandiripally, Gregory, & Cue, 2002). However, not all Mga-regulated genes are located within the *mga* locus. The fibronectin-binding surface proteins SfbX and SOF that enable *S. pyogenes* to interact with the host ECM (Courtney, et al., 1999; Jeng, et al., 2003) are encoded by *sof* and *sfbX* 12 kb away from *mga* in class II SOF^+ strains, and their co-transcription is activated by Mga (Green, et al., 2005). Mga also influences the expression of *sclA,* a gene that is found over 30 kb upstream of the *mga* locus in a subset of serotypes and that encodes the streptococcal collagen-like protein A (*sclA*/*scl1*). This repeat-containing surface molecule found on all *S. pyogenes* strains absorbs human plasma LDL and binds to $\alpha_2\beta_1$ integrins on host cells, which allows for internalization and subsequent re-emergence as a means of immune evasion (Caswell, Lukomska, Seo, Höök, & Lukomski, 2007). As previously indicated, Mga can also regulate the transcription of *speB*, which modulates the production of the secreted SpeB cysteine protease (Podbielski, Flosdorff, & Weber-Heynemann, 1995; Ribardo & McIver, 2006).

Of the carbohydrate-responsive virulence gene regulators, Mga is the best characterized in terms of its mechanism of action. A more thorough overview of Mga binding site and domain architecture can be found in (Hondorp & McIver, 2007). The binding of Mga to upstream promoter regions is essential for high-level transcriptional activation of core regulon genes (McIver & Myles, 2002). Mga binding sites within these promoters vary widely in location with respect to the start of transcription and have thus far been classified into three categories based on biochemical studies ((Almengor & McIver, 2004), reviewed in (Hondorp & McIver, 2007)). Initial DNase I footprinting experiments indicate that Mga protects a large 45- to 59-base-pair non-palindromic region of DNA (McIver, Thurman, & Scott, 1999; McIver, Heath, Green, & Scott, 1995), and these studies suggest a nominal consensus sequence to guide the search for Mga binding sites. A mutagenesis-based analysis of the M1T1 *emm* promoter binding site (Pemm1) identified 34 nucleotides within Pemm1 that had an effect either on Mga binding, on Mga-dependent transcriptional activation, or on both (Hause & McIver, 2012). Among these critical nucleotides, guanines and cytosines within the major groove were disproportionately identified as clustered at the 5' and 3' ends of the binding site, along with runs of nonessential adenines between the critical nucleotides. On the basis of these results, a Pemm1 minimal binding site of 35 bp bound Mga was found at a level comparable to the level of binding of the larger 45-bp site. However, as more sites have been found, the sequence has become less defined, due to lack of conservation between the various promoters, and exhibits only 13.4% identity with no discernible symmetry (Hause & McIver, 2012). Nonetheless, *in vitro* transcription assays have established that Mga is sufficient to activate expression (Almengor, Walters, & McIver, 2006), so while additional factors might modify Mga regulation *in vivo*, they are not required. Despite the

variability in promoter sites, Mga seems to employ the same protein domain(s) to bind locations with little sequence homology, as evidenced by competition of P*emm* and P*scpA* for Mga binding to P*mga* (McIver, Thurman, & Scott, 1999).

Mga has two conserved helix–turn–helix (HTH) domains, HTH-3 (residues 53–72) and HTH-4 (residues 107–126), that map near the N-terminus of the protein (Chen, Bormann, & Cleary, 1993; Podbielski, Flosdorff, & Weber-Heynemann, 1995; McIver & Myles, 2002). These domains have been shown to be required for DNA binding and the activation of transcription for all genes studied (Vahling & McIver, 2006; McIver & Myles, 2002). While HTH-4 is absolutely essential to these processes, HTH-3 appears to serve more of an accessory role during autoactivation. These HTH domains appear to be present in other virulence regulators that do not share homology throughout the entire length of the protein with Mga, such as the RALPs. This has led to the definition of the specific Pfam domains "HTH_Mga" and "Mga," which encompass HTH-3 and HTH-4, respectively (Finn, et al., 2006). An *in silico* analysis comparing Mga to proteins of known structure found in the SCOP database found established Mga DNA-binding domains (Andreeva, Howorth, Brenner, Hubbard, Chothia, & Murzin, 2004; Vahling, 2006), as well as two regions within Mga that were predicted to have strong homology to the dual PTS regulatory domains (PRD) in the antiterminator LicT, the only PRD-containing protein for which a structure has been determined. The overall size and domain structure of Mga, with N-terminal DNA-binding domains and two central PRD domains, closely resembles that of known PRD-containing transcriptional activators such as MtlR (Vahling, 2006). Subsequently, the Pfam database now designates these regions as a "PRD_Mga" domain, which is a member of the PRD superfamily (Finn, et al., 2006). Once the high-level purification of Mga was achieved, gel filtration analyses, analytical ultracentrifugation and co-immunoprecipitation experiments have demonstrated that Mga oligomerizes in solution, and that its ability to oligomerize correlates with transcriptional activation (Hondorp, et al., 2012). Examination of the previously uncharacterized C-terminus detected structural homology to EIIB domains used by the PTS. Truncation analyses of Mga from an M4 and an M1 strain revealed the C-terminal region of Mga is required for oligomerization and *in vivo* transcriptional activity, but not DNA binding (Hondorp, et al., 2012). Conversely, the DNA binding domains of Mga are necessary, but are insufficient to induce the expression of Mga-regulated transcripts.

As mentioned, Mga contains two central PRD domains that are similar to antiterminators (such as LicT), and activators (such as MtlR and LicR) that are involved in the regulation of sugar metabolism (reviewed in (Deutscher, Francke, & Postma, 2006)). The activity of these regulators is modulated by phosphorylation of conserved histidine residues within their PRD domain(s) via the PTS phosphorelay in response to the utilization of different carbohydrate sources, as observed in the *B. anthracis* Mga homolog, AtxA (Tsvetanova, et al., 2007; Hammerstrom, et al., 2015). An M4 strain with a nonfunctional PTS that results from the deletion of the EI-encoding *ptsI* gene exhibited a decrease in Mga activity, and characterization of purified Mga showed that it could be phosphorylated at conserved PRD histidines by EI and HPr components of the PTS *in vitro* (Hondorp, et al., 2013). Further confirmation that Mga activity is directly influenced by the PTS came from examination of Mga transcriptional activity using alanine (unphosphorylated) and aspartate (phosphomimetic) substitution mutations of conserved Mga PRD histidines, which established that a doubly-phosphorylated PRD1 phosphomimetic Mga (D/DMga4) is completely inactive *in vivo* and shuts down the expression of the Mga regulon (Hondorp, et al., 2013). Although the D/DMga4 protein is still able to bind DNA *in vitro*, homo-multimerization of Mga is disrupted and the protein is unable to activate transcription. As a result, carbohydrate availability can be linked to Mga-dependent expression of virulence-related genes. These studies also highlighted the role of a PRD-containing virulence regulator (PCVR) like Mga for pathogenesis, as both non-phosphorylated and phosphomimetic PRD1 Mga mutants were attenuated in a model of *S. pyogenes* invasive skin disease (Hondorp, et al., 2013). A similar study in an M59 serotype background that is highly polymorphic in Mga found that phosphomimetic substitutions at both PRD1 and PRD2 conserved histidine residues are inhibitory to Mga-dependent gene expression and attenuated GAS virulence by influencing Mga-dependent global gene regulation (Sanson, et al., 2015). Conversely, non-phosphorylation-mimicking alanine substitutions

relieved inhibition and the mutants exhibited a wild-type phenotype in a mouse model of necrotizing fasciitis. These studies suggest that adequate regulation (positive and negative) of Mga activity as a PCVR is necessary for virulence and varies between genotypic backgrounds—an important finding, given that PCVRs appear to be widespread among Gram-positive pathogens (Hondorp, et al., 2013).

Mga expression is both autoregulated and influenced by other regulators. Transcription from P*mga* appears not to be autoregulated in response to carbohydrate availability, as sensed by the PTS (Hondorp, et al., 2013). Nonetheless, P*mga* is required for growth phase-regulated expression of the Mga regulon, which indicates that environmental regulation still occurs at the level of *mga* transcription (Okada, Geist, & Caparon, 1993; McIver & Scott, 1997). Such regulation of P*mga* does not appear to occur through the direct influence of two-component signal transduction systems (Ribardo, Lambert, & McIver, 2004), although TCSs are involved in inducing transcription of Mga-regulated virulence genes in other serotypes, as was seen in the TrxRS-mediated activation of P*mga* demonstrated by analysis of a luciferase reporter fusion in a MGAS5005 (M1) strain (Leday, et al., 2008). As previously indicated, CcpA binds to P*mga* and maintains high-level *mga* expression during exponential growth (Almengor, Kinkel, Day, & McIver, 2007). Deletion analysis of P*mga* identified a region overlapping the Mga binding site I that is involved in repression of *mga* transcription (McIver, Thurman, & Scott, 1999). Some RALPs (i.e., RofA, RALP3 and Nra) are known to repress *mga* expression (Beckert, Kreikemeyer, & Podbielski, 2001; Podbielski, Woischnik, Leonard, & Schmidt, 1999; Kwinn, et al., 2007). Another regulator, Rgg/RopB (Federle, 2012), has also been shown to repress Mga expression (Chaussee, et al., 2002); however, direct interaction of these at P*mga* has not been demonstrated. In contrast, RivR (Ralp4) activity in an M1 strain is able to enhance the activation of *mga* and Mga-regulated genes both *in vitro* and *in vivo,* though this is controversial (Roberts, Churchward, & Scott, 2007; Treviño, Liu, Cao, Ramirez-Peña, & Sumby, 2013; Roberts & Scott, 2007). The inactivation of *amrA*, which produces a putative Wzx integral membrane sugar exporter, was found to be essential for exponential-phase expression of *mga* in a serotype M6 strain (Ribardo & McIver, 2003), possibly by altering sugar levels in the cell.

All of these studies suggest a general model for how changes in environmental carbohydrates influence expression of the Mga-regulated virulence repertoire of *S. pyogenes* (Figure 2). While glucose (the preferred carbohydrate source) is abundantly available, CcpA induction combined with reduced PTS activity and Mga autoregulation maintain the high-level expression of Mga and its regulon. As glucose becomes scarce, induction by CcpA decreases and *mga* transcription is reduced, but is maintained by autoregulation. As PTS activity increases to supply the bacterium with alternate carbohydrate sources, Mga activity is repressed by the PTS-dependent phosphorylation of its PRD region. Other factors like Rgg and RALPs, whose regulatory activity is mostly exerted under the stationary phase of growth when carbohydrate energy sources are exhausted, further maintain the repression of Mga until environmental carbohydrate supplies become available. Modulation of RivR by the CovR/S master regulator to influence Mga activity serves to further fine-tune the expression of the Mga regulon. Naturally, this is an oversimplified model that fails to account for the contributions made by different genomic backgrounds, the organization of regulatory regions, or the input of master regulators in controlling the Mga regulon. However, this model might be useful in guiding elucidation of the complex virulence gene regulatory network influencing *S. pyogenes* pathogenesis in response to changes in carbohydrate availability, which is an area of increasing research interest.

Amino acid and nitrogen availability (Rsh, CodY, and Rny)

The primary sites of *S. pyogenes* infection are potentially rich in nutrients in the form of abundant peptides and proteins, but are somewhat lacking in free amino acids (Steiner & Malke, 2000). As a multiple-amino-acid-auxotrophic pathogen (Ferretti, et al., 2001; Davies, Karush, & Rudd, 1965), *S. pyogenes* may encounter starvation for free amino acids when attempting to propagate in these host niches during specific stages of the infection process; most likely when initially contacting the skin or throat as primary infection sites (Steiner & Malke, 2001). Although for *S. pyogenes* found on the skin, the existence of a persistent carrier state seems

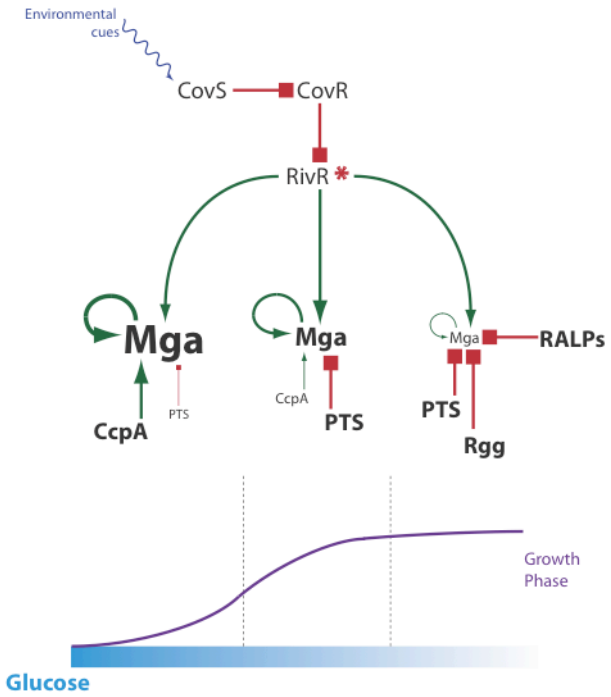

Figure 2. Mga regulation exemplifies phase and metabolite-dependent regulation in *S. pyogenes*: Mga expression and activity is growth phase-dependent and is influenced by carbohydrate availability (indicated by decreasing glucose levels). The regulation of Mga involves direct and indirect activity of metabolite sensing regulators (CcpA), master regulators (CovR, RALPs), and phase dependent regulators (Rgg), as well as autoregulation. The variety of regulators acting upon Mga allows for fine-tuning expression of the broad Mga regulon. *The effect of RivR on Mga activity is unclear, as studies of RivR regulation of Mga have yielded contradictory evidence (Treviño, Liu, Cao, Ramirez-Peña, & Sumby, 2013; Roberts & Scott, 2007).

unlikely, the organism may exist in an asymptomatic state harbored by the oropharynx where it appears to constitute the principal reservoir for *S. pyogenes* in the environment (Pichichero, 1998). The stasis-like carrier state may also typify the physiological state of bacteria that adhere to epithelial cells and succeed in invading them. Additionally, *S. pyogenes* may encounter host nutritional deficiencies when persisting at high cell densities at infection sites or initiating life-threatening invasive disease (such as streptococcal toxic shock syndrome, necrotizing fasciitis, or septicemia) (Steiner & Malke, 2001). Given the polyauxotrophy of *S. pyogenes* and the fact that restriction of an amino acid immediately limits the availability of the corresponding aminoacyl-tRNA (Emilsson & Kurland, 1990), amino acid supply is a central problem for its survival and propagation. Multiple studies indicate that *S. pyogenes* has evolved a stimulus response network that involves peptidases, proteinases, and peptide and amino acid transport systems in order to generate the free amino acids necessary for growth, which counteracts amino acid starvation and enables the pathogen to mount a dynamic response to the protein-rich environment provided by its human host. In this section, we summarize the regulation of this network in relation to the control of *S. pyogenes* virulence.

Rsh: stringent and relaxed responses to amino acid starvation

Originally cloned and sequenced (Mechold, Steiner, Vettermann, & Malke, 1993), as well as structurally and functionally characterized (Hogg, Mechold, Malke, Cashel, & Hilgenfeld, 2004; Mechold, Cashel, Steiner, Gentry, & Malke, 1996; Mechold & Malke, 1997), the stringent factor from *S. dysgalactiae* subsp. *equisimilis* (group C streptococci) has proved to be of seminal importance to the subsequent establishment of the RelA/SpoT homolog (Rsh) superfamily of bifunctional guanosine 5'-3' polyphosphate ((p)ppGpp) synthetases and hydrolases and their distribution and function across all living organisms (Atkinson, Tenson, & Hauryliuk, 2011)). Given the amino acid sequence identities of the order of 95% (97% similarity) between the group C

streptococci query sequence (739 residues) and the available *S. pyogenes* sequences, as well as identical linkage relationships of the respective genomic regions (Ferretti, et al., 2001; Mechold, Steiner, Vettermann, & Malke, 1993), all basic properties established for the group C streptococcus protein can also safely be taken to apply to the *S. pyogenes* homolog. Henceforth, both will be specified as Rsh_{Str}. Like all full-length Rsh proteins, Rsh_{Str} synthesizes the alarmone (p)ppGpp at the expense of ATP and GTP when cognate, but deacylated tRNAs occupy the aminoacyl-acceptor site of the ribosomes (Hogg, Mechold, Malke, Cashel, & Hilgenfeld, 2004; Mechold, Cashel, Steiner, Gentry, & Malke, 1996; Mechold & Malke, 1997). Consequently, ribosomal complexes on mRNA templates are stalled, which prevents wasteful macromolecular syntheses by a rapid shutdown of futile RNA synthesis in stressful metabolic conditions, like amino acid starvation. Alarmone signaling pathways are primarily directed towards transcriptional inhibition of the stable RNAs, such as rRNA and tRNA (the so-called stringent response, reviewed in (Potrykus & Cashel, 2008). Accordingly, under specific nutritional stress conditions, *S. pyogenes* synthetase-deficient Rsh_{Str} insertion mutants with rsh_{Str} truncated at codons 220 or 129 show survival rates about three orders of magnitude lower than wild types. The basic reason for this deficiency is the energetically wasteful continuation of RNA synthesis during amino acid deprivation (the so-called relaxed response (Steiner & Malke, 2000; Mechold & Malke, 1997; Potrykus & Cashel, 2008)).

As shown by functional Rsh_{Str} analyses, this enzyme has also a strong (p)ppGpp-hydrolytic activity that gives rise to GTP/GDP plus pyrophosphate (Mechold, Cashel, Steiner, Gentry, & Malke, 1996). The first-order rate constants of (p)ppGpp turnover reflect short half-lives of 20 and 60 sec for the penta- and tetraphosphates, respectively, of the alarmone, which enables the cells to recover quickly from the stasis-like survival state under improved nutritional conditions (Mechold, Cashel, Steiner, Gentry, & Malke, 1996). Defined Rsh_{Str} fragments created by cloning and functionally analyzed by enzymatic activity assays showed that the two opposing activities reside in the 1-385 residue N-terminal half, bridged by an exposed hinge region to the C-terminal half, which is suggested to be involved in regulatory intramolecular interactions or intermolecular interactions with the ribosome (Atkinson, Tenson, & Hauryliuk, 2011; Mechold, Murphy, Brown, & Cashel, 2002). Dissecting the N-terminal half more precisely by a nested deletion analysis, combined with enzymatic activity assays of purified peptide fragments, revealed non-overlapping minimal fragments of residues 1-198 and 211-347 for full hydrolytic and synthetic activities, respectively (Mechold, 2009).

One challenging problem posed by the opposing enzymatic activities of Rsh_{Str} concerned the prevention of futile cycling of the alarmone metabolism, which would waste ATP and hamper the rapid adjustment of (p)ppGpp levels as dictated by the prevailing niches of infection *in vivo* and indeed demonstrated *in vitro* (Mechold & Malke, 1997; Mechold, Murphy, Brown, & Cashel, 2002) and Figure 3). However, the resolved X-ray structure of the catalytic Rsh_{Str} region (residues 1-385), which is the first to be established for any Rsh homolog, presented this fragment in distinct hydrolase-OFF/synthetase-ON and hydrolase-ON/synthetase-OFF conformations (Hogg, Mechold, Malke, Cashel, & Hilgenfeld, 2004). The structure localized the opposing enzymatic activities >30 A apart and rendered them mutually exclusive by a combination of induced fit principles and nucleotide ligand-induced intramolecular allosteric signaling between the opposing active sites (Hogg, Mechold, Malke, Cashel, & Hilgenfeld, 2004; Mechold, 2009). As a result, structural antagonism rules out the *in vivo* possibility of simultaneous (p)ppGpp synthesis and hydrolysis.

Continuing this line of research, (Mechold, Potrykus, Murphy, Murakami, & Cashel, 2013)) have recently developed strategies that enable them to fine-tune the differential accumulation of pppGpp and ppGpp by activating different Rsh_{Str} synthetic fragments in combination with *E. coli* pppGpp 5'-gamma phosphate hydrolase to get rid of the pentaphosphate and generate only ppGpp. They found that ppGpp is a more efficient regulator than pppGpp with respect to a plethora of five stress responses, with the most important one in the present context being rRNA synthesis regulation. Reducing the aura of the alarmone even further, multiple groups found that both nucleotides bind to a site at the interface between the beta' and omega RNAP subunits (Ross, Vrentas, Sanchez-Vasquez, Gaal, & Gourse, 2013; Zuo, Wang, & Steitz, 2013). This finding supports a model where ppGpp connects the core and the shelf of RNAP to form a mobile clamp around the DNA. By

controlling the opening and closing of the clamp through an allosteric mechanism, ppGpp modulates RNAP activity. However, since the structural results were obtained with RNAP holoenzyme from *E. coli*, it remains to be seen if RNAP from *S. pyogenes* will behave in a similar fashion.

Apart from its primary function of stable RNA synthesis inhibition, (p)ppGpp also affects negatively the transcription of important groups of protein-encoding *S. pyogenes* genes, including regulators, virulence factors and genes for transport systems (Malke, Steiner, McShan, & Ferretti, 2006). Of pivotal importance is the strong negative stringent control of rsh_{str} itself. Exposure to isoleucine plus valine starvation results in 40-fold changes in rsh_{str} expression in an rsh_{str} mutant, relative to the wild type. This high degree of negative autoregulation may be necessary for a gene with broad connectivity in order to sustain cellular homeostasis. Among 31 additional genes studied for their expression during the stringent response (and found to be strongly negatively controlled) were the virulence factors *graB (see above)* and *speH* encoding the superantigen pyrogenic exotoxin H. An approximately tenfold expression increase under relaxed conditions was also observed for the important branched-chain amino acid (BCAA, see below) transporter gene *braB*, which reflects a cost-effective means for the repression of a futile transport process under nutrient-rich conditions.

CodY: (p)ppGpp-independent response to amino acid starvation.

Experiments in which both *S. pyogenes* wild type and rsh_{Str} mutant strains were exposed to BCAA starvation led to the unexpected discovery of an rsh_{Str}-independent mode of global transcriptional regulation (Steiner & Malke, 2000; Steiner & Malke, 2001). This regulatory response to the deprivation of the essential amino acids isoleucine and valine is characterized by the up-regulation of a wide array of housekeeping genes, as well as accessory and dedicated virulence factors in both isogenic genotypes. Up-regulation under these conditions also applies, for example, to the CovRS regulation of virulence gene expression in a global fashion. Importantly, of the operons affected by the rsh_{Str}-independent response, the oligopeptide permease (*opp*), FAS regulatory system (*fasBCAX*) and streptolysin S operon (*sag*), exhibit internal transcriptional termination under nutritionally rich conditions. However, BCAA-starvation promotes full-length transcription of these operons, which supports the level of coordinate transcription of functionally related genes and contributes to the efficacy of these systems under stressful conditions. When all effects are considered, the rsh_{Str}-independent response to BCAA limitation appears to counteract the stringent response and enables *S. pyogenes* dynamically to exploit the protein-rich niches of their host to cope with stasis states under nutritional stress conditions.

The results summarized above were generated before anything was known about CodY action in *S pyogenes.* BCAAs had been shown to activate the pleiotropic repressor CodY in *Lactococcus lactis* (Guédon, Serror, Ehrlich, Renault, & Delorme, 2001), as well as in *Bacillus subtilis* (Shivers & Sonenshein, 2004). The *codY* of *S. pyogenes* was subsequently inactivated in strain NZ131 (M49) and experiments were performed to find possible instances of the *codY* mutants mimicking rsh_{Str}-independent stimulation of transcription upon BCAA deprivation under a variety of culture conditions, such as growth media and growth phases (Malke, Steiner, McShan, & Ferretti, 2006; Malke & Ferretti, 2007). Given the broad regulatory connectivity of *codY* among the Firmicutes (Sonenshein, 2007), CodY action could be thought to involve both direct and indirect effects. Direct action was suggested by finding the 15-base pair CodY binding sequence, as defined in *Lactococcus lactis* (den Hengst, et al., 2005; Guédon, Sperandio, Pons, Ehrlich, & Renault, 2005) upstream of several NZ131 genes, including the *opp* operon, the *braB* gene, and the negatively autoregulated *codY* gene itself (Malke & Ferretti, 2007). These findings obtained *in silico* were substantiated by electrophoretic mobility shift assays (EMSAs), which provided direct evidence for the function of the *codY* box in *S. pyogenes* (Kreth, Chen, Ferretti, & Malke, 2011). However, how much degeneracy of the consensus sequence is tolerated for function is still not known, given that a putative CodY-binding site with 4 mismatches (including loss of the inverted repeat of the box) was identified in the *mga* promoter (Flores, et al., 2013).

Concerning the fact that high BCAA levels render CodY a more active repressor, a unique feature of the *S. pyogenes* homolog deserves further consideration. Northern analysis showed for two strains, NZ131 (M49) and

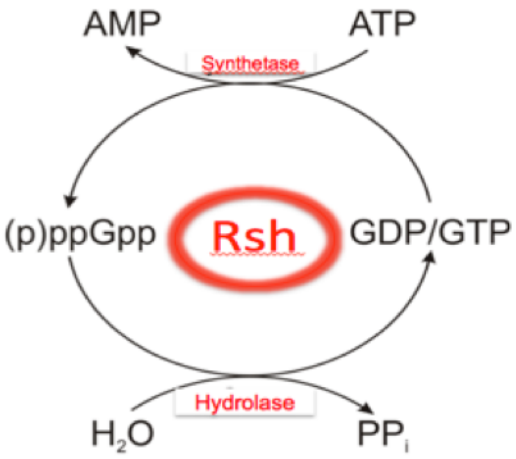

Figure 3. Synthesis and hydrolysis of (p)ppGpp catalyzed by bifunctional Rsh_{Str}

SF370 (M1), that *codY* is transcribed both monocistronically from its own promoter and dicistronically from the promoter of the co-oriented upstream *aat* gene that encodes aspartate aminotransferase (Malke, Steiner, McShan, & Ferretti, 2006). This implies that monocistronic transcript levels are down regulated at low BCAA levels (namely, under nutritional stress conditions) and are counterbalanced by the BCAA-independent dicistronic mode of *codY* transcription, with the caveat that nothing is known about the control of *aat* transcription.

Using quantitative RT-PCR, (qPCR), a selected set of about 50 NZ131 (M49) genes was studied for differential expression under various conditions. This included regulators with known targets, established virulence factors, physiologically important transporters, and metabolic enzyme genes (Malke, Steiner, McShan, & Ferretti, 2006; Malke & Ferretti, 2007; Malke, McShan, & Ferretti, 2009). The results revealed that CodY and Rsh_{Str} did not significantly affect the expression of each other; instead, they appeared to act independently, as indicated by an rsh_{Str}-*codY2* double mutant that showed a composite transcription pattern of the gene sets for the single mutants (Malke, Steiner, McShan, & Ferretti, 2006). Additional support for an independent action of the two regulatory systems was provided by their additive effects in the double mutant regarding the expression patterns of *braB* and *graB*, which are already strongly affected in both single mutants under specific conditions (Malke, Steiner, McShan, & Ferretti, 2006). Overall, specific transcription units from all four groups previously mentioned were under CodY control, often in a medium and growth-phase dependent manner. In particular, growth in human blood apparently involves environmental cues that are absent in laboratory media, which leads to partially discordant CodY-directed transcription profiles. The impact on transcription regulators (*covRS*, *fasX*, *mga*, *sptRS*) amplifies the connectivity of CodY and provides bona fide evidence for the indirect CodY regulation of diverse sets of genes. Virulence genes (*cfa*, *emm49*, *graB*, *hasA*, *ideS*, *nga*, *prtS*, *sagA*, *scl*, *scpA*, *ska*, *slo*, *sof*, *speH*), were shown to be under the control of CodY, presumably because their direct regulators (mainly *covRS* and *mga*) are in the CodY regulon. Therefore, CodY tends to up-regulate the positive regulator Mga and to down-regulate the negative regulator CovRS, which indirectly enhances the expression of a broad range of virulence factors. Notably, a number of these up regulated virulence factors (such as Cfa, Ids, Nga, PrtS, ScpA, and Slo) also serve to release nutrients from the host (Malke & Ferretti, 2007). Transporter gene expression (*braB*, *dppA*, *malX*, *opp*, *pbuX*, *ptsG*, *pyrP*) turned out to be highly specific to the individual systems, with *braB* and *opp* being most strongly affected in a cost-effective manner, as accounted for by the culture conditions.

Regarding the metabolic enzyme genes, CodY-mediated control of the *arcABC* operon involved in the catabolism of arginine is particularly relevant, because it serves to integrate basic nitrogen metabolic and virulence pathways by yielding ammonia and ATP, as well as leading to host-generated nitric oxide production. Ammonia production supports survival of the cells in acidic conditions, such as those found within

phagolysosomes (Degnan, et al., 2000), and nitric oxide mediates host innate immunity (Cusumano, Watson, Jr., & Caparon, 2014). Experiments carried out in near-natural conditions, namely with NZ131 (M49) and its isogenic *codY1* mutant cultured in heparinized human blood and sampled at different points during the course of growth, found that *arcABC* transcript levels up regulated in the *codY1* mutant decrease dynamically during growth (Malke, McShan, & Ferretti, 2009) and imply that strong CodY repressor activity occurs preferentially during the first hours of exposure to blood, when effective BCAA concentrations are still available. This activity does not appear to be directly or indirectly influenced by CodY action on the *arc* operon regulators *argR* and *crp* (Malke, McShan, & Ferretti, 2009).

Interaction of the global CodY and CovRS regulatory systems

Given that CodY and CovRS show a broad overlap regarding their target genes, their regulons were dissected using NZ131 and its *codY1*, *covRS* and *codY1-covRS* mutants grown in C medium (Loughman & Caparon, 2006b) to mid-exponential (ME) and early stationary (ST) phase. In contrast to the nonpolar *codY1*, *covRS* disrupting the operon at codon 174 is polar and inactivates both *covR* and the downstream *covS*, which prevents the possible cross talk of CovS with other regulators (Steiner & Malke, 2000). Transcriptome analysis using DNA microarrays (Kreth, Chen, Ferretti, & Malke, 2011) showed that CodY controls the transcription of about 17% of the core genome (1,605 genes, excluding prophage), which is a finding comparable to the 15% controlled by CovRS (Graham, et al., 2002). Regarding the action of the two systems on virulence gene control, CovR-directed repression generally appears to be stronger than CodY-mediated activation. This can be seen in Figure 4 by looking at the phenotypes of the four strains, with respect to capsule production (*hasABC)*.

The most intriguing results concerning the relationship between the actions of CodY and CovRS were observed upon robust correlation analysis of the DNA microarray results (Kreth, Chen, Ferretti, & Malke, 2011). This revealed a strong and highly significant negative correlation between the action of CodY and CovRS ($r(1603) = -0.40$; $p<0.0001$) in ME phase cells. The negative correlation is relieved in the double mutant, but stays slightly negative ($r(1603) = -0.16$; $p<0.0001$), which indicates that CovRS-directed repression overrides CodY-mediated gene activation. When ST phase cells were subjected to the same analysis, the negative correlations between the actions of the two systems ceased to operate. This was particularly evident under expression threshold parameters (Kreth, Chen, Ferretti, & Malke, 2011) where the low r values did not reach significance. Therefore, CodY and CovRS appear to act in opposite directions, with the expression-balancing effect being largely restricted to actively growing cells. This leads to intermediate levels of not only virulence gene transcripts, but also lessens full transcriptional control of a substantial fraction of the whole genome. This could be due to CodY and Mga governing about 17% and 10%, of the genome, respectively (Hondorp & McIver, 2007; Kreth, Chen, Ferretti, & Malke, 2011). In light of the Red Queen principle, the interaction of CodY and CovRS may indicate that the host-pathogen association has coevolved to become less harmful to either partner.

Virulence-related metabolic regulators

Transcriptional regulators that control virulence factor expression not only respond to environmental and intracellular metabolites, but can also influence the expression of metabolism-related genes in order to maintain the necessary homeostasis to promote colonization and survival of *S. pyogenes* within a host niche.

VicR/S

VicR/S is one of the TCSs found in *S. pyogenes* and its orthologs in other Gram-positive pathogens appear to be essential (Fabret & Hoch, 1998; Lange, et al., 1999; Martin, Li, Sun, Biek, & Schmid, 1999). Work from several groups has indicated that *vicR* is essential to some *S. pyogenes* serotypes, including the 5448 (M1T1) strain (Le Breton, et al., 2015); however, the construction of an insertional inactivation mutant of *vicR* in an MGAS5005 (M1T1) strain was successful (Liu, et al., 2006). Characterization of this strain revealed that VicR induces expression of 13 genes, including those of virulence factors like the Mac/IgG endopeptidase (Mac/IdeS), a

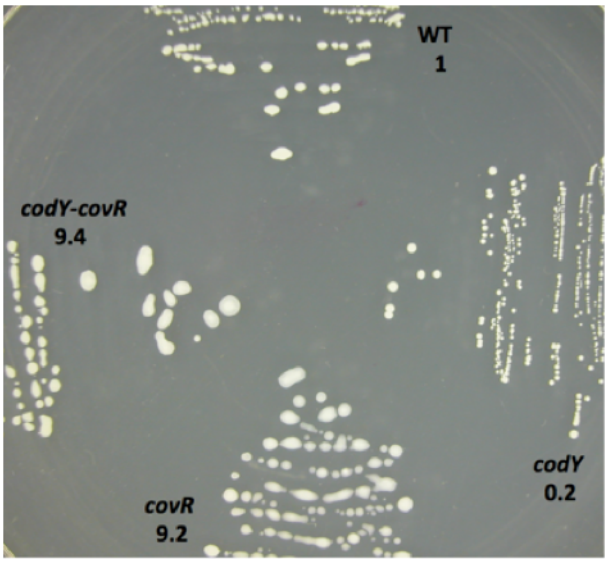

Figure 4. Comparison of the colony morphology of NZ131 *codY* and *covR* mutants: colonies of wild-type NZ131 were compared to that of the indicated mutants grown on C-agar (Loughman & Caparon, 2006b). Figures reflect relative amounts of capsule formation, i.e. transcript levels of *hasA*. Reproduced from (Malke, McShan, & Ferretti, 2009), with permission.

putative cell wall hydrolase gene (*pcsB*), and an operon that encodes a putative PTS mannose/fructose family EII (*spy1058-1060*) for carbohydrate transport. VicR also represses the expression of 5 genes, including ones that encode the osmoprotectant transporter OpuA (Liu, et al., 2006). The VicR-deficient strain showed no growth defects in rich culture media or any changes in susceptibility to phagocytic killing. However, the mutant grew poorly in non-immune human blood and serum and showed attenuated virulence in an intraperitoneal model of infection, which suggests that VicR/S influences virulence only in a specific host tissue environment. Admittedly, the insertional inactivation of *vicR* examined was unstable *in vivo* (Liu, et al., 2006). Nonetheless, further confirmation of the role of VicR/S as a transcriptional regulator influencing virulence through modulation of a metabolism-related gene came from deletion by allelic exchange of one of the genes in the PTS EII operon (*spy1060*) regulated by VicR, which recapitulates the attenuated virulence phenotype of the *vicR* insertional inactivation. Successful deletion of *vicS* partially replicates the growth defects and attenuation *in vivo* of the VicR-deficient strain (Liu, et al., 2006).

Virulence-related control of Carbohydrate and Amino acid metabolism (CovR/S, CcpA and Rgg/RopB)

Besides virulence genes and other regulators, the CovR regulon includes multiple genes involved in carbohydrate catabolism and nitrogen utilization (Graham, et al., 2002; Shelburne, et al., 2010; Dalton, Collins, Barnett, & Scott, 2006), and shares both virulence and metabolic targets for regulation with the CcpA regulon (Shelburne, et al., 2010; Shelburne, et al., 2008; Kinkel & McIver, 2008). Both CcpA and CovR influence transcription of *arcA*, which encodes a protein involved in arginine utilization, and *amyA*, which encodes an actively secreted carbohydrate degrading protein by binding with high affinity to the promoter regions of *arcA* and *amyA* (Shelburne, et al., 2010). Additional metabolic genes whose transcription is influenced by CovR and CcpA in an MGAS2221 (M1T1) strain are operons involved in sialic acid production (*spy0212-08*), cyclodextrin (*spy1062-67*) sucrose (*spy1538-43*) and lactose (*Lac.2*) transport and catabolism, a cellobiose (*spy1079-83*) transporter, as well as operons involved in arginine deiminase (*spy1271-75*) and histidine (*spy1770-78*) degradation pathways (Shelburne, et al., 2010). Additionally, CcpA was observed to affect other metabolic operons not influenced by CovR, such as the putative mannose/fructose PTS operon influenced by VicR in MGAS5005 (*spy1058-60*), and modulated expression of its metabolic operons to a greater degree than CovR.

This highlights a more influential role in metabolism by a metabolite responsive regulator, in comparison to that of a master regulator like CovR.

The transcriptional regulator Rgg/RopB, which influences expression of several virulence-associated proteins in a growth-phase dependent manner as described elsewhere, has been shown to affect both amino acid and carbohydrate catabolism genes. Examination of the catabolic activities of an NZ131 (M49) strain and an isogenic *rgg* mutant (NZ131*rgg*$^-$) during growth in complex and defined media revealed that, as opposed to the wild-type strain, the *rgg*-inactivated strain fermented arginine (even in the presence of glucose), metabolized serine and asparagine during the exponential phase of growth, and produced NH_3 and ornithine as a result (Chaussee, Somerville, Reitzer, & Musser, 2003). Altered metabolism of arginine and serine in the mutant was associated with increased transcription of genes that encode arginine deiminase (*arcA*) and a putative serine dehydratase (*sdhA*). Proteomic analysis of cytoplasmic content isolated from the NZ131*rgg*$^-$ strain during exponential and stationary phases of growth showed that the loss of Rgg/RopB function abrogated the growth phase-associated regulation of enzymes involved in the metabolism of arginine (ArcABC), histidine (HutI), and serine (SdhA), as evidenced by synthesis of these proteins in the exponential phase of growth in the NZ131*rgg*$^-$ strain, but not in the wild type, which only expressed them during the stationary phase (Chaussee, Callegari, & Chaussee, 2004). Additionally, proteins associated with thermal and oxidative stress responses, including ClpE and ClpL, were present in samples isolated from the NZ131*rgg*$^-$, but not in samples from the wild-type strain, which proved to be less tolerant to elevated temperature and puromycin. The previous findings were supported by comparison of wild type and NZ131*rgg*$^-$ transcriptomes using DNA microarrays, which identified genome-wide differences in transcript abundance during exponential and stationary phases of growth. The inactivation of *rgg* disrupted the coordinate expression of genes associated with the metabolism of non-glucose carbon sources (e.g. fructose, mannose, and sucrose), impairing the ability of NZ131*rgg*$^-$ to grow using these compounds as its primary/sole carbon source (Dmitriev, McDowell, Kappeler, Chaussee, Rieck, & Chaussee, 2006). Thus, growth phase-dependent regulation of virulence mediated by Rgg/RopB occurs in conjunction with modulation of the *S. pyogenes* metabolic program to use growth phase-appropriate energy sources (such as metabolizing glucose, instead of amino acids like arginine and serine, during exponential growth).

Metalloregulation and virulence (MtsR, PerR, CiaHR)

Transition metal ions are involved in a variety of biochemical processes in bacteria necessary for colonization, survival, and proliferation in their environmental niche, which present pathogens with the challenge of overcoming metal ion deprivation within host tissue environments. Metalloregulatory, or "metal-sensing" proteins have evolved in bacteria to mediate metal ion homeostasis by modulating the expression of genes that encode metal ion transport systems upon binding their cognate metal ion, and emerging literature indicates that, in pathogens like *S. pyogenes*, such proteins have also been adapted to act as virulence regulators (Merchant & Spatafora, 2014).

Such is the case for MtsR, a transcriptional regulator of the DtxR family of metalloregulators, expressed from a gene proximal to the free iron and manganese cation-specific transporter-encoding *mtsABC* operon (Hanks, et al., 2006). Examination of metalloregulatory activity in *S. pyogenes* revealed that the deletion of *mtsR* abolishes the Mn^{2+} and Fe^{3+}-induced repression of *mtsA* expression, as well as the Fe^{3+}-dependent decrease in expression of the heme-specific transporter gene (*htsA*) (Hanks, et al., 2006). Additionally, MtsR represses the streptococcal iron acquisition (*sia*) operon during cell growth under conditions of high metal levels by specifically binding the promoter regions of these genes in an iron- and manganese-dependent manner (Hanks, et al., 2006; Bates, Toukoki, Neely, & Eichenbaum, 2005), which clearly establishes the function of MtsR as a metal-responsive transcriptional repressor. The first observation that this metalloregulatory protein is involved in virulence gene regulation in *S. pyogenes* was made in an M3 serotype strain subclone (MGAS9887), which was isolated from a patient with an invasive infection, and that showed a decreased ability to cause necrotizing fasciitis (Beres, et al., 2006). DNA–DNA microarray hybridization-based comparative genomic resequencing and polymorphism

sequencing of the M3 isolates determined that MGAS9887 possesses a single base pair insertion in the *mtsR* gene just downstream of the start codon, which results in a truncated MtsR protein. The deletion of *mtsR* results in the deregulation of iron uptake, making *S. pyogenes* hypersensitive to both streptonigrin and hydrogen peroxide (Bates, Toukoki, Neely, & Eichenbaum, 2005) and attenuated for virulence in intramuscular and intraperitoneal models of infection (Bates, Toukoki, Neely, & Eichenbaum, 2005; Olsen, et al., 2010). Further characterization of the MtsR transcriptome in an *mtsR*-deficient MGAS315 (M3) strain revealed, in addition to the expected differential expression of genes involved in metal ion transport and oxidative stress, the repression of *prsA*, which encodes a peptidyl-prolyl isomerase (Olsen, et al., 2010). PrsA is required for proper maturation of the SpeB protease that plays an important role in the pathogenesis of necrotizing fasciitis. Isogenic mutants overexpressing PrsA or deficient in SpeB recapitulated the decreased necrotizing fasciitis phenotype of MtsR-deficient strains, which confirms the role of MtsR in regulating SpeB production.

The regulation of virulence genes by MtsR appears to vary among *S. pyogenes* serotypes and tissue sites of infection. Epidemiological studies ascribe a representative number of pneumonia, bacteremia, and synovitis cases to the naturally occurring *mtsR* mutant (MGAS9987), which indicates that the loss of MtsR regulation in an M3 serotype background only affects virulence in the context of necrotizing fasciitis (Merchant & Spatafora, 2014). Examination of an *mtsR* knockout strain in an NZ131 (M49) strain revealed differential expression of 64 genes involved in metal ion transport, nucleic acid synthesis, and regulation, many of which coincided with those affected in the M3 serotype strain (Toukoki, Gold, McIver, & Eichenbaum, 2010). However, there were a number of dysregulated transcripts exclusive to each strain. Among the MtsR-influenced virulence genes particular to the NZ131 strain were *mga, emm*, and *ska.* Further involvement of MtsR in *S. pyogenes* virulence relates to the management of oxidative stress, which, as detailed in another section of this chapter, must be overcome in order to colonize and persist in multiple host niches. Suffice it to say that the MtsR-dependent maintenance of iron homeostasis supports *S. pyogenes* virulence by aiding resistance to oxidative stress.

As previously indicated, the metalloregulator MtsR influences virulence gene expression in the context of necrotizing fasciitis in certain strains, in addition to directly repressing the transcription of metal transport operons (*mtsABC, htsABC* and *siaABCD*). However, virulence-related regulators besides MtsR also influence these operons. PerR is a transcriptional regulator primarily involved in oxidative stress resistance, a role described in another section of this chapter. PerR activity as a peroxide-responsive transcriptional regulator is inextricably linked to its function as a metalloregulator, as indicated by decreased iron uptake into *S. pyogenes* cells and reduced transcription of *mtsA* from the *mtsABC* operon in a PerR-deficient AP1 (M1) strain (Ricci, Janulczyk, & Björck, 2002). In an M3 isolate, PerR was shown to repress the three known iron and heme acquisition systems of *S. pyogenes*: the MtsABC iron transporter (Janulczyk, Pallon, & Björck, 1999; Sun, Ge, Chiu, Sun, & He, 2008), the heme/ferrichrome transport system (FhuGBDA or FtsDCBA) (Hanks, et al., 2006), and the Shr/Shp/SiaABCD (also dubbed HtsABC) transporter involved in hemoglobin acquisition (Bates, Montañez, Woods, Vincent, & Eichenbaum, 2003; Lei, et al., 2003; Lei, et al., 2002; Grifantini, Toukoki, Colaprico, & Gryllos, 2011). At the same time, PerR up regulated genes of the AdcRCB transport complex involved in Mn^{2+}/Zn^{2+} import in streptococci (Grifantini, Toukoki, Colaprico, & Gryllos, 2011; Dintilhac & Claverys, 1997). The role that regulation of these metal cation transporters has in oxidative stress responses relating to virulence is specified later.

Host environment responsive regulators

As has been indicated throughout this chapter, multiple transcriptional regulators of *S. pyogenes* influence their targets in a strain-specific manner (such as in RALPs (Kreikemeyer, Beckert, Braun-Kiewnick, & Podbielski, 2002)) or a tissue site-specific manner (such as in MtsR, Mga, or VicR). Here we describe the role of different *S. pyogenes* virulence-regulators in the context of the host environments and responses in which experimental data indicate that they are of the greatest relevance to colonization and survival.

Oxidative and Acid Stress

In addition to the proteins that co-opt host processes for the pathogen's benefit or subvert host immune responses, virulence factors also include proteins that function to protect the bacterium from environmental stressors in the host, especially those resulting from mechanisms of host immunity. *S. pyogenes* produces a variety of proteins to sense and respond to oxidative and acid stresses, the chemical stressors primarily encountered within its human host, and both dedicated and general regulators tightly control expression of these proteins.

PerR

Oxidative stress is the product of exposure to reactive oxygen species (ROS) that are generated from atmospheric oxygen or produced by a phagocyte oxidative burst. These include superoxide anions (O_2^-), hydrogen peroxide (H_2O_2), and highly toxic hydroxyl radicals (OH^-), all of which can damage nucleic acids, proteins, and cell membranes. Like other lactic acid bacteria, *S. pyogenes* can also produce peroxide endogenously as a byproduct of carbohydrate metabolism (Gibson & Caparon, 1996) and consequently can generate hydroxyl radicals in an iron-rich environment as a result of the Fenton reaction (Tsou, et al., 2008). As a result, bacteria have evolved distinct inducible responses to handle peroxide and superoxide stress (Tsou, et al., 2008; Farr & Kogoma, 1991). Superoxide dismutase (SodA) mitigates superoxide stress by converting O_2^- into H_2O_2 and O_2 (Fridovich, 1997; Gerlach, Reichardt, & Vettermann, 1998), and a critical role for SodA in the aerobic growth of *S. pyogenes* has been proposed (Gibson & Caparon, 1996). However, *S. pyogenes* does not encode catalase, so peroxidases like alkyl hydroperoxidase/alkyl hydroperoxide reductase (*ahpCF*), and glutathione peroxidase (*gpoA*) act to neutralize endogenous and exogenous peroxides, which contribute to its aerotolerance and ROS detoxification *in vitro* (Brenot, King, & Caparon, 2005; King, Horenstein, & Caparon, 2000). Iron chelating proteins required for iron homeostasis, such as the iron/DNA-binding protein Dpr/MrgA (Dps-like peroxide resistance protein), have also been shown to contribute to virulence through their involvement in resistance against oxidative DNA damage (Tsou, et al., 2008; Brenot, King, & Caparon, 2005); by chelating free intracellular Fe^{2+}, Dpr/MrgA prevents its reaction with peroxide (Fenton reaction), which minimizes the production of highly reactive hydroxyl radicals. However, the individual contributions of these factors to bacterial fitness *in vivo* is only moderate, as revealed by subcutaneous and intraperitoneal infection of mice with *ahpC*- and *gpoA*-deficient strains (Brenot, King, & Caparon, 2005; Brenot, King, Janowiak, Griffith, & Caparon, 2004). As indicated above, control of iron metabolism is also part of the oxidative stress response in *S. pyogenes*, since iron is both an essential cofactor and, when unregulated, a hazardous metal that participates in the production of ROS (Ricci, Janulczyk, & Björck, 2002; Tsou, et al., 2008).

Control of the peroxide stress response in *S. pyogenes* occurs mainly through the transcriptional regulator PerR, a homolog of the H_2O_2- and metal ion-responsive ferric uptake regulator (Fur) and other Fur-like proteins found in *Bacillus subtilis* and *Staphylococcus aureus* (Bsat, Herbig, Casillas-Martinez, Setlow, & Helmann, 1998; Horsburgh, Clements, Crossley, Ingham, & Foster, 2001). PerR was first identified in *S. pyogenes* as required for an inducible peroxide resistance response (King, Horenstein, & Caparon, 2000). Characterization of a PerR-deficient AP1 (M1) strain in which the DNA- and metal-binding domains of *perR* were deleted revealed that PerR influences iron metabolism and acts as a repressor of the peroxide response (Ricci, Janulczyk, & Björck, 2002). Further analysis revealed that the loss of PerR expression results in highly attenuated virulence in both subcutaneous and intraperitoneal models of infection (Brenot, King, & Caparon, 2005), is associated with reduced resistance to phagocytic killing in human blood and by murine macrophages *in vitro*, and produces severely attenuated virulence in a baboon model of *S. pyogenes* pharyngitis (Gryllos, et al., 2008b). PerR-dependent gene expression appears to be linked to resistance to the phagocyte oxidative burst, as increased phagocytic killing of a *perR*-deficient strain was abrogated in the presence of diphenyleneiodonium chloride (DPI), a general oxidative burst inhibitor (Gryllos, et al., 2008b).

Dissection of the PerR regulon of *S. pyogenes* initially yielded confounding results. Unlike the PerR protein of *Bacillus subtilis* that up-regulates the expression of genes that encode peroxidases (e.g. *ahpC*) (Helmann, et al., 2003; Mostertz, Scharf, Hecker, & Homuth, 2004), some peroxide responsive transcripts in *S. pyogenes* were directly repressed by PerR via binding of their promoter regions (e.g. *mrgA*) (Brenot, King, & Caparon, 2005). In contrast, other genes (e.g. *ahpCF*, *sodA*) were simultaneously expressed in both a PerR-dependent (Gryllos, et al., 2008b) and PerR-independent (Grifantini, Toukoki, Colaprico, & Gryllos, 2011) manner. Additional transcripts repressed in a PerR- and/or peroxide-dependent manner included genes involved in carbohydrate metabolism (such as the Lac.2 operon), DNA metabolism (such as purine synthesis loci *purM, purN,* and *purE*), metal homeostasis (such as *siaABC, mtsABC,* and *pmtA*), and amino acid/peptide transport (such as the oligopeptide permease operon, *oppABCDF*) (Grifantini, Toukoki, Colaprico, & Gryllos, 2011; Gryllos, et al., 2008b). The reason for these seemingly contradictory results is the fact that the PerR-dependent expression was examined by comparing the transcriptomes controlled by PerR in peroxide (Grifantini, Toukoki, Colaprico, & Gryllos, 2011) with those identified by comparison of wild-type and *perR*-deficient transcriptomes in the absence of stress (Gryllos, et al., 2008b), which suggests that the oxidative state of the cell influences the extent of the PerR regulon. This was further suggested by an analysis of metal binding in PerR activity, which revealed that PerR transcriptional regulation is dependent on iron for maximal sensitivity to peroxide (Grifantini, Toukoki, Colaprico, & Gryllos, 2011). Characterization of the crystal structure of PerR combined with mutational analysis demonstrated that metal binding by PerR occurs at a structural site and a distinct regulatory site, the latter of which is critical to *S. pyogenes* virulence (Makthal, et al., 2013). Metal binding at the regulatory site is not required for DNA binding per se; rather, it promotes higher affinity PerR/DNA interactions and PerR conjugated with iron (as opposed to manganese) at this site responds to peroxide stress directly and dissociates from operator sequences.

Given that PerR does not upregulate any known peroxidase(s) or MrgA, and that PerR represses DNA and protein metabolism as well as iron/heme uptake, a mechanism was proposed for the existence of dual PerR regulons (Grifantini, Toukoki, Colaprico, & Gryllos, 2011). In this model, a transient decrease in gene expression and decreased metabolic function upon oxidative challenge would allow for DNA and protein damage repair before normal transcription resumes. Reduced iron/heme uptake (from *siaABC, mtsABC* repression) coupled to increased Mn^{2+} import, as indicated by observed PerR-dependent up regulation of the *adc* operon (Grifantini, Toukoki, Colaprico, & Gryllos, 2011), would lead to the replacement of Fe^{2+} as an enzymatic cofactor with the Fenton-insensitive Mn^{2+}, thereby permitting optimal bacterial metabolism in oxidative environments. This would also translate into increased levels of Mn^{2+}-bound PerR and an ensuing gradual decrease in transcriptional responses, initially driven by the oxidation of Fe^{2+}-bound PerR species in the presence of peroxide. As a result of PerR presenting different modes of metal-dependent DNA binding, PerR appears to control two different regulons in both the presence and absence of oxidative stress (Grifantini, Toukoki, Colaprico, & Gryllos, 2011; Gryllos, et al., 2008b; Makthal, et al., 2013).

CiaH/R

Recent mutational analysis of the sensor proteins of TCSs in *S. pyogenes* uncovered the involvement of the CiaH sensor kinase in acid and oxidative stress (Tatsuno, Isaka, Okada, Zhang, & Hasegawa, 2014). A *ciaH*-deficient M1 clinical isolate (1529) was significantly sensitive to hydrogen peroxide and displayed reduced survival when grown in acidic media (pH 6.0) in an atmosphere with 5% CO_2. Though full characterization of the CiaH/R regulon is pending, an interesting result of this study was the observation that loss of *ciaH* under the conditions tested resulted in decreased expression of *nrdR*, a regulator of ribonucleotide reductase genes, and *polA1*, a putative DNA polymerase I previously reported to contribute to peroxide stress defenses in *S. pyogenes* (Tatsuno, Isaka, Okada, Zhang, & Hasegawa, 2014; Toukoki & Gryllos, 2013). A prior study examining CiaH/R activity in an M49 serotype strain found that the loss of *ciaH* expression resulted in the increased transcription of multiple divalent cation transporters and six transcripts of genes generally involved in stress response or genetic competence (*arcA*, *csp*, *dnaK*, *epuA*, *hsp33*, and *recA*), while 11 members of the PTS and four genes that encode

ribosomal proteins showed decreased transcript amounts, with maximal expression of CiaHR occurring during the transition phase of growth (Riani, et al., 2007). Among virulence-related genes of this strain background, the expression of hemolysin (*hlyX*), hyaluronidase (*hylA*), and protein F2 (*prtf2*) were increased, while *sfbX* and DNase (*mf2*) transcripts were decreased in the *ciaH* mutant. Nonetheless, characterization of the CiaH/R regulon *in vivo* or under stress conditions remains to be performed.

Virulence-related biofilm regulation (Srv, Rgg2/3)

Srv

The streptococcal regulator of virulence (Srv) was first identified, as its name implies, when disruption of the *srv* gene encoding it in the MGAS5005 (M1T1) strain resulted in significantly attenuated virulence in a murine intraperitoneal (*i.p.*) model of infection (Reid, Montgomery, & Musser, 2004). Srv is a member of the Crp/Fnr family of regulators, with high amino acid homology to the PrfA transcriptional virulence activator of *Listeria monocytogenes*, and its inactivation in MGAS5005 results in a drastically altered *S. pyogenes* transcriptome during exponential growth (Reid, et al., 2006). Loss of Srv reduces expression of genes that code for proteins involved in translation, posttranslational modification, protein turnover, and amino acid and lipid transport and metabolism. Other transcriptional regulators like CovR/S and Mga were down regulated in the Srv-deficient mutant during exponential growth, and multiple virulence-factor encoding genes were dysregulated, including *speC*, *ska*, *scpA*, *hma*, and most significantly, *sic* and *speB*, whose protein products were reduced and increased, respectively (Reid, et al., 2006). Further investigation of the phenotypic effects of *srv* inactivation revealed that absent the expression of Srv, MGAS5005 exhibits significant reduction in biofilm formation under both static and flow culture conditions (Doern, et al., 2009). This biofilm phenotype was directly linked to the previously observed constitutive production of SpeB protease that results from *srv* disruption, as the inhibition of SpeB protease activity restored biofilm formation in the Srv-deficient strain.

Biofilm are 3-dimensional communities of bacteria encased in a protective matrix of extracellular protein, DNA, and polysaccharide(s) that bacteria will form when colonizing a host or surface, primarily to defend against environmental insults (such as antibiotics, chemical stressors, or agents of host immunity) (Ogawa, et al., 2011). The role in host colonization and disease, as well as the mechanisms of assembly or dispersal of these structures in *S. pyogenes*, will be detailed elsewhere; for the purposes of this chapter, MGAS5005 and other strains have been observed to reside within biofilms both *in vitro* and *in vivo*. Characterization of Srv-dependent biofilm formation showed that a *srv*-deficient strain (MGAS5005Δ*srv*) produces significantly larger lesions than wild-type bacteria in infected animals. This phenotype stems from readily detectable levels of SpeB protease and the consequent lack of biofilm formation in infected tissue, since both the chemical inhibition of SpeB and genetic inactivation of *speB* in a MGAS5005Δ*srv* strain abrogates the severe lesion phenotype (Connolly, Roberts, Holder, & Reid, 2011a). Most intriguing is the fact that Srv-mediated control of SpeB production and, by extension, biofilm formation, is independent of CovR/S-mediated SpeB regulation. While the loss of a functional CovS is associated with decreased SpeB production, due to CovR repression of *speB* transcription (Treviño, et al., 2009; Sumby, Whitney, Graviss, DeLeo, & Musser, 2006), constitutive SpeB production and biofilm dispersal due to inactivation of *srv* is observed in both CovS-inactive (MGAS5005) and CovS-active (clinical M1 isolate RGAS053 and M3 serotype MGAS315) strains (Connolly, Braden, Holder, & Reid, 2011b). Based on these data, a model of Srv function in *S. pyogenes* pathogenesis proposes that Srv control of SpeB production may be a mechanism to regulate biofilm dispersal, and could provide a means by which localized colonization can disseminate to other tissue sites, as well as a transition from a mild infection to severe invasive disease. However, in an entirely different NZ131 (M49) background a different set of transcriptional regulators (Rgg2/3) appears to play a primary role in *S. pyogenes* biofilm formation.

Rgg2/3

These proteins are members of the Rgg family of transcriptional regulators, which are widespread among low-G+C Gram-positive bacteria (Firmicutes) and represented by several paralogs within each species (Federle M. , 2012; Cook & Federle, 2014). Rgg2 and Rgg3 are the first quorum-sensing receptors that influence virulence described in *S. pyogenes* (Chang, LaSarre, Jimenez, Aggarwal, & Federle, 2011), and will be detailed in a subsequent section. Limiting the scope of description here to the effect Rgg2 and Rgg3 have on biofilm formation; the addition of synthetic analogs of the pheromones sensed by Rgg2/3 to cultures of M49 NZ131 bacteria resulted in a near doubling of biofilm biomass (Federle, 2012; Chang, LaSarre, Jimenez, Aggarwal, & Federle, 2011). Rgg2 was required to produce such biofilms, in conjunction with the oligopeptide permease (Opp), and Rgg2-mediated biofilm formation in response to pheromone was greatly enhanced in a strain deficient in Rgg/RopB, an activator of SpeB protease described next. As indicated before, SpeB was shown to facilitate *S. pyogenes* biofilm dispersal, but whether the protease also interferes with peptide signaling of Rgg2/3 and consequent biofilm induction is unknown. Therefore, it appears that the Rgg2/3 circuit promotes biofilm development, while a separate circuit (namely, Rgg/RopB-dependent SpeB production) prevents or disassembles biofilms (Federle, 2012).

Growth phase/quorum sensing-mediated virulence gene control (Rgg/RopB, Rgg2/3, Sil, TrxSR)

A theme running through this chapter is that *S. pyogenes* continuously senses the conditions of its surrounding environment and simultaneously regulates the expression of its genomes to maintain and enhance its survival. Batch culture is the simplest method to investigate such bacterial adaptation to the depletion of sources of energy and resources for growth, as well as the accumulation of metabolic end products. *S. pyogenes* exhibits two primary growth phases in culture: exponential and stationary. The former is characterized by preferential use of glucose as the source of energy that is fermented to lactic acid, continuous cell division, expansion of the bacterial population and the expression of a regulatory program corresponding to the prevailing metabolic status of the cell (Chaussee, Dmitriev, Callegari, & Chaussee, 2008). The stationary phase of growth is evidenced by the depletion of glucose, acidification of the media, the arrest of cell division, and population growth and transition into a radically different regulatory program. Examination of the growth phase-dependent changes in gene expression in *S. pyogenes* has revealed that, unlike other bacteria that employ a variety of alternative sigma factors to modulate gene expression in response to changes in the environment (Helmann & Moran, Jr., 2001; Beyer-Sehlmeyer, Kreikemeyer, Hörster, & Podbielski, 2005), *S. pyogenes* alters the growth phase-associated patterns of gene expression through interactions among transcriptional regulators. Therefore, growth phase-dependent regulation of *S. pyogenes* virulence involves global regulators (such as CovR/S or RALPs), metabolite responsive regulators (namely, CodY and Mga) and dedicated regulators that have been observed to have genome-wide effects in response to changing growth phases.

One such dedicated virulence regulator, which was first identified by its influence on growth-phase dependent SpeB production, is the Rgg-family transcriptional regulator Rgg/RopB, which induces maximal expression of the extracellular SpeB protease (Chaussee, Ajdic, & Ferretti, 1999; Lyon, Gibson, & Caparon, 1998) during stationary phase growth (Unnikrishnan, Cohen, & Sriskandan, 1999). Proteomic and qPCR approaches to analyze the influence of Rgg/RopB on the expression of other extracellular proteins in a SpeB-deficient NZ131 (M49) strain found that, during the stationary phase of growth, the loss of Rgg/RopB expression led to a decreased transcription of genes that encode lysozyme, autolysin and ClpB (a heat shock protein), but produced more streptodornase (DNase encoded by *sdb*/*mf*) and a DNA entry nuclease (Chaussee, Watson, Smoot, & Musser, 2001). As described in an earlier section, transcriptome analysis using DNA microarrays revealed that Rgg/RopB affects the stationary-phase expression of amino acid metabolic genes. Further characterization of *rgg* inactivation in the NZ131 (M49) strain found Rgg/RopB also controls amino acid and non-glucose carbohydrate metabolism genes (Chaussee, Somerville, Reitzer, & Musser, 2003; Chaussee, Callegari, & Chaussee, 2004;

Dmitriev, McDowell, Kappeler, Chaussee, Rieck, & Chaussee, 2006). These studies also uncovered that transcripts that encode virulence factors involved in cytolysin-mediated translocation of NAD-glycohydrolase (*spn*), including the immunity factor (*ifs*) and streptolysin O (*slo*), were more abundant in the *rgg*-deficient strain, which correlated to increased levels of NADase (SPN) and SLO activity in both exponential and stationary growth phases (Dmitriev, McDowell, Kappeler, Chaussee, Rieck, & Chaussee, 2006). These data suggested that Rgg/RopB both directly and indirectly exerts growth-phase dependent control of virulence proteins and that it represses the transcription of *spn*, *ifs*, and *slo* during the exponential phase of growth, as well as controlling the degradation of the secreted proteins during the stationary phase by inducing the expression of the SpeB protease.

Growth-phase–dependent changes in gene expression in *S. pyogenes* involve connections between Rgg and more globally acting regulatory networks. The inactivation of *rgg* in NZ131 (M49) increases the transcription of several virulence-associated genes, including *speH* (which encodes a superantigen), *scl1/sclA* (which encodes an adhesion protein), *ska*, *hasABC*, *mf*, *mf3*, *grab*, and *mac*, in addition to targets of the Mga regulon (*emm*, *scpA*, *sagA*, and *slo*) (Chaussee, et al., 2002). The altered expression of these virulence genes correlates with to changes in the transcription of several regulatory genes, including *mga*, *covR/S*, and *fasBCAX;* indicating that Rgg/RopB influences other global regulators to exert its genome-wide modulation of virulence. Further evidence of this is in the increased transcription of the TCS genes *lytR* and *lytS* in an *rgg*-deficient strain during the exponential growth phase; in the stationary phase of growth, the transcripts of FasB, CpsY, and the RALP4 (RivR) regulator were less abundant in the mutant strain (Dmitriev, McDowell, Kappeler, Chaussee, Rieck, & Chaussee, 2006). However, interserotype and intraserotype variations are a common feature of the Rgg/RopB regulon. The sequencing of *rgg*/*ropB* in 171 invasive serotype M3 strains identified 19 distinct alleles (Carroll, et al., 2011). The observed *rgg*/*ropB* polymorphisms alter regulator function, as inactivation of the gene in strains producing distinct Rgg/RopB variants had dramatically divergent effects on global gene expression. Isoallelic *S. pyogenes* strains that differed by as little as a single amino acid in Rgg/RopB all exhibited differing transcript levels of *speB*. Comparison of parental, *rgg*/*ropB*-inactivated, and *rgg*/*ropB* isoallelic strains in mouse infection models manifested differences in virulence and disease manifestations (Carroll, et al., 2011). Additionally, *rgg*-deficient mutants in three different strains, which represented M1 (SF370 and MGAS5005) and M49 (CS101) serotypes, all exhibited different changes in gene expression in the stationary phase of growth (Dmitriev, McDowell, & Chaussee, 2008). Specifically, the inactivation of *rgg* alters transcription of regulatory genes in SF370 and CS101 strains, but not in MGAS5005. These studies identified *speB* and an adjacent hypothetical gene (*spy2040*) as the only transcripts similarly affected by *rgg* inactivation in all three strains. Nevertheless, a strain-specific Rgg/RopB subregulon was also detected, which could vary by as little as a single gene (*nrdD*) in the MGAS5005 strain, and by as much as 43 genes in SF370 (Dmitriev, McDowell, & Chaussee, 2008).

Given such variation among strains for Rgg/RopB-dependent gene regulation, it was important to define the mechanism of Rgg/RopB-mediated control of transcription in *S. pyogenes*. Chromatin immunoprecipitation followed by DNA microarray (ChIP-chip) analysis identified 65 DNA binding sites for Rgg in the NZ131 chromosome: 35 of them within noncoding DNA and 43% of which were adjacent to genes previously identified as regulated by Rgg (Anbalagan, McShan, Dunman, & Chaussee, 2011). EMSA demonstrated Rgg binding to noncoding DNA upstream of *speB*, and the genes that encode PulA, Spd-3, a predicted transcriptional regulator (*Spy49_1113*), prophage-associated genes that encode a putative integrase (*Spy49_0746*) and a surface antigen (*Spy49_0396*), both *in vivo* and *in vitro*. Rgg-mediated transcriptional regulation of these genes via an active promoter was confirmed using a luciferase reporter system (Anbalagan, McShan, Dunman, & Chaussee, 2011). Further characterization of Rgg-mediated promoter activation found that Rgg is unable to bind to the *speB* promoter to induce transcription when bound to LacD.1 during exponential growth, but as levels of glycolytic intermediates decrease upon entry into stationary phase, a change in LacD.1 conformation results in dissociation from Rgg and activation of SpeB expression (Loughman & Caparon, 2006a). This LacD.1-dependent effect on the DNA binding specificity of Rgg in response to changes in environmental carbohydrates was not limited to SpeB production, as demonstrated for *emm49*, *sof*, *sfbX49*, and *speH* genes in NZ131 (M49)

using *in vitro* DNA-binding and *in vivo* transcriptional fusion assays (Anbalagan, Dmitriev, McShan, Dunman, & Chaussee, 2012). These results further support the paradigm that growth-phase–dependent gene modulation in *S. pyogenes* is based on the interactions of multiple transcriptional regulators, which vary greatly among strains, and which necessitates further examination of the manner in which regulatory networks are structured in different serotypes in order to understand strain differences and similarities when infecting a host.

Rgg2/3

Recent research into transcriptional regulation by Rgg homologs in streptococci suggests that Rgg-family regulators also serve as cytoplasmic receptors for intercellular signaling peptides that resemble the quorum-sensing pathways of other Gram-positive bacteria (Fontaine, et al., 2010; Ibrahim, et al., 2007a; Mashburn-Warren, Morrison, & Federle, 2010). Signaling peptides, which are commonly called autoinducers or pheromones, are detected either extracellularly by transmembrane sensor kinases, or intracellularly by import to the cytoplasm, where they engage a signal transduction pathway or a transcription factor to influence gene regulation (reviewed in (Cook & Federle, 2014)). Such signaling peptide-based intercellular communication (such as quorum sensing) among Firmicutes is an established paradigm, according to which, ribosome-dependent polypeptides are produced as inactive pro-peptides, are secreted from the cell by the general secretory (Sec) system or through designated ABC transporters, and are processed into active signaling molecules by a variety of proteases (Cook & Federle, 2014). The newly-defined activity for Rgg proteins in streptococci as quorum sensing receptors comes from experimental analysis of genes that encode Rgg members and adjacent open reading frames (ORFs) that encode short, secreted peptides, as exemplified by the *S. mutans* and *S. thermophilus* ComR protein, which, together with the short peptide ComS, positively regulates competence development (Ibrahim, et al., 2007a; Mashburn-Warren, Morrison, & Federle, 2010). Including Rgg/RopB, *S. pyogenes* has four identifiable genes encoding Rgg-family proteins: *comR* (*spy49_0032*), *rgg/ropB* (*spy49_1691*), *rgg2* (*spy49_0415*), and *rgg3* (*spy49_0449c*) (Chang, LaSarre, Jimenez, Aggarwal, & Federle, 2011). Additionally, the analysis of unannotated small ORFs throughout Gram-positive genomes identified ORFs coding for short hydrophobic peptides (*shp*) adjacent to both *rgg2* and *rgg3* in every sequenced *S. pyogenes* genome (Ibrahim, et al., 2007b). The *shp* genes were shown to encode 22 and 23 amino acid peptides that are processed to mature pheromones, that serve to induce expression of the related neighboring genetic loci (i.e. *rgg2/3*), and that positively regulate their own transcription in a NZ131 (M49) strain (Chang, LaSarre, Jimenez, Aggarwal, & Federle, 2011). As mentioned above, the expression of the *shp* genes promotes biofilm biogenesis in *S. pyogenes*, an activity mediated by the regulatory activity of Rgg2 and Rgg3. The function of the peptides depends on a trans-membrane peptidase (Eep) for processing, and the oligopeptide permease (Opp) for importation (Chang, LaSarre, Jimenez, Aggarwal, & Federle, 2011), as previously observed in other Gram-positive bacteria (Solomon, Su, Shyn, & Grossman, 2003; Doeven, Abele, Tampé, & Poolman, 2004). Though Rgg2/3 lack recognizable primary-sequence similarity to any other quorum-sensing components, structural prediction algorithms reveal a potentially similar secondary and tertiary structure to PlcR and PrgX, two prototypical members of the RNPP protein family found throughout Gram-positive bacteria that serve as receptors for imported signaling peptides (Federle, 2012). Rgg2 and Rgg3 each control the transcription of the promoters driving that drive both *shp* genes, but do so with antagonistic activities. In the absence of pheromones, Rgg3 binds to DNA at both *shp* promoters and represses their transcription; but when pheromones are present, Rgg3 releases DNA and transcription initiates (Chang, LaSarre, Jimenez, Aggarwal, & Federle, 2011). Rgg2 appears to act only as a transcriptional activator when the *shp*-encoded pheromones are transported into the cell. Regulation of the peptide-responsive promoters by the antagonist Rgg2 and Rgg3 transcriptional regulators involves competition for highly conserved, shared binding sites located proximal to the −35 nucleotide in the target promoters, which results in concentration-dependent, exclusive occupation of the binding site that can be skewed in favor of Rgg2 *in vitro* by the presence of the pheromones (Lasarre, Aggarwal, & Federle, 2013). Therefore, the net response to pheromones is to induce *shp* and *rgg2/3* promoter expression, which results in amplification through a positive feedback loop. These findings provide further evidence that the Rgg family of transcriptional regulators function

as quorum-sensing effector proteins and comprise the first functional quorum-sensing pathway conserved in *S. pyogenes*.

Sil

The streptococcal invasion locus, or Sil, is a bacteriocin-like peptide-based quorum sensing system identified in approximately 25% of *S. pyogenes* strains (Michael-Gayego, Dan-Goor, Jaffe, Hidalgo-Grass, & Moses, 2013). This locus was originally identified by a transposon insertion into the *silC* gene of the JS95 (M14) strain that resulted in attenuated virulence in a mouse model of necrotizing fasciitis. Sil is composed of genes encoding a TCS (*silAB*), an ABC transporter (*silDE*) and the *silC*/*silCR* locus (Hidalgo-Grass, et al., 2002). The *silCR* locus, which overlaps a majority of the *silC* gene but is transcribed from the reverse strand, encodes the peptide pheromone silCR. The SilAB and silDE/CR polypeptides appear to be co-transcribed from two promoters, termed P1 and P3 respectively, with the latter induced upon addition of SilCR in a SilA-dependent fashion, which controls several putative bacteriocin-related genes (Eran, et al., 2007; Belotserkovsky, et al., 2009). As indicated before, the Sil system is not present or functional in a majority of *S. pyogenes* strains. SilC is not found in M1 or M3 serotype strains; the ATG start codon has been mutated in M14 strains and the putative pheromone transporter SilD is truncated in M18 strains (Hidalgo-Grass, et al., 2004). Though SilCR was originally reported to limit infection by promoting neutrophil-mediated clearance and preventing systemic spread in mice inoculated with an M14 strain (Hidalgo-Grass, et al., 2004), a conflicting study later found that the addition of the SilCR peptide induced expression of *scpC*, *sagA*, and *siaA in vitro* and of the latter two genes *in vivo* in an M1 strain, which resulted in impaired lesion healing in infected mice (Salim, de Azavedo, Bast, & Cvitkovich, 2008). These conflicting results and the paucity of *S. pyogenes* strains with an active Sil indicate that its function in virulence may be either strain- or serotype-specific.

TrxSR

Another transcriptional regulator recently linked to quorum sensing in *S. pyogenes* is the TCS encoded by the *trxSR* operon. Originally studied due to its homology to a virulence-related TCS in *S. pneumoniae*, an insertional mutation in the response regulator gene, *trxR*, in a MGAS5005 (M1T1) strain led to a significant reduction in lesion size, lesion severity, and lethality in a murine skin infection model (Leday, et al., 2008). Examination of TrxR activity showed that CovR directly represses the *trxR* promoter *in vitro* and TrxR induces transcription of Mga-regulated virulence genes by activation of P*mga*, as demonstrated by analysis of a luciferase reporter fusion. Complementation of the *trxR*-deficient MGAS5005 mutant restored expression of Mga regulon genes and restored virulence in the mouse model to wild type levels (Leday, et al., 2008). Investigation of Sil activity during adherence to host cells uncovered that secretion of Slo and Sls produces endoplasmic reticulum stress, which in turn results in increased asparagine (ASN) synthetase expression and production of ASN (Baruch, et al., 2014). Sensing of the released ASN by both a JS95 (M14) and a MGAS5005 (M1) strain alters the expression of ~17% of *S. pyogenes* genes, of which about one-third are regulated by TrxSR. Given that MGAS5005 lack an active Sil, the induced expression of the streptolysin toxins and the down-regulation of proliferation-related transcripts in the absence of ASN was determined to be TrxSR-dependent (Baruch, et al., 2014). These results were further confirmed by the loss of ASN-mediated activation of Sil in a *trxR*-deficient JS95 strain, the loss of ASN-mediated regulation of TrxSR in a *trxR*-deficient MGAS5005 strain, and the observation that treatment with asparaginase both arrests *S. pyogenes* growth in human blood and blocks proliferation in a mouse model of human bacteremia. Therefore, TrxSR appears to constitute a quorum sensing-like system in *S. pyogenes*, which regulates gene expression in response to sensed ASN produced by host cells under stress.

FasBCA/X

The fibronectin/fibrinogen binding/hemolytic activity/streptokinase (Fas) regulator of *S. pyogenes* was identified in the M49 serotype strain, based on homologies to the histidine protein kinase (HPK) and response regulator components of *Staphylococcus aureus* (Agr) and *S. pneumoniae* (Com) quorum-sensing systems (Kreikemeyer,

Boyle, Buttaro, Heinemann, & Podbielski, 2001). Despite their homology to the Com system, auto-inducing peptides thus far detected in culture supernatants do not function with the Fas regulator (Chang, LaSarre, Jimenez, Aggarwal, & Federle, 2011; Belotserkovsky, et al., 2009), which makes this unlikely to be another quorum-sensing system. The *fasBCA* operon is found in all tested M serotypes and is transcribed as a polycystronic message that expresses two predicted HPKs (FasB and FasC) and one response regulator (FasA). Insertional mutagenesis that disrupts *fasBCA* and *fasA* revealed that the expression of the Fas operon down-regulates transcription of genes that encode *S. pyogenes* adhesins (*fbp54*, *mrp*) and concomitantly induces the expression of genes that encode secreted virulence factors (*sagA* and *ska*) in a growth-phase-dependent manner (Kreikemeyer, Boyle, Buttaro, Heinemann, & Podbielski, 2001). A FasA-dependent 300 nucleotide monocistronic transcript that does not encode a peptide sequence, designated *fasX*, was also identified downstream of *fasBCA*. Deletion mutagenesis of *fasX* resulted in a similar phenotype to that of the *fasBCA* or *fasA* insertional mutations, whereas complementation in trans of the *fasX* deletion restored the wild-type *fasBCA* regulation, which suggests that *fasX*, encodes a putative non-translated RNA that is the main effector molecule of the Fas regulon (Kreikemeyer, Boyle, Buttaro, Heinemann, & Podbielski, 2001). Two virulence factor-encoding mRNAs have been confirmed as targets of FasX regulation, with the first being streptokinase (*ska*), which FasX base-pairs to at the first nine nucleotides of the *ska* mRNA in an interaction that increases the stability of the mRNA, and which results in a 10-fold increase in *ska* transcript abundance and streptokinase protein expression (Ramirez-Peña, Treviño, Liu, Perez, & Sumby, 2010). FasX must remain bound to its mRNA target to enhance stability, and the reversible nature of the FasX:*ska* mRNA interaction implies that that induction by FasX can be attenuated by decreasing FasX transcription or increasing FasX turnover. The other confirmed FasX-regulated virulence factor is the first gene in the pilus biosynthesis operon, *cpa*, which encodes the collagen-binding minor pilus protein located at the pilus tip (Liu, Treviño, Ramirez-Peña, & Sumby, 2012). FasX base pairs to 16 of the first 17 nucleotides of *cpa* mRNA in order to repress pilus biosynthesis gene expression by inhibiting *cpa* mRNA translation and reducing the access of ribosomes to the *cpa* mRNA ribosome binding site. Such opposing regulatory activity on adhesins and streptokinase, a known agent of *S. pyogenes* dissemination, suggests that FasX is a virulence regulator that modulates the transition from localized colonization to systemic spread (Miller, Cao, Pflughoeft, & Sumby, 2014). Further evidence of this is the observation that complementation of a hypervirulent M3 strain to correct a naturally occurring mutation in *fasC* significantly reversed the streptokinase activity characteristic of the strain by enhancing the levels of FasX and increasing *ska* transcription (Cao, et al., 2014). Examination of the consequence of said complementation on virulence is pending, but results will hopefully shed further light on the role of the Fas regulator in virulence.

Saliva

Multiple studies have examined virulence factor expression and transcriptional changes of *S. pyogenes* in body fluids, including saliva (Graham, et al., 2005; Shelburne, et al., 2005a; Shelburne, 3rd, et al., 2005b). An MGAS5005 strain exposed to human saliva produces a variety of factors in a growth-phase dependent manner, including Sic, SpeB, streptococcal pyrogenic exotoxin A, Mac, streptococcal phospholipase A2, the lantibiotic salivaricin A (SalA), DNases, streptolysin S, and the 67-kDa myosin crossreactive protein (*spy0470*) (Shelburne, et al., 2005a; Shelburne, 3rd, et al., 2005b). Transcriptome profiling during exponential and stationary growth phases in blood identified a two component regulatory system, termed SptR/S, as essential to the persistence of *S. pyogenes* in human saliva (Shelburne, et al., 2005a). SptR/S acts as a transcriptional inducer of numerous virulence genes, including *spd*, *spd3*, *sic*, *hasA,* and *speB*, as well as genes that encode complex carbohydrate transporters and utilization enzymes. During exponential growth in saliva transcripts involved in pathogen-host interactions (such as *emm* and *sic*), oxidative stress response (e.g., *ahpC* and *mtsA*), ATP generation and pH balancing (such as the arginine deiminase operon) are up regulated, in accordance with the nutritional limitations and the low pH found in saliva; whereas in the stationary growth phase, starch-degrading and carbohydrate-metabolism genes are differentially regulated, either positively (*malX*, *malE*, *amyA*, *agaD*) or negatively (*pulA*, *malE*) (Shelburne, et al., 2005a). Additional virulence regulators observed to display activity in

the saliva environment include RofA and several stress-response regulators (PerR, HrcA, Spy0583 and Ihk/Irr), which are all up regulated. Additional studies that examined the role of other virulence regulators found that CcpA and CovR influence gene expression in response to saliva, as it was observed that a *ccpA*-deficient MGAS2221 (M1) strain failed to induce the expression of *speB*, *sagA*, and *arcA* upon exposure to saliva and that a *covR*-deficient mutant in the same background exhibited reduced induction of these genes, as well as *spyCEP*, *slo*, and *amyA*, when grown in human saliva (Shelburne, et al., 2010).

Salivaricin A is one of the *S. pyogenes* factors induced in the presence of saliva. The salivaricin (*sal*) locus of *S. pyogenes* is ~85% homologous to the *sal* locus of *Streptococcus salivarius*, the commensal bacterium in which SalA was originally identified. Paradoxically, only M4 serotype strains produce active SalA and are immune to the lantibiotic produced by *S. salivarius*, yet the *sal* locus is highly conserved (>92% homology) among sequenced *S. pyogenes* serotypes (Phelps & Neely, 2005; Upton, Tagg, Wescombe, & Jenkinson, 2001), suggesting an alternative function for the *sal* operon in *S. pyogenes* pathogenesis. In general, lantibiotic loci are transcribed as operons. In addition to the prelantibiotic peptide, these operons encode modifying enzymes to produce the mature lantibiotic, an immunity protein or specialized ABC transporter to provide resistance to the lantibiotic and a TCS to regulate expression of the operon (Willey & van der Donk, 2007). Transcription of the *sal* locus in *S. pyogenes* was determined to occur from two promoters, one of which is upstream of the operon. The other one is an internal promoter in the coding region of *salY*, directly upstream of the *salKR* genes that encode the putative TCS regulating expression of the *sal* locus (Namprachan-Frantz, Rowe, Runft, & Neely, 2014). Transposon insertions into *salY* and *salK*, as well as in frame deletion of *salK*, resulted in attenuated virulence of an HSC5 (M14) strain in the zebrafish infection model (Phelps & Neely, 2005). Additionally, the product of *salR* was found to repress activity of the *salKR* promoter, as did SalA, whereas human serum increased expression from the promoter (Namprachan-Frantz, Rowe, Runft, & Neely, 2014). Altogether, this indicates that regulation of the *sal* operon is complex and involves multiple inputs, while its activity influences *S. pyogenes* virulence through some as yet uncharacterized mechanisms.

Another regulator found to be involved in virulence in the context of human saliva is the maltose repressor, MalR, which, like CcpA, is a LacI/GalR family member. Insertional inactivation of MalR in the MGAS2221 strain reduced colonization of the mouse orophyarnx, but did not detrimentally affect invasive infection (Shelburne, et al., 2011), which suggests a specificity of MalR activity for the mucous membrane environment. The observed MalR transcriptome is limited to only 25 genes, and a highly conserved MalR DNA-binding sequence required for DNA interaction *in vitro* was identified. Inactivation of one of the transcriptional targets of MalR, the cell wall anchored carbohydrate binding and degrading enzyme encoded by *pulA*, significantly reduced *S. pyogenes* adhesion to human epithelial cells and mouse oropharyngeal colonization (Shelburne, et al., 2011).

Blood

Severe invasive infection by *S. pyogenes*, which involves its dissemination into blood and other sterile deep tissue sites, is most frequently caused by strains of serotypes M1T1, M3, and M12 (O'Loughlin, et al., 2007). Clinical isolates of these invasive strains exhibit hypervirulence and an enhanced capacity to invade soft tissues and evade neutrophil responses, in contrast with the more commonplace pharyngitis isolates (Kansal, et al., 2010; Li, et al., 2013). Thus, it has been of great importance to elucidate the virulence determinants required for *S. pyogenes* dissemination and survival in blood.

Characterization of the MGAS5005 (M1) transcript profile during growth in human blood revealed that the transcriptome of *S. pyogenes* undergoes extensive modulation in this environment (Graham, et al., 2005). Three-quarters (n = 1467) of encoded genes were differentially transcribed, with changes in expression that indicate the broad metabolic adaptation of *S. pyogenes* to the blood environment, shutting down glycolysis and activating amino acid-fermentation pathways. Induced virulence genes included Mga-dependent (such as *emm* and *sic*) and Mga-independent genes (*hasABC*, *sagA*, *mac*, *slo*, *speA*, *speC*, and *smeZ*), whereas *speB* and *crgR* transcripts

were less abundant. In terms of regulation, exposure to blood resulted in the coordinated accumulation of *mga*, *rofA*, *fasBCA* and *ihk/irr* transcripts, as well as a temporal increase in *covR/S* expression. From these data, a regulatory model of *S. pyogenes* adaptation to the blood environment, composed of three phases, was proposed: persistence, adaptation, and dissemination. Persistence is mainly characterized by immune evasion through the up-regulation of *mga* and *fasBCA*, as well as down-regulation of *crgR*. Adaptation is typified by initiating cell contact, proteolysis, and peptide scavenging (along with up-regulation of *covRS* and *ihk*/*irr*) and dissemination was defined as tissue destruction, cell necrosis, and apoptosis, accompanied by the up-regulation of RALPs and pyrogenic toxin antigens.

Further analysis of *S. pyogenes* virulence gene regulation in blood involved the examination of 51 gene transcripts in a *codY*-deficient NZ131 (M49) strain, which revealed differential responses for 26 genes that occasionally differed from responses seen in laboratory media (Malke & Ferretti, 2007). Differentially expressed regulators in this study included *covR*, *fasX*, *mga*, and *sptR*/*S*. Virulence genes with altered expression included *cfa*, *emm49*, *grab*, *hasA*, *ideS*, *nga*, *prtS*, *scl*, *scpA*, *ska*, *slo*, *sof*, and *speH*, as did genes encoding amino acid transporters and metabolic enzymes. Degenerate derivatives of the CodY binding box potentially serving as a cis-regulatory element for CodY regulation were identified in the upstream regions of 15 genes of the NZ131 genome, and these genes featured sequence motifs identical to the NZ131 CodY box in all completely sequenced *S. pyogenes* genomes. However, these genes consisted almost exclusively of metabolic and transporter encoding ORFs rather than virulence factors or regulators, which suggests that the observed differential transcription of the majority of virulence genes was caused by the indirect regulation by CodY of the *S. pyogenes* regulatory network.

Efforts have been recently made to identify the essential genes necessary for the survival of *S. pyogenes* in blood. Transposon-site hybridization (TraSH) analysis of a complex *mariner*-based transposon mutant library in a 5448 (M1T1) strain subjected to negative selection in human blood identified 81 genes that are important to *S. pyogenes* fitness in the blood environment (Le Breton, et al., 2013). This approach found regulatory genes already known to play a role in the survival of *S. pyogenes* in blood, such as *mga*, *perR*, and *ralp3*, as well as genes previously reported for their contribution to sepsis in other pathogens, such as genes involved in *de novo* nucleotide synthesis (*purD*, *purA*, *pyrB*, *carA*, *carB*, *guaB*), sugar metabolism (*scrB*, *fruA*), zinc uptake (*adcC*), and transcriptional regulators (*cpsY*). Further work to characterize the contribution of these genes to *S. pyogenes* behavior in blood will be important to identifying the major determinants of invasive infection.

The contribution of CovR/S regulation to *S. pyogenes* dissemination and survival in blood is also being actively investigated, due to this regulator's association with invasive infection. Mutations in *covS* that attenuate the activity of its gene product toward CovR are a common cause of hypervirulence and enhancement of soft tissue invasion and innate immune evasion in invasive clinical isolates (Ikebe, et al., 2010; Shea, et al., 2011; Maamary, et al., 2012; Tatsuno, Okada, Zhang, Isaka, & Hasegawa, 2013; Garcia, et al., 2010; Masuno, et al., 2014) and animal passaged clones that arise from experimental invasive infection in mice (Sumby, Whitney, Graviss, DeLeo, & Musser, 2006; Walker, et al., 2007; Engleberg, Heath, Miller, Rivera, & DiRita, 2001). One outstanding feature of hypervirulent *S. pyogenes* strains with inactivated *covS* ($CovR^+S^-$) is the lack of detectable SpeB in culture supernatants (Maamary, et al., 2010). Intact *covS* greatly enhances the *speB* expression that is necessary for establishing early infectivity, after which the down-regulation of *speB* expression promotes virulence (Cole, Barnett, Nizet, & Walker, 2011). This switch in *speB* expression is further facilitated by *covS* gene mutations that allow CovR to repress *speB* expression, thereby permitting other proteins (such as the products of *sda1*, *ska*, and *slo*) to avoid degradation by SpeB and sustaining their activities as *S. pyogenes* virulence factors (Aziz, et al., 2004; Cole, et al., 2006; Liang, et al., 2013). The selection for $CovR^+S^-$ strains suggests that only colonies maximally discharging their virulence factors are able to survive in host compartments that are particularly hostile to *S. pyogenes* (namely, blood), whereas at sites without host immune surveillance, CovS is important for alleviating the energy burden of producing virulence gene products and allows for long-term colonization.

The consistency of gene mutations in *covS* that engender hypervirulent strains was examined by comparing the invasive globally disseminated 5448 ($CovR^+S^+$, M1T1) strain and the non-virulent engineered strain AP53 ($CovR^+S^+$) in a number of mouse-passage studies, where the same wild type bacteria were used to infect different mice (Mayfield, et al., 2014). Infection and dissemination sites were screened for SpeB-deficient clones also exhibiting increased *sda1* and *ska* expression and activity. Characterization of the isolated clones showed many types of mutations that occur in the *covS* gene (such as frame-shift insertions, deletions, and in-frame small and large deletions), which indicates that, when establishing infection, the entire *covS* gene is susceptible to mutations and that clones that contain CovS-inactivating mutations affect numerous phenotypes, which leads to serious invasive disease. Most interestingly, characterization of CovS function in clinical isolates with single amino acid substitutions demonstrated that point mutations partially, but not completely, impaired the function of the *covS* alleles (Tatsuno, Okada, Zhang, Isaka, & Hasegawa, 2013). Unlike *covS*-knockout (Δ*covS*) mutants, *S. pyogenes* strains that exhibit partial loss of CovS function stemming from *covS* alleles with point mutations were not impaired for growth in culture. As a result, complete loss of CovS function may confer greater virulence at the expense of the ability to respond to environmental cues recognized by CovS, which explains the abundance of point mutations among hypervirulent clinical isolates. Both the consequences of different *covS* mutations on *S. pyogenes* adaptation to host environments like blood and the mechanism driving these mutations require further study, though recent evidence hints that the latter entails interaction with host innate immunity, as described in the next section.

Host innate immunity (Ihk/Irr, CovR/S)

Polymorphonuclear leukocytes (PMNs) and macrophages constitute host innate cellular immunity, engaging in pathogen removal once bacteria spread from their primary infection sites to deeper tissues or reach the blood stream. These cell types kill invading pathogens by producing extracellular factors, like neutrophil extracellular traps (NETs) and antimicrobial peptides (CAMPs) or by phagocytosing and destroying bacteria through phagosome acidification, ROS production via the oxidative burst, and/or the delivery of CAMPs and cytolytic toxins through granule fusion (reviewed in (Okumura & Nizet, 2014)). *S. pyogenes* evades or survives killing by these host immune processes in order to colonize and persist in the host through a variety of virulence factors under tight control by some of the regulators already discussed in this chapter, as well as through other dedicated transcriptional regulators.

Ihk/Irr

This TCS of *S. pyogenes* has been mentioned throughout the chapter, given that its expression is influenced in multiple environments (e.g. blood, saliva) and by other regulators (such as Nra). Multiple lines of evidence indicate that Ihk/Irr is a central regulator of *S. pyogenes* responses to innate cellular host immunity. It was initially identified in the investigation of Mga regulation (McIver, Subbarao, Kellner, Heath, & Scott, 1996) and later as part of the CovR/S regulon (Federle, McIver, & Scott, 1999), displaying homology with the ArlS/R virulence-gene regulating TCS of *Staphylococcus aureus* (Fournier, Klier, & Rapoport, 2001). Ihk/Irr exhibits an essential role in evasion of polymorphonuclear leukocyte (PMN)-mediated killing and is highly expressed during *S. pyogenes*-induced acute pharyngitis (Voyich, et al., 2003). In addition to the observed up regulation of virulence-factors encoded by *sic*, *mac*, *speH*, *endoS*, *smeZ*, *speB* and *srtA*, expression of the *ihk* and *irr* genes is also induced during phagocytosis by human PMNs. Deletion of the response regulator encoded by *irr* in the JRS4 (M6) strain analyzed did not enhance phagocytosis or production of ROS by PMNs in the presence of *S. pyogenes*, which suggests that the Ihk/Irr regulon enhances the survival of *S. pyogenes* within phagocytes (Voyich, et al., 2003). Further characterization of Ihk/Irr in JRS4 by genome-wide microarray-based analysis of the Irr-dependent transcriptome showed Ihk/Irr controls the expression of genes involved in cell wall synthesis, oxidative stress responses (such as *trx/thioredoxin*, *nrdH, nrdR*), and of several virulence factor-encoding genes, including *fbp* (fibronectin-binding protein), *mf*, *mf3* (both DNases), and *sagA* (streptolysin S) (Voyich, et al., 2004). In this same study, expression of *ihk* and *irr* transcripts was highly up regulated by *S. pyogenes* exposure to

neutrophil primary granules and was moderately induced by hydrogen peroxide challenge, while the loss of *irr* expression resulted in induction (*vicR*) or repression (*codY*) of virulence-related regulator transcripts. Additionally, an *irr*-deficient strain in JRS4 (M6) was attenuated for virulence *in vivo*, as mice infected with the wild type strain formed abscesses more rapidly and that were of larger size, as compared to mice inoculated with the *irr* mutant, and infection with the *irr*-deficient strain was cleared more quickly from the blood (Voyich, et al., 2004). Examination of the *S. pyogenes* transcriptome using a 5448 (M1T1) strain challenged with human monocyte-derived macrophages demonstrated Ihk/Irr is involved in the early stages of adaptation to the environment within phagocytic cells (Hertzén, et al., 2012). In this study, intracellular bacteria exhibited increased expression of *ihk* and *irr*, in addition to higher expression of genes involved in cell wall synthesis and energy production, but only in the early stages of infection, as over time, *ihk/irr* transcript levels diminished with a concomitant rise in *covR/S* transcripts. An Ihk/Irr deficient mutant in the 5448 background produced lower intracellular bacterial counts from infected macrophages (Hertzén, et al., 2012), which demonstrate that Ihk/Irr TCS is required for *S. pyogenes* survival of innate cellular host immunity across strain backgrounds and phagocytic cell types.

CovR/S

Since it influences many of the transcripts involved in innate immune evasion (Federle, McIver, & Scott, 1999; Heath, DiRita, Barg, & Engleberg, 1999; Levin & Wessels, 1998; Treviño, et al., 2009), a great deal of research has examined CovR/S function in the interaction of *S. pyogenes* with host innate immunity. These studies uncovered that mutations in *covRS* associated with the loss of SpeB protease production and enhanced synthesis of the hyaluronic acid capsule are responsible for hypervirulent phenotypes (Li, et al., 2013; Aziz, et al., 2004; Cole, et al., 2006; Engleberg, Heath, Vardaman, & DiRita, 2004). Along with the lack of SpeB production ($SpeB^-$) as a defining trait of *S. pyogenes* variants with *covRS* mutations, concomitant expression of the *sda1*-encoded DNase was observed to be critical to the selection of *covRS* mutations in 5448 (M1T1) during infection in mice (Walker, et al., 2007). High-level expression of *sda1* facilitates the degradation of DNA in NETs, which promotes resistance to neutrophil-mediated killing at the initial site of infection (Buchanan, et al., 2006; Sumby, et al., 2005), while the deletion of *sda1* abolished selection for *covRS* mutation *in vivo* following subcutaneous mouse infection (Walker, et al., 2007). In addition to Sda1, the capsule synthetase gene *hasA* and the M protein gene *emm* are required for the selection of $SpeB^-$ variants (Cole, et al., 2010). As previously mentioned, the loss of the broad-spectrum cysteine protease activity of SpeB inhibits the degradation of Sda1 and several additional virulence proteins, like streptokinase and the M1 protein, which are therefore able to interact with host plasminogen and accumulate plasmin activity on the bacterial cell surface to facilitate systemic spread (Aziz, et al., 2004; Cole, et al., 2006; Kansal, McGeer, Low, Norrby-Teglund, & Kotb, 2000). However, introduction of the Sda1-encoding prophage into an SF370 strain did not facilitate the selection of $SpeB^-$ mutants *in vivo* (Venturini, et al., 2013), and hypervirulent variants with *covS* mutations arise in strains that lack *sda1* (Tsatsaronis, et al., 2013). Genotype-dependent variations aside, the association of virulence factors required for phagocyte evasion with *covS*-deficient hypervirulent isolates suggests that interaction with host innate cellular immunity may be involved in the selection of *S. pyogenes* invasive strains. Screening infection sites for $SpeB^-$ isolates in neutrophil-depleted mice inoculated with MGAS2221 ($CovR^+S^+$, M1T1) revealed that neutrophils are the primary selection pressure for *covRS* mutation, as the lack of neutrophils drastically reduced the number of *covS*-deficient clones recovered from animal passaging (Li, et al., 2014). Additionally an isolate with a null *covS* mutation from said passaging displayed enhanced survival and an enhanced capacity to evade innate immune responses. Further work is needed to characterize the mechanism(s) through which *S. pyogenes* -phagocyte interaction selects for invasive genotypes and the ways in which this contributes to *S. pyogenes* epidemiology.

Non-immune cells

The host cells infected by *S. pyogenes* are predetermined by the major entry ports the bacterium uses to enter the human body, thus making tonsillar epithelial cells and skin keratinocytes the primary targets in cases of

infection of mucous membranes and the skin (Fiedler, Sugareva, Patenge, & Kreikemeyer, 2010). A prerequisite for colonization of these sites is the need to specifically adhere to epithelial cell surfaces, a task for which adhesion- and pilus-encoding genes found in a genotype-specific pattern within the FCT region are predominantly responsible. As previously indicated in this chapter, different members of the RALP family act as the primary regulators that influence the expression of FCT region genes in a serotype-dependent manner, but they are not the only ones to regulate them.

MsmR

MsmR is an AraC/XylS-type regulator that is identified in the FCT region and is shown to counteract Nra activity in the M49 serotype (Nakata, Podbielski, & Kreikemeyer, 2005). Microarray analysis of an *msmR* mutant in the M49 background showed that the genes located within or adjacent to the FCT region (*prtF*, *cpa*, *spy0128*, *prtF2*, *nga*, *spy0166*, *slo*, and *spy0170*), as well as genes of the Mga region (*scl*, *spy2006*, *fbaA*, *scpA*, *sof sfbX*, and *hasA*) were downregulated in the *msmR* mutant. Thus, MsmR acts as a positive regulator of all fibronectin-binding protein genes (*prtF2*, *fbaA*, *sof*, and *sfbX*) in serotype M49 (Nakata, Podbielski, & Kreikemeyer, 2005). In addition, the genes that encode cytolysin-mediated translocation system proteins, such as *nga* and *slo*, are upregulated by MsmR. Most of the upregulated genes in the *msmR* deficient mutant encode for prophage-associated proteins and stress-response factors, and the regulatory effects observed for MsmR on FCT region genes are in opposition to those of Nra/Ralp regulators (Nakata, Podbielski, & Kreikemeyer, 2005). MsmR activity, like that of the RALPs, appears to be strain-specific, as examination of an Alab49 (M53) strain showed that MsmR acts as a repressor of *nra* and pilus gene transcription, rather than as an inducer of such genes, as observed in the M49 genotype (Luo, Liziano, & Bessen, 2008). This specificity appears to correlate to the regulator repertoire present in a strain, as replacement of the native *nra*-lineage allele and its respective upstream region with a *rofA*-lineage allele at the *nra*/*rofA* locus in the Alab49 strain produced a chimeric strain in which the polarity of MsmR activity was reversed, which made it an activator instead of a repressor of pilus gene expression, and without altering Nra regulatory activity on same said genes (Lizano, Luo, Tengra, & Bessen, 2008). This result highlights the importance of examining the activity of virulence gene regulators in *S. pyogenes* in the context of the regulatory network they constitute, as it shows that the exchange of orthologous forms of a regulatory gene can generate new phenotypes by altering the circuitry of the transcriptional regulatory network.

FasX

As previously described, the Fas regulator affects expression of S. pyogenes adhesins and streptokinase, which are factors involved in localized colonization and dissemination, respectively. To gain further insight into Fas function in the interaction with host epithelial cells, researchers used Affymetrix human genome DNA-arrays to measure the temporal and global transcriptional responses of HEp-2 cells infected with a fasX-deficient mutant and its M49 isogenic parent strain (Klenk, et al., 2005). A total of 86 HEp-2 cell genes were differentially transcribed upon infection, and included an increased expression of genes encoding fibronectin and integrin-α5 proteins involved in S. pyogenes host cell adherence and internalization. Activity of the Fas regulator appeared to promote high adherence and internalization rates, massive cytokine gene transcription and cytokine release, host cell apoptosis via a caspase-2 activation pathway, and cytotoxicity (Klenk, et al., 2005); whereas the absence of FasX decreased secreted IL-8 levels to below those of non-infected cells, which highlights the involvement of the Fas regulator in defining localized colonization vs. systemic spread in S. pyogenes infection.

Alternative Mechanisms of Gene Regulation

mRNA half-life of the *S. pyogenes* transcriptome as influenced by RNase Y

Knowledge of global mRNA half-lives in bacterial species in general and pathogens in particular is limited, but can provide valuable insights on the adaptability of a species to changing environmental conditions. In *Bacillus*

subtilis and *Staphylococcus aureus*, numerous transcription units are up or down regulated in mutants that carry a defective *rny* gene, which encodes the endoribonuclease RNase Y responsible for the initiation of mRNA degradation (Lehnik-Habrink, et al., 2011; Marincola, et al., 2012). Recently, a NZ131 *rny* mutant was assessed using a novel approach (steepest-slope method) for precise mRNA half-life determination on a transcriptome-wide scale (Chen, Itzek, Malke, Ferretti, & Kreth, 2013). The results encompassed 1,485 *S. pyogenes* genes (87.1% of the core genome) and revealed median and mean transcriptome half-lives of 0.89 min and 1.24 min, respectively, in the wild-type strain. The corresponding values for the *rny* mutant amount to 1.81 min and 2.79 min, respectively. In the wild type, the overwhelming majority of these genes (85%) show "unstable“ mRNAs with half-lives shorter than 2.0 min, and only a small minority (2.3%) exhibit half-lives longer than 5.0 min (“stable” mRNAs). In contrast, the *rny* mutant gave corresponding percentage changes that changed to 56% and 12%, respectively. These data compare well with a 2-fold increase of overall mRNA stability, as affected by a non-functional *rny* gene. Compared to other bacterial species, which show longer transcriptome half-lives, the high mRNA turnover rate in *S. pyogenes* suggests that this species is particularly capable of adaptation to fast-changing environments during the course of the infection process (Figure 5).

mRNA stability is also differentially affected in important functional groups of *S. pyogenes* genes (Chen, Itzek, Malke, Ferretti, & Kreth, 2013). For example, Clusters of Orthologous Groups (COGs (Tatusov, Koonin, & Lipman, 1997)) involved in energy production and translation are about 2- to 3-times more stable than the transcriptome average in the wild type *S. pyogenes* .The vast majority of mRNA in all 20 COGs studied showed a 1.7- 3.3-fold increase in the *rny* mutant. However, the stability profile of the different functional groups stays about the same in wild type and mutant strains, indicating that RNase Y does not preferentially affect certain functional groups. Resolving the general picture of *rny*-directed mRNA half-life control to the level of individual target genes leads to the identification of mRNAs, the half-lives of which are not significantly influenced by RNase Y (*upp*, *ska*, *cfa*; (Chen, Itzek, Malke, Ferretti, & Kreth, 2013)).

The unexplored methylome

It has recently become possible to quickly and reliably determine the complete methylation profile of bacterial genomes (Murray, et al., 2012) as part of the single molecule, real-time (SMRT) sequencing procedure (Flusberg, et al., 2010). The methylated bases, created by DNA methyltransferases and present practically in all bacterial genomes, not only serve functions in restriction-modification systems (Euler, Ryan, Martin, & Fischetti, 2007), but also regulate the transcription of virulence genes (Casadesús & Low, 2006). Such genome-wide epigenetic data have not yet been published for *S. pyogenes*, but promise to open up an entirely new era of investigations into its pathogenicity once they are available. Issues that immediately come to mind include the problem of whether or not the methylome is environment-dependent, or, vice versa, if a given methylome determines the tissue specificity of infection. Likewise, it will be interesting to find out whether the methylome of carrier strains differs from that of their invasive counterparts, which might explain how CodY turns a gene activator. Clearly, we need to gain knowledge about the epigenetic regulation of transcription, and can foresee exciting new fields of research arising in the near future.

Overall Perspectives

A survey such as this of existing knowledge on virulence gene regulation in *S. pyogenes* yields several overarching observations about the nature of virulence control in this strictly human pathogen. First, as introduced by the concept of the Red Queen argument, the regulatory networks of *S. pyogenes* have evolved in concert with its host niche. A clear example of this is the organization of various pathogenicity regions and regulons in the *S. pyogenes* genome (such as the FCT region, ERES, and CovRS regulon) whose structure and regulation correlates with the tissue environment and isolate in which it is found. Second, that the function of any given regulator and its contribution to virulence must be considered in the context of its interacting network, as multiple regulators have been shown to have varying, or even opposing, activity that depends on the

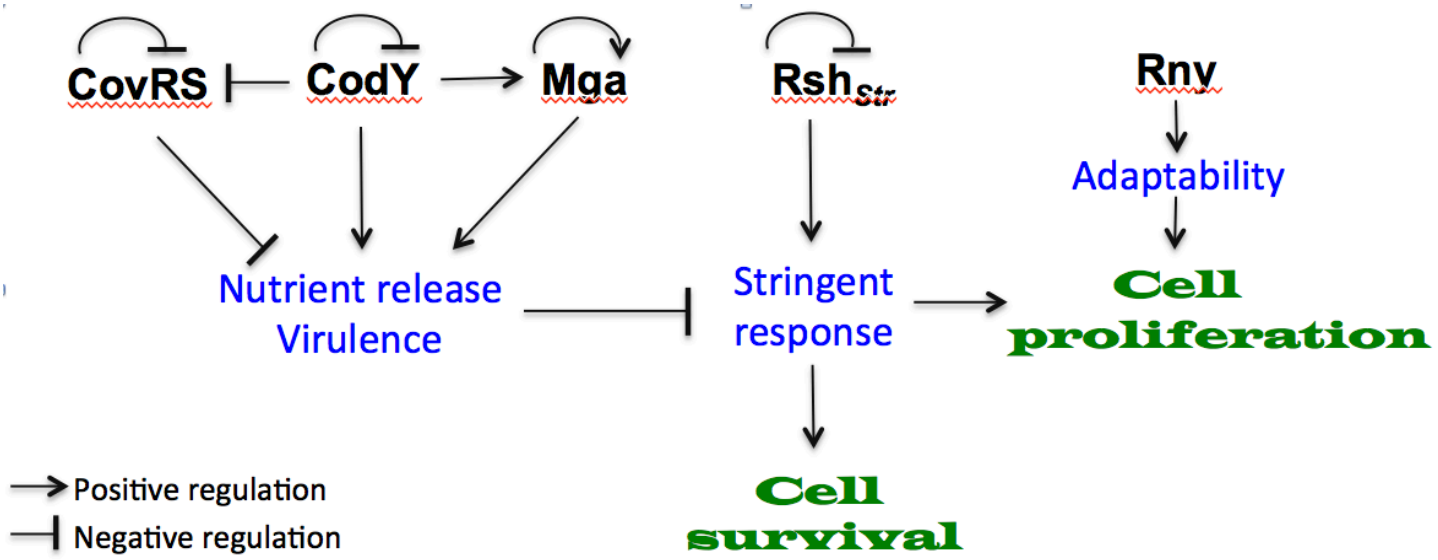

Figure 5. Independence and interaction of the regulatory systems important for amino acid starvation, mRNA stability, and global virulence in *S. pyogenes*.

strain genotype (such as CcpA or RALPs). Third, that the control of metabolic function and virulence are inextricably linked in a complex feedback network. This should come as no surprise, as the ultimate purpose of virulence factors is to ensure and enhance the survival of a pathogen in its environment, which in the case of *S. pyogenes* is the human host. Therefore, *S. pyogenes* has connected sensing of internal and environmental resources (such as carbohydrates and amino acids) to regulation of virulence proteins that act to evade host immunity, as well as proteins involved in nutrient acquisition (Figure 6). Finally, in order to further the development of more effective therapeutics, it is necessary to study the *S. pyogenes* "regulome" as a system in constant flux, as it is very susceptible to mutation and dependent upon the context of the surrounding environment. Faster and more practical techniques for whole-genome characterization are becoming available, which make this sort of investigation less unwieldy, and it appears that this is the area of greatest promise to define the mechanisms that determine the success of *S. pyogenes* as a pathogen. Throughout this chapter, we have called attention to areas of research in *S. pyogenes* pathogenesis that either require deeper examination (such as the contribution of CovS-inactivating mutations to invasive disease) or that have the potential for uncovering novel regulatory relationships important to virulence (namely, PTS-dependent regulation of Mga activity). Research in *S. pyogenes* virulence regulation has the potential to drive the development of novel therapeutics to treat disease (such as targeting the stringent response or immune evasion signaling), as well as those in which innovative approaches promise to broaden our understanding of gene regulation in *S. pyogenes* pathogenesis.

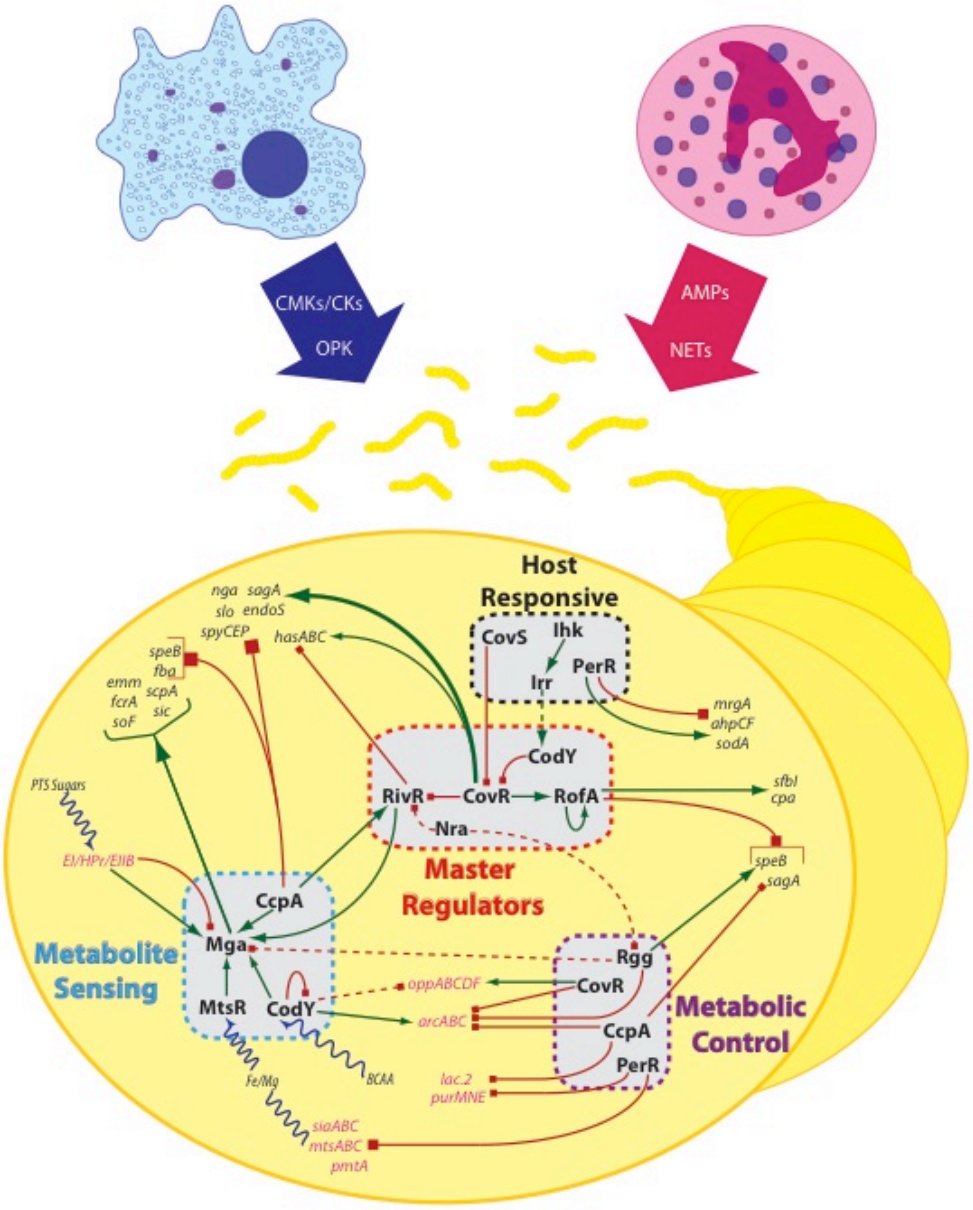

Figure 6. *S. pyogenes* employs a complex regulatory network to interact with host immunity: *S. pyogenes* responds to host immunity by engaging all categories of gene regulators (dashed boxes). Monitoring and control of *S. pyogenes* metabolism is integral to virulence responses. Carbohydrates, amino acids and metals all influence expression of large virulence regulons (Mga, CcpA), directly (CcpA), and indirectly (EI/HPr/EIIB, MtsR, CodY). Metabolism is linked to virulence by regulators that function in several categories and act as metabolic sensors and regulators (CcpA), metabolite sensors and master regulators (CodY), or host environment responsive and metabolic regulators (PerR). Regulators can induce (RofA) or repress (CodY) their own activity, and regulate a multitude of targets directly (CovR) or indirectly (CodY, Rgg, Nra). Regulators of the same family influence diverse targets including each other, sometimes in opposing ways (RivR, Nra, RofA). The graphic presents only one level of regulation and a sampling of targets, as the regulators shown influence a large proportion of the *S. pyogenes* genome and their activity varies, depending on genotype and environmental background (e.g. RALPs, CcpA, PerR). Transcriptional activation (green arrows) and repression (red boxes), direct (solid line) and indirect (dashed line) are shown. Virulence genes (*black italics*), metabolic genes (*pink italics)*, and regulators (bold text) are indicated. Squiggly arrows denote metabolite influence on regulators; *BCAA* = Branched chain amino acids; CMKs/CKs=chemokines/cytokines; OPK=opsonophagocytic killing; AMPs=antimicrobial peptides; NETs=neutrophil extracellular traps.

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Secreted Extracellular Virulence Factors

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Abstract

Group A streptococci produce a wide variety of secreted extracellular products, many of which are considered virulence factors. The number of these potential virulence factors exceeds that of many other pathogens, and probably correlates to this organism's ability to be a successful pathogen. This chapter reviews those virulence factors that are secreted and released from the streptococcus, including: streptokinase, proteinases, esterase, the hemolysins SLO and SLS, CAMP factor, DNases, hyaluronidases, complement inhibitor, superoxide dismutase, and immunoglobulin degrading enzymes. Many of these presumed virulence factors may also function as digestive enzymes that provide the bacteria with nutrients from the host.

The group A streptococci produce a variety of extracellular products, many of which are secreted proteins that are often considered to be virulence factors. Historically, many of these proteins have been studied, either individually or in isolation, to determine their mechanism of action in evading host defenses, as well as to determine possible candidate antigens for a potential vaccine. The total number of these potential virulence factors exceeds that of many other pathogens—and probably correlates with this organism's ability to be a successful pathogen. How each or any of these virulence factors functions in the disease process is not fully understood; it is unlikely that all these factors play a role in all diseases. The role of various virulence factors in some group A streptococcal diseases has recently been reviewed (Reglinski & Sriskandan, 2014; Walker, et al., 2014).

Recent genomic studies have provided a great deal of new information about these extracellular products at the cell-system level since many of their genes have previously been individually cloned in order to study the specific proteins and their role in pathogenesis. The number of secreted proteins produced can vary among serotypes and strains, since some genomes either do not contain the specific gene for a particular protein, or the gene has mutations that do not allow for proper transcription and/or translation. Additionally, differences in the functional regulatory networks between different strains will influence the number and types of products produced. Another reason for the variation in secreted proteins among different strains is that some of the extracellular protein genes are chromosomally located, while others are encoded in prophages that are located in the genome.

This chapter reviews those virulence factors that are secreted and released from the streptococcus; that is, the released extracellular products (Figure 1, Table 1). Other virulence factors, those that are cell bound or surface associated, are discussed elsewhere (Hynes, 2004), as well as in various chapters of this book (as in the chapters by Fischetti, Chaussee, Vega, and Cattoir). Additionally, the mechanism of transport and secretion of the various virulence factors are also discussed elsewhere in this work. Most (but not all) of the prophage encoded genes specify superantigens, such as the streptococcal exotoxins SpeA, SpeC, SpeH, SpeI, SpeK, and streptococcus superantigen (SSA) are described further in the chapter on superantigens.

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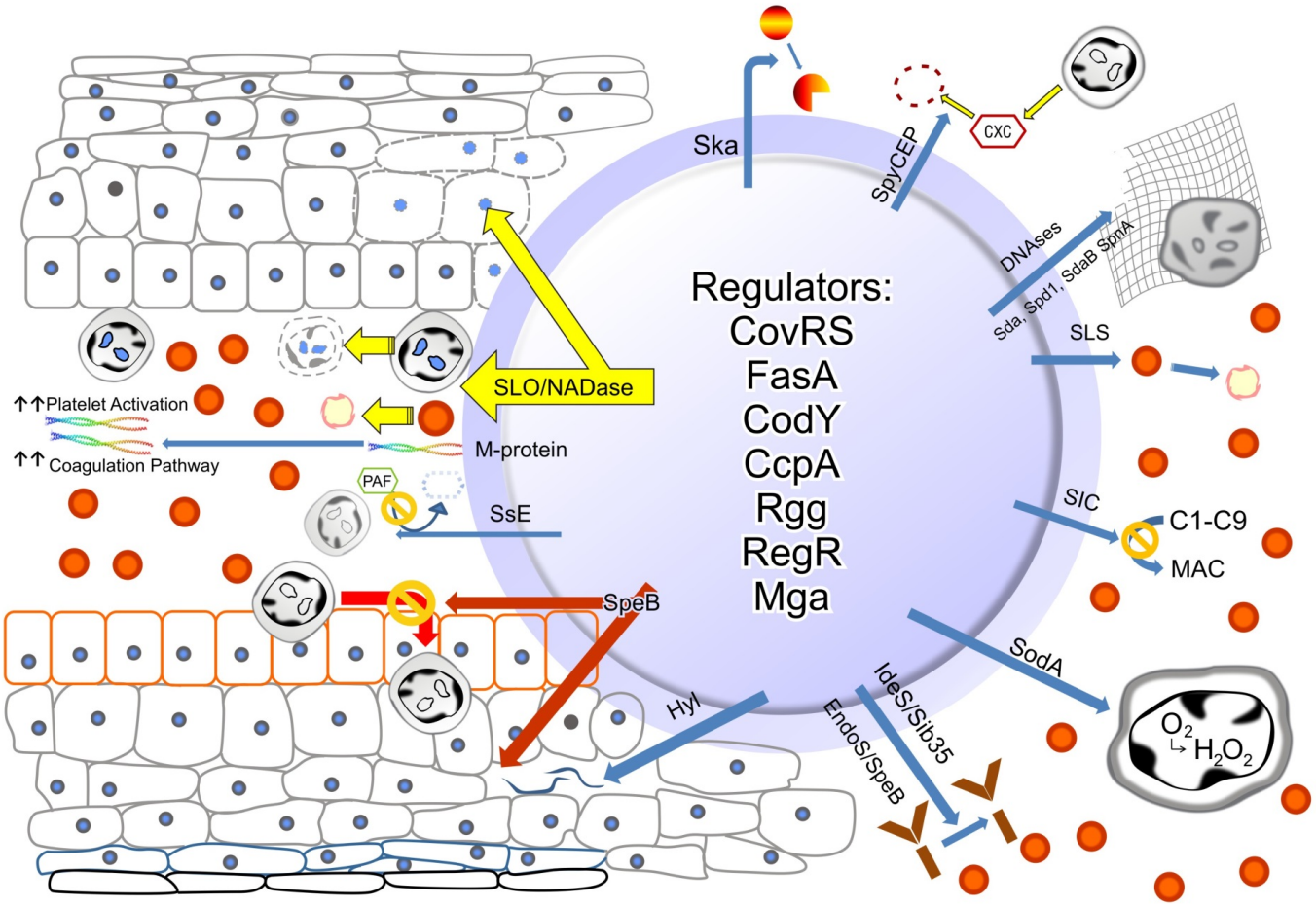

Figure 1: Group A streptococci produce an arsenal of extracellular virulence factors that have a variety of effects on tissues, cells, and components of the immune response. SLO and NADase destroy tissues, red blood cells, and immune cells; SLS lyses a variety of cell types; SpeB is able to degrade tissues, inhibit immune cell migration, and degrade host immune response proteins; SsE degrades platelet-activating factor, which results in reduced immune cell migration; Ska converts plasminogen to plasmin; SpyCEP degrades CXC-type cytokines; DNAses destroy DNA-based neutrophil traps; SIC inhibits formation of the complement mediate membrane attack complex; SodA converts oxygen radicals to hydrogen peroxide; hyaluronidases degrade the cement component of the extracellular matrix; immunoglobulin binding proteins (IdeS, Sib35, EndoS) degrade antibodies; and soluble M protein induces platelet activation and the coagulation pathway.

Table 1: Secreted Extracellular Virulence Factors based on hypothesized effects*

Action	Gene	Locus tag	Protein ID
Fibrinolysis			
Streptokinase	*ska*	SPy_1979	NP_269944.1
Degradation of epithelial intercellular junctions			
Streptolysin S	*sag*A	SPy_0738	NP_268963.1
Bacterial Spread			
Hyaluronidase	*hyl*A	SPy_1032	NP_269203.1

Table 1 continued from previous page.

Action	Gene	Locus tag	Protein ID
Streptokinase	*ska*	SPy_1979	NP_269944.1
Proteolytic Enzymes			
Cysteine proteinase	*spe*B	SPy_2039	NP_269985.1
Serum opacity factor	*sof*	SPY49_RS08325	WP_012561050.1
IgG-degrading enzyme	*ideS*	SPY49_RS03465	WP_009880309.1
C5a peptidase	*scp*A	SPy_2010	NP_269970.1
Neutrophil Modulation			
Cell envelope protease	*cep*A	SPy_0416	NP_268723.1
Secreted esterase	*sse*	SPy_1718	NP_269743.1
NAD glycohydrolase	*nga*	SPy_0165	NP_268544.1
Streptolysin O	*slo*	SPy_0167	NP_268546.1
Streptolysin S	*sag*A	SPy_0738	NP_268963.1
SpdI	*spd*1	SPy_0712	NP_268944.1
SdaD2	*sda*D2	M5005_Spy_1415	YP_282778.1
Superoxide dismutase	*sod*A	SPy_1406	NP_269500.1
Glutathione peroxidase	*gpo*A	SPy_0605	NP_268861.1
Streptococcal exotoxinA	*spe*A	SPYM18_RS01810	WP_009880239.1
Cell pore formation			
NAD glycohydrolase	*nga*	SPy_0165	NP_268544.1
Streptolysin O	*slo*	SPy_0167	NP_268546.1
Streptolysin S	*sag*A	SPy_0738	NP_268963.1
CAMP factor	*cfa*	SPy_1273	NP_269402.2
Complement activation			
Streptokinase	*ska*	SPy_1979	NP_269944.1
Complement inactivation			
C5a peptidase	*scp*A	SPy_2010	NP_269970.1
Inhibitor of complement	*sic*	SPy_2016	NP_269972.1
Immunoglobulin Modulation			
SibA	*sibA*	M5005_Spy_0017	WP_010921769.1
IdeS	*ide*S	SPY49_RS03465	WP_009880309.1
MAC-1, Sib35, MspA	*mac*	M5005_SPY_0668	YP_282031.1
EndoS (EndoS$_2$)	*ndo*S	M5005_Spy_1540	WP_011285695.1
EndoS2	*ndo*S2	SPY49_RS06990	WP_012560921.1

* Many locus designations from strain SF370, Genomic Sequence: NC_002737.2

Streptokinase

Streptokinase (Ska) is a single-chain 414 amino acid protein, secreted by group A, C, and G streptococci, that has an activity similar to two host proteins (urokinase-type and tissue-type plasminogen activators), in that it non-enzymatically converts inactive plasminogen to proteolytically active plasmin. Plasminogen is a single-chain glycoprotein zymogen of plasmin, a serine protease. Plasminogen is a key component of the fibrinolytic system and is found in plasma and extracellular fluids, with activation to plasmin having a number of effects. Although streptokinase is a plasminogen activator protein, it is not itself a protease. Streptokinase activates plasminogen non-enzymatically; the binding of streptokinase to plasminogen induces a conformational change that results in the formation of an active site in the streptokinase-plasminogen complex. Within this complex, the intermolecular cleavage of plasminogen to plasmin occurs. The enzymatically active complex then proteolytically converts its substrate, plasminogen, to plasmin (Boxrud & Bock, 2004; Boxrud, Fay, & Bock, 2000). Figure 2 presents a hypothesized mechanism of plasminogen (Pg) activation by streptokinase (SK) using a two-cycle catalytic pathway (Nolan, Bouldin, & Bock, 2013).

The streptokinase gene from a group C strain was the first streptococcal gene to be cloned and sequenced (Malke & Ferretti, 1984; Malke, Roe, & Ferretti, 1985) and was shown to be 85% identical in amino acid sequence to that of the group A streptokinase (Huang, Malke, & Ferretti, 1989b), with heterogeneity seen in the genes (Huang, Malke, & Ferretti, 1989a). Since that time, streptokinase has been shown to be composed of three distinct domains: α (aa 1–150), β (aa 151–287) and γ (aa 288–414) (Kalia & Bessen, 2004; Wang, Lin, Loy, Tang, & Zhang, 1998) separated by coiled-coil regions with the N- and C- termini of the protein being disordered flexible structures (Wang, Lin, Loy, Tang, & Zhang, 1998). The β-domains of streptokinases from different strains of group A streptococci show sequence variability (55% identity), while the α- and γ-domains showed higher levels of conservation (77% and 84% identity) (Zhang, Liang, Glinton, Ploplis, & Castellino, 2013). Phylogenetic studies indicate that sequence variation in the β-domains divides the streptokinases into 2 clusters (SK1 and SK2); within the SK2 cluster, the sequences can be further subdivided into SK2a/SK2b (Kalia & Bessen, 2004; McArthur, et al., 2008; Zhang, Liang, Glinton, Ploplis, & Castellino, 2013). Phylogenetic studies also found that the streptokinases form discrete lineages associated with tissue specific *emm* patterns of the organism (McArthur, et al., 2008). SK2a-expressing strains are nasopharngeal isolates, many of which express fibrinogen binding M protein, while SK2b is secreted by skin-tropic strains that express plasminogen-binding M protein (Kalia & Bessen, 2004; Zhang, Liang, Glinton, Ploplis, & Castellino, 2013). The sequence variation observed among the different streptokinases leads to structural β-domain differences that lead to functional differences in SK1, SK2a, and SK2b plasminogen activation. Variations in the streptokinase sequences of different isolates of *S. pyogenes* results in distinct plasminogen activation pathways being used by the pathogen, which can directly affect the pathogenesis of this organism (Cook, Skora, Gillen, Walker, & McArthur, 2012). Isogenic chimeric strains (streptokinase and M protein) were shown to influence streptococcal virulence by changes in plasminogen activation and plasmin binding; virulence was partially explained by disparate plasminogen activation, coupled with the M protein of the strain (Chandrahas, et al., 2015). In addition to the different activation pathways, it is also important to note that streptokinase expressed by human isolates is only active against human plasminogen (Marcum & Kline, 1983; Sun, et al., 2004). The importance of activation of (human) plasminogen was shown experimentally by using transgenic mice. Mice expressing human plasminogen were more susceptible to group A streptococci expressing streptokinase than mice expressing their own plasminogen, or those without streptokinase expression (Marcum & Kline, 1983; Sun, et al., 2004).

The interaction of streptokinase with the plasminogen activation systems of the host is considered to be a virulence mechanism for the pathogenesis of this organism (Khil, et al., 2003; Sun, et al., 2004; Sun, et al., 2012; Walker, McArthur, McKay, & Ranson, 2005). Not only does *S. pyogenes* produce streptokinase for activating plasminogen, but the organism also binds plasminogen and plasmin to the cell surface via numerous cell wall-associated proteins such as M proteins, glyceraldehyde-3-phosphate, and streptococcal enolase (Cole, Barnett,

Nizet, & Walker, 2011; Gase, Gase, Schirmer, & Malke, 1996; Lähteenmäki, Kuusela, & Korhonen, 2001; Malke, Mechold, Gase, & Gerlach, 1994). These cell surface-bound proteins, along with streptokinase, result in the availability of both soluble and cell-bound plasmin activity to the invading pathogen. Plasmin also binds and cleaves fibrinogen, the product of which (fibrinogen D domain fragments) can interact with the cell surface fibrinogen receptors, and in doing, so mediate the acquisition of plasmin onto the bacterial surface (Sanderson-Smith, De Oliveira, Ranson, & McArthur, 2012).

The generation of plasmin from plasminogen at the infection site may result in the activation of host matrix metalloproteinases that lead to fibrinolysis and degradation of extracellular matrix and basement membrane components, which allow streptococci to spread from its primary or initial site of infection into surrounding sites (Sun, et al., 2004). The initial host response is to confine the infection, but by degradation of the extracellular matrix, tissue barriers, and degradation of fibrin networks, plasmin plays a role in spreading the bacteria to other sites in the body (Walker, McArthur, McKay, & Ranson, 2005). In addition, streptokinase has been shown to induce inflammation by complement activation, which may play a role in post-infectious sequelae of *S. pyogenes*; streptokinase has also been suggested to play a role in acute poststreptococcal glomerulonephritis (APSGN) (Nordstrand, Norgren, Ferretti, & Holm, 1998; Ohkuni, et al., 1991). While playing this apparently important role in virulence, streptokinase may also be degraded by another virulence factor, the cysteine proteinase SpeB (discussed below), which is produced by the same organism (Sumby, Whitney, Graviss, Deleo, & Musser, 2006).

Expression of the streptokinase gene, *ska*, is negatively controlled by the CovRS regulatory system (Walker, et al., 2014), with phosphorylated CovR repressing the expression of *ska* (Treviño, et al., 2009); *ska* expression is upregulated in *cov*RS mutants (Sumby, Whitney, Graviss, Deleo, & Musser, 2006). In addition to the CovRS system, streptokinase expression is also controlled by the FasBCA regulatory system in *S. pyogenes*, and similarly in *S. equisimilis* (Kreikemeyer, Boyle, Buttaro, Heinemann, & Podbielski, 2001; Malke & Steiner, 2004; Steiner & Malke, 2002). This regulation is through FasA acting indirectly by controlling FasX, a sRNA that post-transcriptionally regulates streptokinase production. It does this by enhancing *ska* transcript stability, which results in an approximately 10-fold increase in streptokinase activity (Ramirez-Peña, Treviño, Liu, Perez, & Sumby, 2010).

Cysteine Proteinase

The cysteine proteinase, SpeB, is also known as streptococcal pyrogenic erythrogenic toxin B or streptococcal cysteine proteinase. The proteinase was originally thought to be two distinct proteins, one with pyrogenic activity and the other with proteinase activity. However, when it was cloned, it was found that "both activities" were encoded by the same gene, which is designated *speB* (Bohach, Hauser, & Schlievert, 1988; Gerlach, Knöll, Köhler, Ozegowski, & Hríbalova, 1983). Cloning allowed for the analysis of the protein in the absence of any contaminating streptococcal proteins, and showed the proteinase precursor to be both pyrogenic and mitogenic (Gerlach, Knöll, Köhler, Ozegowski, & Hríbalova, 1983). Although the SpeB protein is not an exotoxin, the SpeB designation has been retained over the years and is still used in the current literature. The streptococcal cysteine proteinase is produced as an inactive zymogen (40 kDa) that undergoes autocatalytic cleavage and conversion to a mature 28 kDa active enzyme (Chen, et al., 2003; Liu & Elliott, 1965a; Musser, Stockbauer, Kapur, & Rudgers, 1996). In addition to cleavage, the protein also needs to be reduced (cysteine residue) in order to be active (Liu & Elliott, 1965b; Musser, Stockbauer, Kapur, & Rudgers, 1996). In culture, it is produced in the late log to the early stationary phase, in response to starvation (Chaussee, Phillips, & Ferretti, 1997). The crystal structure of the zymogen form of the enzyme revealed it to be a distant homolog of the papain superfamily (Kagawa, et al., 2000). Additionally, the structure revealed an integrin-binding motif located on the protein surface of some isolates (M1 and 20% of other isolates) and suggested a link to the pathogenesis of invasive streptococcal strains (Kagawa, et al., 2000). This proteinase is one of a number of proteolytic enzymes produced by *S. pyogenes*; others

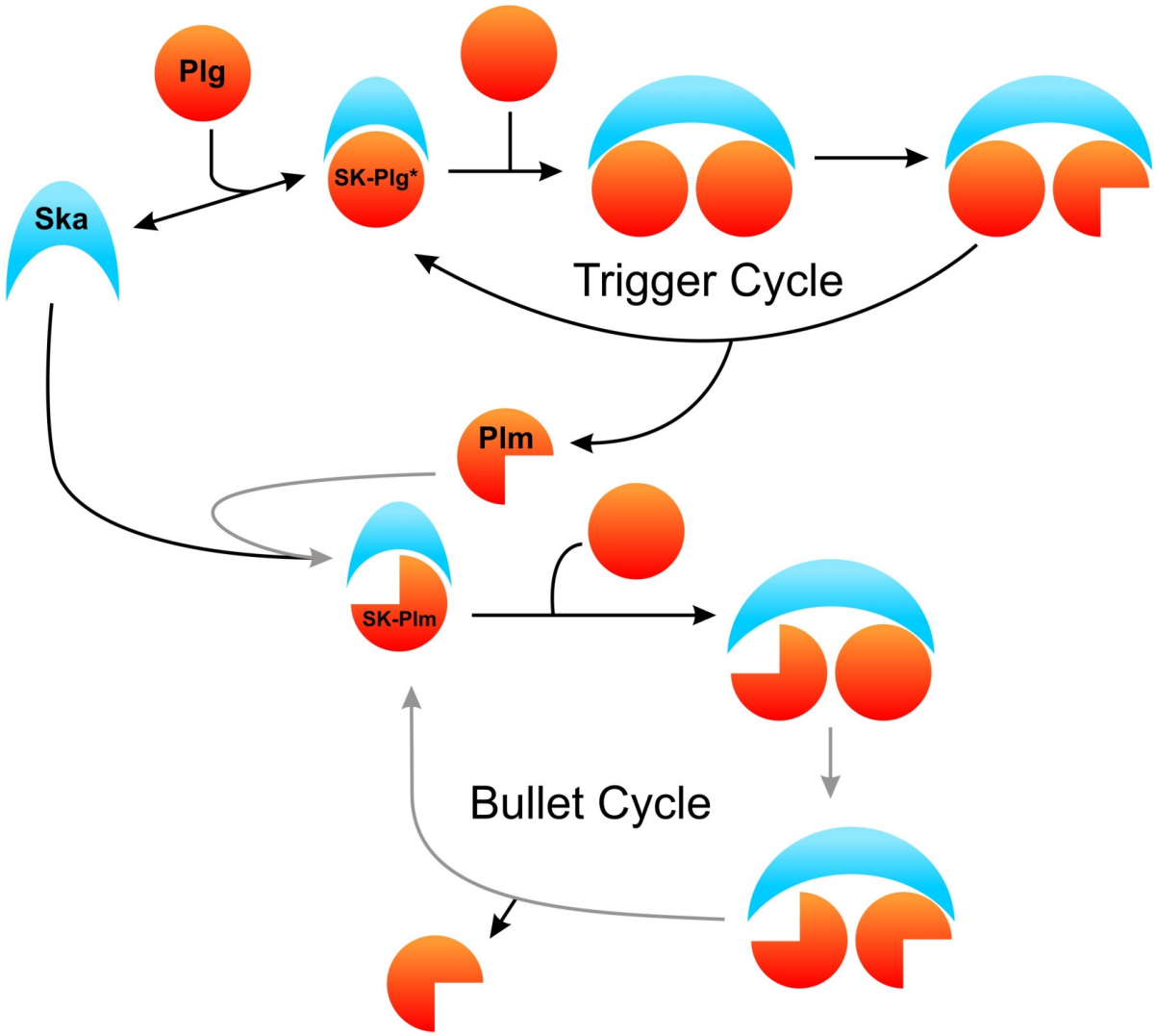

Figure 2: Activation of plasmin by streptokinase. Trigger cycle: Binding of plasminogen (Plg) by streptokinase (Ska) results in the formation of an activated SK-Plg* complex that binds another Plg molecule, which is cleaved to plasmin (PlM). Free Plg and Plm complete for Ska, with Plm binding being more favorable, due to the higher affinity of Plm (over Plg) for Ska, outcompeting the Plg binding, which results in termination of the trigger cycle and initiation of the bullet cycle. Bullet cycle: The resulting SK-Plm complex then binds Plg, which results in proteolytic activation into Plm.

include the C5a peptidase, the cell-associated aspartic apoproteinase or opacity factor (see the chapter on antibiotic resistance), a serine protease, and other cysteine proteinases, such as IdeS (Rasmussen & Björck, 2002).

The cysteine proteinase of *S. pyogenes* is one of the most studied of this organism's virulence factors, but its role in pathogenesis is still not fully understood (Nelson, Garbe, & Collin, 2011). The highly conserved gene for the proteinase is found in all strains of group A streptococci (Chaussee, Phillips, & Ferretti, 1997; Yu & Ferretti, 1991) and has been implicated in multiple studies as having a role in virulence, yet other studies suggest that it has no obvious role. The proteinase has broad specificity, as it is able to degrade a number of host proteins (such as cytokines, chemokines, complement components, various immunoglobulins, protease inhibitors found in the serum, and extracellular matrix components), as well as streptococcal proteins (Nelson, Garbe, & Collin, 2011).

One role of proteinases in bacterial virulence is attributed to their abilities to facilitate or enhance bacterial spread by degradation of tissue structure; this degradation allows for either the organism or its products to spread in the host. In the case of the streptococcal cysteine proteinase, different roles in virulence have been

suggested, including effects on degradation of host extracellular matrix material, effects on the immune system, and effects on proteins of the producer strain; however, other results contradict some of these findings (Hynes W. , 2004; Nelson, Garbe, & Collin, 2011; Walker, et al., 2014). The proteinase has been shown to be required for growth in saliva (Shelburne, 3rd, et al., 2005), the establishment of skin infections (Cole, et al., 2006; Svensson, et al., 2000), and virulence in a mouse model (Cole, et al., 2006). In the case of *S. pyogenes* M1T1, survival at the site of local infection requires SpeB (Cole, et al., 2006). However, SpeB also disrupts the interaction of *S. pyogenes* M1T1 with the human plasminogen activation system, which results in decreased systemic spread; decreased streptococcal proteinase activity allows for increased surface binding of plasmin, which can result in an increase in the systemic spread of the organism (Cole, et al., 2006; Sumby, Whitney, Graviss, Deleo, & Musser, 2006).

The cysteine proteinase also degrades human cathelicidin LL-37, a cationic peptide component of the innate immune system (Nyberg, Rasmussen, & Björck, 2004); this peptide is also degraded by surface-bound plasmin (Hollands, et al., 2012) that is partially controlled by the level of SpeB. Retention of proteinase activity at the bacterial surface protects against killing by the antibacterial peptide LL-37 (Nyberg, Rasmussen, & Björck, 2004). The enzyme can also be retained on the bacterial surface by being trapped by alpha$_2$-M-binding protein bound to the protein G-related alpha$_2$-M-binding protein (GRAB) (Rasmussen, Müller, & Björck, 1999); GRAB also protects virulence factors from degradation by both host and bacterial proteases. The proteinase also promotes cleavage of H-kininogen, which has a role in kinin activation and inflammation (Herwald, Collin, Müller-Esterl, & Björck, 1996). Another function assigned to the cysteine protease has been as an immunoglobulin-degrading enzyme that is capable of degrading IgG, IgA, IgM, IgD and IgE (Collin & Olsén, 2001a); however, recent results indicate that SpeB shows no immunoglobulin degrading activity under physiological conditions (Persson, Vindebro, & von Pawel-Rammingen, 2013). SpeB has also been suggested to play a role in APSGN, perhaps as the much sought-after nephritis-associated antigen; evidence suggests that SpeB is the major antigen involved in the pathogenesis of most cases of APSGN (Batsford, Mezzano, Mihatsch, Schlitz, & Rodríguez-Iturbe, 2005).

SpeB has many conflicting immune-response–related activities: it induces inflammation but possesses anti-inflammatory properties, it inhibits recruitment of neutrophils to an infection site while preventing degradation of neutrophil traps, it cleaves IgG while inhibiting other antibody-degrading enzymes, and it both activates and inhibits complement activation (Nelson, Garbe, & Collin, 2011). One question that still remains is whether the proteinase would be active under the physiological conditions found during an infection (Nelson, Garbe, & Collin, 2011).

Cysteine proteinase production is positively regulated by the two-component regulatory system CovRS (Walker, et al., 2014), with non-phosphorylated CovR repressing the expression of *speB* (Treviño, et al., 2009). Mutations in the CovRS system result in lack of production of the proteinase, and such variants have been shown to have an enhanced ability to evade innate immune responses (Li, et al., 2014). Recent results have shown that the divalent metals zinc and copper post-translationally inhibit SpeB activity (Chella Krishnan, Mukundan, Landero Figueroa, Caruso, & Kotb, 2014). The authors suggest that availability of zinc and/or copper in the bacterial microenvironment may modulate the SpeB activity that protects other virulence factors essential for bacterial survival and dissemination within the host. The proteinase has the ability to change the phenotype of the producing strain, not only by indirectly affecting transcription, but also by various post-translational events, such as releasing proteins from the bacterial surface, modifying them to altered forms (active or inactive), or degrading them to terminate activity (Nelson, Garbe, & Collin, 2011).

Cell envelope proteinase

Streptococcus pyogenes secretes a serine proteinase, SpyCEP, (*Streptococcus pyogenes* cell envelope protease, *prt*S) capable of cleaving and inactivating neutrophil chemokines in a specific manner (Lawrenson & Sriskandan, 2013); in particular the chemokine CXCL-8/IL-8, which the host uses to recruit polymorphonucleocytes when confronted with a microbial challenge. SpyCEP is able to cleave all human CXC chemokines that contain the

ELR motif (Lawrenson & Sriskandan, 2013; Zingaretti, et al., 2010); this specificity makes it unique among the many proteinases produced by group A streptococci. SpyCEP cleaves CXCL8/IL-8 at the C-terminal α-helix; this results in diminished function of this host-manufactured signal protein (Kurupati, et al., 2010).

SpyCEP has been determined to exist in secreted, as well as cell-associated forms; it is produced as a large immature form, 170kDa, which is composed of two polypeptide fragments (Zingaretti, et al., 2010). The protein undergoes an autocatalytic cleavage, but the two fragments of SpyCEP act in concert to form an active protease (Zingaretti, et al., 2010). SpyCEP is produced throughout exponential growth, as it is found in both the supernatant and in the cell wall (Turner, Kurupati, Jones, Edwards, & Sriskandan, 2009). The way in which the proteinase is released from the cell wall is unknown, but there is no evidence for either an autocatalytic release or cleavage mediated by an alternative protease (Lawrenson & Sriskandan, 2013). SpyCEP transcription appears to be under the regulation of the CovR/CovS two-component regulatory system (Sumby, et al., 2008).

A variety of pathogenic streptococci produce homologs of SpyCEP, including *S. equi*, *S. pneumoniae*, *S. agalactiae*, and *S. suis* (Lawrenson & Sriskandan, 2013). In addition, the C5a peptidase of *S. pyogenes* is considered to be a homolog of SpyCEP. The C5a peptidase, ScpA, is a cell-bound peptidase anchored to the cell wall by sortase A (Raz & Fischetti, 2008) that inactivates the complement factor C5a, which is responsible for stimulating polymorphonuclear leukocytes to migrate to the site of infection (Kagawa & Cooney, 2013). As discussed in the chapter on vaccines, C5a peptidase has shown promise as a potential vaccine component.

The widespread distribution of homologs of this proteinase suggests more than a passing involvement in pathogenesis, although studies with mutants that lack SpyCEP have found differing results (Hidalgo-Grass, et al., 2006; Kurupati, et al., 2010; Sjölinder, et al., 2008; Sumby, et al., 2008). Studies have demonstrated that the expression of SpyCEP both impedes bacterial clearance and assists in bacterial spread to the regional lymph node, as well as in systemic circulation. When SpyCEP was introduced into and expressed by *L. lactis*, hallmark features of a group A streptococcal-induced systemic infection occurred in a mouse model using the strains expressing SpyCEP, but not the wild-type *L. lactis* strain (Kurupati, et al., 2010).

Esterase

Streptococcus secreted esterase, SsE, is an extracellular product that appears to have a role in virulence and pathogenesis, being essential for invasive infections and systemic dissemination (Zhu, Liu, Sumby, & Lei, 2009). SsE is a carboxylic acid esterase similar to that found in other organisms that hydrolyzes platelet-activating factor (PAF). PAF is a receptor and serves as a phospholipid mediator manufactured by endothelial cells, neutrophils, macrophages, and granular eosinophiles. PAF mediates IL-12-induced chemotaxis of natural killer cells and neutrophils, and can induce the migration of neutrophils to an exposed endothelium. SsE has been demonstrated to diminish the solicitation and the recruitment of neutrophils, which provides a mechanism for bacterial evasion of the host immune system (Liu, et al., 2012). The esterase has been shown to be important in the virulence of group A streptococci, as it has an important role in subcutaneous infections and dissemination from the skin, though its role has yet to be fully determined (Zhu, Liu, Sumby, & Lei, 2009).

Two distinct variants of SsE have been reported, and are described as either complex I or complex II. Complex I esterases are produced by serotypes M1, M2, M3, M5, M6, M12, and M18, while complex II proteins are produced by serotypes M4, M28, and M49. The two SsE complexes share greater than 98% identity in their amino acid sequence, but can have a sequence variation of up to 37% between the complexes. SsE appears to be under the control of the CovR/CovS two-component regulatory system (Liu, Liu, Xie, & Lei, 2013), as it is negatively controlled by the regulator (Zhu, Liu, Sumby, & Lei, 2009).

Streptococcal Hemolysins

Streptococcus pyogenes secretes two well-known hemolysins, streptolysin O and streptolysin S, which have effects on a variety of cell types. Other putative hemolysins, including CAMP factor, may also be encoded in the genomes of group A streptococci (Ferretti, et al., 2001), but their role in bacterial growth and virulence remains to be determined.

Streptolysin O

Streptolysin O (SLO) is a pore-forming, cholesterol-dependent, oxygen-labile, thiol-activated cytotoxin (Tweten, 2005). Similar types of hemolysins are produced by a variety of other pathogens, and the structure of SLO is similar to these other cholesterol-dependent cytolysins, but there are also some differences (Feil, Ascher, Kuiper, Tweten, & Parker, 2014). One difference is in the binding of the cytolysins to cholesterol-rich membranes, where there is a structural difference in the membrane-binding interface between SLO and perfringolysin O (Farrand, et al., 2015). The SLO hemolysin is 69 kDa in size, which is subject to N-terminal cleavage by the cysteine proteinase (Pinkney, et al., 1995). The hemolysin is produced with a 70-residue N-terminal region that is required for the translocation of another streptococcal product, the NAD-glycohydrolase (*nga*) into host cells (Madden, Ruiz, & Caparon, 2001); with *slo* and *nga* being co-transcribed (Madden, Ruiz, & Caparon, 2001). Streptolysin O pore formation occurs in stages, including cholesterol-dependent binding of monomeric forms to the cell membrane, followed by oligomerization, which results in the development of pores (Bhakdi & Tranum-Jensen, 1985; Bhakdi, Tranum-Jensen, & Sziegoleit, 1985). In addition to cholesterol, the membrane-binding domain of SLO also implicates a glycan (galactose) receptor involvement in binding and pore formation (Mozola & Caparon, 2015; Shewell, et al., 2014). These pores result in disruption of the integrity of host cell membranes and induce apoptosis (Timmer, et al., 2009). An alternate pathway utilized by *S. pyogenes* adhering to cells does not involve the galactose receptor, but an unknown receptor that associates with the streptococcal NAD-glycohydrolase (NADase, Nga, or SPN); this results in translocation of the NADase and orients the SLO, which allows for pore formation (Mozola & Caparon, 2015). Streptolysin O has also been shown to induce intracellular Ca^{2+} oscillations that result from the depletion of intracellular stores and activation of store-operated Ca^{2+} in host cells, the mechanisms of which remain unknown (Usmani, et al., 2012).

An interesting function of SLO is that it is required for the transfer of streptococcal NAD-glycohydrolase into epithelial cells (Madden, Ruiz, & Caparon, 2001). The NADase of group A streptococci is encoded by the *nga* gene, which is found next to the *slo* gene, and was originally reported as being an unusual glycohydrolase with three activities. However, on reevaluation, it was shown to possess only the β-NAD hydrolytic activity (Ghosh, Anderson, Chandrasekaran, & Caparon, 2010). Like SLO, NADase is encoded on a 36 kb chromosomal region of M1T1 that was acquired prior to the global dissemination of the invasive clone commonly found in developed countries (Walker, et al., 2014).

The structure of NADase showed two functional domains; the amino terminal domain responsible for translocation via the SLO-mediated system, and the carboxyl terminal domain that contains the NADase activity (Ghosh & Caparon, 2006). However, pore formation, per se, by SLO is not required for the transport of the NADase (Magassa, Chandrasekaran, & Caparon, 2010). The role of NADase is not fully understood, but data suggests its function in pathogenesis is as a NAD-glycohydrolase (Ghosh, Anderson, Chandrasekaran, & Caparon, 2010) when injected across the membrane into the cytoplasm, using the cytolysin mediated translocation pathway. Once in the cytoplasm, the toxin functions to deplete the intracellular pool of NAD (Ghosh, Anderson, Chandrasekaran, & Caparon, 2010; Yoon, et al., 2013). In *S. pyogenes*, the SLO operon encodes genes for NAD-glycohydrolase (*nga*) and its inhibitor (*ifs*) (Kimoto, Fujii, Hirano, Yokota, & Taketo, 2006; Madden, Ruiz, & Caparon, 2001). As bacterial cells are also susceptible to the action of NADase, the immunity factor for the glycohydrolase functions as a competitive inhibitor of enzyme function (Kimoto, Fujii, Hirano, Yokota, & Taketo, 2006). The intracellular enzyme-inhibitor complex is dissociated during transport

through the streptococcal membrane and into the host cell (Yoon, et al., 2013). Although all strains appear to possess the *nga* gene, some isolates produce a protein that lacks NADase activity (Ajdic, McShan, Savic, Gerlach, & Ferretti, 2000). NADase without activity had a toxic effect on *E. coli* but not on *S. pyogenes*; the reason for this is currently unknown, and has been attributable to an unknown NADase-independent function of the protein. Similarly, little is known about any role of this form of the protein in pathogenesis, although NADase has been associated with cytotoxic activity (Michos, et al., 2006); no association is seen between NADase subtype (active or inactive) and particular disease category or invasiveness (Riddle, Bessen, & Caparon, 2010). Additionally, no association was found between the NADase-inactive *nga* allele and virulence in a mouse infection model (Tatsuno, Isaka, & Hasegawa, 2013). Notably, there does appear to be an association between tissue tropism and NADase subtype where *emm*-types that act as generalists (those that infect both throat and skin) tend to be NADase-active, while the specialists (those that infect either throat or skin) are associated with production of NADase-inactive forms (Riddle, Bessen, & Caparon, 2010).

Delivery of NAD-glycohydrolase to the cytoplasm of human cells results in major changes in host cell biology that enhance streptococcal pathogenicity and intracellular survival (Bricker, Cywes, Ashbaugh, & Wessels, 2002; O'Seaghdha & Wessels, 2013). Mutants deficient in SLO and/or NADase also showed impaired survival in macrophages, with both proteins being necessary for resistance to macrophage-mediated killing; survival was mediated by preventing the acidification of the phagolysosome (Bastiat-Sempe, Love, Lomayesva, & Wessels, 2014). When taken up by a macrophage, the phagosome that contains bacteria fuses with a cellular lysosome, creating a phagolysosome, which normally results in destruction of an invading bacterium. With *S. pyogenes*, SLO and NADase are secreted into the phagolysosome; SLO prevents acidification, while the NADase is translocated into the macrophage cytosol, via a specific translocation mechanism that involves the N-terminal domain of the protein, where it hydrolyzes NAD and thereby interferes with the cell's ability to repair damage to the phagolysosome membrane (Bastiat-Sempe, Love, Lomayesva, & Wessels, 2014).

In addition to acting as a means of introducing NADase into host cells, other roles for SLO in *S. pyogenes* pathogenesis have been reported. SLO mutants appear attenuated for virulence (Walker, et al., 2014), with mutants varying in their ability to have an effect or cause disease, with higher expression levels being seen in invasive isolates compared to non-invasive isolates (Ato, Ikebe, Kawabata, Takemori, & Watanabe, 2008). SLO activity enhances mucosal inflammation through tissue destruction (Brosnahan & Schlievert, 2011; Reglinski & Sriskandan, 2014); it also induces co-aggregation of platelets and neutrophils, which may be important in tissue viability in severe infections that involve tissue destruction (Bryant, et al., 2005). Expression of SLO is also required for induction of caspase-1-dependent release of IL-1β, which is important in the development of an inflammatory and immune response (Harder, et al., 2009). Additionally, SLO activates human polymorphonuclear neutrophils that can result in an exaggerated host response (Nilsson, et al., 2006), as well as modulates cytokine synthesis in human peripheral blood mononuclear cells (Stevens & Bryant, 1997). In addition, unlike the other major streptococcal hemolysin (SLS), SLO induces an immune response during infection, and antistreptolysin O antibodies are still used to confirm streptococcal infections (Sheeler, Houston, Radke, Dale, & Adamson, 2002). SLO is extremely toxic, but its immunogenicity could make it useful in vaccine development. Mutated SLO that lacks hemolytic activity has been shown to reduce virulence and has a decreased capacity to destroy immune cells in mouse models of infection. When the mutated version of the toxin was used to immunize mice, the toxoid provided protection against the wild-type strain by antibody-mediated neutralization (Chiarot, et al., 2013).

S. pyogenes can be internalized by epithelial cells as part of the host defense; SLO has been shown to interfere with this internalization process through membrane perturbation and disruption of the clathrin-dependent uptake pathway (Logsdon, Håkansson, Cortés, & Wessels, 2011). Mutation of single amino acids within the structural domains of SLO can affect activity; a double mutant was found to have no toxicity with an impaired ability to bind to eurkayotic cells and was unable to form the required oligomeric structures in the membrane (Chiarot, et al., 2013).

Full expression of SLO and the NADase in non-immune human blood requires the regulator CodY, with decreased transcript levels of each protein being seen in a CodY mutant in an incubation-time–dependent manner (Malke & Ferretti, 2007).

Streptolysin S

Streptolysin S, SLS, is the second type of hemolysin produced by *S. pyogenes*, and was originally extracted from streptococcal cells grown in the presence of serum, hence its name. It is a member of a family of proteins known as the thiazole/oxazole-modified microcins, which are produced by a number of pathogens that show hemolytic exotoxin activity (Molloy, Cotter, Hill, Mitchell, & Ross, 2011). SLS is an oxygen stable cytotoxin that forms hydrophilic pores in a variety of cell types from both the innate and adaptive immune systems, including erythrocytes, leukocytes, and platelets. In addition, pores are formed in sub-cellular organelles (Ginsburg, 1972; Miyoshi-Akiyama, et al., 2005; Molloy, Cotter, Hill, Mitchell, & Ross, 2011; Ofek, Bergner-Rabinowitz, & Ginsburg, 1970). However, while protoplasts and spheroplasts are lysed by SLS, bacteria with intact cell walls are not (Bernheimer, 1966). Although its toxicity is not fully understood, it is believed that SLS acts through the accumulation of proteins in the membrane, which leads to transmembrane pore formation that results in osmotic lysis, in a mechanism similar to that mediated by complement (Carr, Sledjeski, Podbielski, Boyle, & Kreikemeyer, 2001).

SLS is a 2.7 kDa ribosomally synthesized, post-translationally, and extensively modified (prior to export) peptide (Molloy, Cotter, Hill, Mitchell, & Ross, 2011) encoded by a nine-gene operon locus (*sagA* to *sagI*) (Nizet, et al., 2000). The modifications result in the formation of a peptide with an unusual structure (a heterocyclic compound) that is only cytolytic when associated with a cell surface or in the presence of "carrier" molecules (Molloy, Cotter, Hill, Mitchell, & Ross, 2011). It has recently been shown that some HIV protease inhibitors are able to stop SLS production by blocking a proteolytic cleavage, by SagE, in SLS production (Maxson, et al., 2015). Agents such as these can provide reversible control of production, which may allow for investigation of the role of SLS in virulence without the need for genetic manipulation of the bacteria. The peptide, either because of its small size or modified structure, is not immunogenic when produced during an infection (Dale, Chiang, Hasty, & Courtney, 2002), however, antibodies raised to a synthetic peptide were able to neutralize the hemolytic activity of SLS (Dale, Chiang, Hasty, & Courtney, 2002).

Streptolysin S contributes to the pathogenesis of *S. pyogenes* in a variety of ways, including cytotoxicity, activation inflammatory response, and inhibition phagocytosis (Ginsburg, 1999). SLS-deficient mutants generated from clinical isolates by transposon mutagenesis showed reduced virulence, as compared to the parental strains (Betschel, Borgia, Barg, Low, & De Azavedo, 1998). Both of the streptolysins (SLO and SLS) have been reported to enhance virulence, with SLS enhancing the virulence of acapsular *S. pyogenes* (Sierig, Cywes, Wessels, & Ashbaugh, 2003). Although SLS mutants show reduced virulence in lab situations (tissue culture or animal models), non-hemolytic mutants that result from naturally occurring mutations or deletions in the SLS biosynthetic operon are still able to cause severe soft tissue infections (Jantsch, et al., 2013), pharyngeal infections, and otitis media (Yoshino, et al., 2010).

SLS is considered to be one of the two major secreted virulence factors of *S. pyogenes*, along with the cysteine protease, SpeB. The role of each of these in virulence is still somewhat unclear, but recent results have indicated that they act synergistically in mouse models; SLS is important to mouse mortality, while SpeB is more important to local tissue damage (Hung, et al., 2012). One possible function for SLS is facilitation of invasion of the pathogen through degradation of epithelial intercellular junctions (Sumitomo, et al., 2011); this degradation would occur with the assistance of streptococcal proteinases.

CAMP Factor

The CAMP factor of *S. pyogenes*, encoded by the *cfa* gene, was first identified during the sequencing of the genome of an M1 strain (Ferretti, et al., 2001), and was confirmed to be an active CAMP factor (Gase, Ferretti, Primeaux, & McShan, 1999). The CAMP reaction is a synergistic process that involves a sphingomyelinase from *Staphylococcus aureus* and a cohemolysin (CAMP factor) from various streptococci; this reaction was first reported to occur with *S. agalactiae* (Podbielski, Blankenstein, & Lütticken, 1994). The CAMP factor appears to be widespread among the streptococci, as it is present in groups A, B, C, G, M, P, R, and U (Gase, Ferretti, Primeaux, & McShan, 1999). The group A streptococcal CAMP factor is widespread among isolates; 82% of strains tested produced an active CAMP factor with 99% of the strains that contained the gene (Gase, Ferretti, Primeaux, & McShan, 1999). The CAMP factor is at least partially controlled by CodY, with its expression being decreased in a CodY mutant during growth in blood (Malke & Ferretti, 2007). The carbon catabolite repressor protein, CcpA, also has a regulatory effect on *cfa* regulation, as it is growth-phase regulated with increased expression in the stationary phase (Kietzman & Caparon, 2010). Another nutritional related regulatory protein, CvfA, can also affect *cfa* expression (Kang, Caparon, & Cho, 2010).

DNases

S. pyogenes is known to produce up to four DNases as extracellular products. A potent DNase produced by the M1T1 globally disseminated clone (Nasser, et al., 2014; Walker, et al., 2014) is the bacteriophage-encoded SdaD2, and the major DNase that contributes to virulence (Sumby, et al., 2005). This enzyme protects *S. pyogenes* from neutrophil-mediated killing by degrading DNA-based neutrophil extracellular traps (Buchanan, et al., 2006; Walker, et al., 2007). These traps are an innate response that binds bacteria to prevent them from spreading, and in doing so, ensures the presence of antimicrobial agents to kill or degrade the bacteria and their virulence factors (Brinkmann, et al., 2004). The SdaI DNase may play a role in *S. pyogenes* hyper-invasiveness in some genetic backgrounds, but not all, which indicates that severe diseases can result from the production of a balance of different virulence factors (Cole, Barnett, Nizet, & Walker, 2011; Venturini, et al., 2013). SdaI also shows a novel innate immune system evasion mechanism, where the enzyme destroys CpG-rich DNA and suppresses the TLR-9 mediated response (Uchiyama, Andreoni, Schuepbach, Nizet, & Zinkernagel, 2012).

The expression of *sda*1 is negatively regulated by CovRS (Walker, et al., 2014); it is upregulated in a *cov*RS mutant (Sumby, Whitney, Graviss, Deleo, & Musser, 2006). However, under oxidative stress, *sda*1 expression is PerR-dependent (Wang, et al., 2013); PerR, the peroxide regulator, is a transcription factor involved in metal homeostasis and oxidative stress in *S. pyogenes* (Brenot, Weston, & Caparon, 2007; Wang, et al., 2013).

Another bacteriophage-encoded DNase is SpdI1, a type 1 extracellular, non-specific metal-dependent nuclease expressed during prophage induction (Korczynska, Turkenburg, & Taylor, 2012). This DNase is able to degrade both single- and double-stranded DNA, as well as RNA (Korczynska, Turkenburg, & Taylor, 2012). It is also co-expressed with the phage-encoded streptococcal pyrogenic exotoxin C (SpeC) (Broudy, Pancholi, & Fischetti, 2002). The prophage-encoded Spd1 was found in *emm*12 strains isolated from an ongoing scarlet fever outbreak in Hong Kong (Tse, et al., 2012; Walker, et al., 2014).

Other nucleases include streptodornase B or mitogenic factor 1 (SdaB or Mf-1) and the bacteriophage encoded Spd-3 found in the chromosome of the M49 strain NZ131 (McShan, et al., 2008). Note that with these genes that if the global regulator CodY is deleted, SdaB is more abundant, but Spd-3 is less abundant (McDowell, Callegari, Malke, & Chaussee, 2012). Another transcriptional regulator, Rgg (also known as RopB) represses transcription of streptodornase B and *spd*-3 by directly controlling the expression of these genes (Anbalagan & Chaussee, 2013; Anbalagan, McShan, Dunman, & Chaussee, 2011). The role of DNases in *S. pyogenes* infections remains to be fully investigated.

SpnA (*Streptococcus pyogenes* nuclease A) is a cell-bound nuclease (Hasegawa, et al., 2010) reported to be involved in the escape of the bacteria from neutrophil extracellular traps (Chang, Khemlani, Kang, & Proft, 2011). This nuclease, like the others, plays a role in streptococcal survival and appears to be expressed during infection, since antibodies to the protein are detected in convalescent serum (Chang, Khemlani, Kang, & Proft, 2011).

After years of declining streptococcal infections, a resurgence of severe infections occurred in the late 1980s and early 1990s. This resurgence was the result of a global dissemination of an M1T1 clone, which accounted for a significant portion of isolates from developed countries (Walker, et al., 2014). This invasive clone was derived from a progenitor strain by acquisition of bacteriophages containing an extracellular DNase, which were then acquired in a stepwise manner by the superantigen A1 variant of the streptococcal pyrogenic exotoxin (SpeA); this was followed by evolution to the A2 variant of the toxin, prior to acquisition of a 36 kb region encoding genes for SLO and NAD-glycohydrolase (Cole, Barnett, Nizet, & Walker, 2011; Maamary, et al., 2012; Nasser, et al., 2014; Venturini, et al., 2013).

Hyaluronate lyase / hyaluronidase

S. pyogenes encodes proteins that are classified as hyaluronidases. These include the chromosomally-encoded hyaluronate lyase, HylA, and the bacteriophage-encoded hyaluronidases, HylP (Hynes, 2004); these phage-encoded enzymes are also hyaluronate lyases (El-Safory, Lee, & Lee, 2011). The hyaluronidase-type enzymes have been classified as virulence factors, based on their ability to aid in the spread of the organism or its proteins and toxins. Another protein that was originally classified as a hyaluronidase, Spy1600, in the genome of M1 strain SF370 (Ferretti, et al., 2001) and other sequenced strains, is a β-N-acetylglucosaminidase and not a hyaluronidase (Sheldon, et al., 2006).

Chromosomally encoded hyaluronate lyase

Certain strains of *S. pyogenes* express and secrete an active hyaluronate lyase (Hynes & Walton, 2000). Production of active hyaluronate lyase was originally reported to be associated with certain serotypes, particularly M-types 4 and 22 (Crowley, 1944). Subsequent work indicated that the production was strain-associated, rather than being related to serotype (Benchetrit, Avelino, & de Oliveira, 1984). Genomic studies showed that the *hyl*A gene is present in all *S. pyogenes* strains sequenced; however, not all of these produce an enzymatically active product (Hynes, Dixon, Walton, & Arigides, 2000; Hynes, Hancock, & Ferretti, 1995; Starr & Engleberg, 2006). There are differences in the *hyl*A genes, with three gene structures having been reported: full length, truncated, and those that contain a deletion (Hynes, Johnson, & Stokes, 2009); this is different from the enzymatically active N-terminal degradation products seen with *S. agalactiae* (Gase, Ozegowski, & Malke, 1998). An additional difference reported in the group A streptococcal hyaluronate lyases is a point mutation that changes amino acid 199 of HylA from an aspartic acid residue to a valine residue; this change results in a loss of enzymatic activity (Hynes, Johnson, & Stokes, 2009). The aspartic acid residue is present in *emm*4 and *emm*22 HylA protein; namely, those serotypes that produce active enzymes. Of interest is that both these serotypes lack the ability to produce a hyaluronic acid capsule, due to the loss of the hasABC genes required for capsule production (Flores, Jewell, Fittipaldi, Beres, & Musser, 2012; Henningham, et al., 2014). This could partially address the often-asked question as to why an organism would produce both a protective shield (hyaluronic acid capsule) and an enzyme (hyaluronate lyase) capable of destroying that protection. It has been suggested that hyaluronate lyase may be an anti-virulence factor for those organisms involved in severe invasive diseases by making it more susceptible to phagocytosis (Hynes, Johnson, & Stokes, 2009; Starr & Engleberg, 2006). Despite the loss of the capsule-producing ability by serotypes 4 and 22, they are still pathogenic and are able to proliferate in blood (Flores, Jewell, Fittipaldi, Beres, & Musser, 2012; Henningham, et al., 2014). It appears that these serotypes have developed alternative methods of virulence and that a hyaluronic acid capsule is not needed to colonize the upper respiratory tract, to cause mucosal infections, or even to cause invasive infections (Flores,

Jewell, Fittipaldi, Beres, & Musser, 2012). In these cases, the hyaluronate lyase activity may be more important for the diffusion of bacterial toxins and other proteins, rather than for the overall spread of the organism (Starr & Engleberg, 2006). Recent evidence suggests that hyaluronate lyase may also limit infections because of increased uptake of bacterial cells by macrophages; an effect not only of the bacterial capsule, but also controlled by host hyaluronan (Schommer, Muto, Nizet, & Gallo, 2014). Additionally, Henningham et al. found a mutual exclusivity of capsule and hyaluronate lyase activity (Henningham, et al., 2014). If an active *hyl*A gene was introduced into an M1 strain, capsule expression was abolished (Henningham, et al., 2014); if capsule genes were added to an M4 isolate, the amount of capsule was significantly less in the presence of an active *hyl*A gene. Currently, there is no evidence of a direct relationship between hyaluronate lyase activity and disease.

In addition to being a possible virulence factor, hyaluronate lyase may be important for supplying nutritional needs to a producing cell, at least in those strains that are capable of producing active enzyme (Starr & Engleberg, 2006). Regulation of genes in *S. pyogenes* in response to nutritional stress is in part controlled by CodY and this gene, at least in part, regulates *hyl*A expression; levels of HylA being decreased in a CodY mutant (McDowell, Callegari, Malke, & Chaussee, 2012). In *Streptococcus pneumoniae,* hyaluronidase production is regulated by the global LacI/GalR family regulator, RegR (Chapuy-Regaud, et al., 2003). A similar RegR gene appears to control the expression of *hyl*A in *S. pyogenes* (Sloan & Hynes, 2015).

Bacteriophage-encoded hyaluronate lyase

Many (if not all) strains of *S. pyogenes* carry one or more prophage genomes that encode a number of potential virulence factors (Suvorov, Polyakova, McShan, & Ferretti, 2009). The prophage genomes encode a hyaluronate lyase, which, when produced, is associated with the phage tail fibers. The role of the phage hyaluronate lyases may be to allow the phage to penetrate the hyaluronic acid capsule and allow for its attachment to appropriate receptors (Baker, Dong, & Pritchard, 2002; Hynes & Ferretti, 1989; Hynes, Hancock, & Ferretti, 1995; Niemann, Birch-Andersen, Kjems, Mansa, & Stirm, 1976). The fact that temperate phages are able to establish infections in encapsulated strains may have played an intriguing role in the development of streptococcal virulence. These phages would be capable of infecting, lysogenizing, and laterally transferring virulence factors into encapsulated strains, resulting in increased virulence as proposed for the evolutionary events that led to the development of the epidemic M1 clone (Nasser, et al., 2014). In contrast to their lysogenic cousins, virulent phages are unable to infect encapsulated strains of group A streptococci unless the capsule is removed (McClean, 1941). This leaves the virulent phages capable of infecting, and killing, unencapsulated and less virulent isolates.

The phage-encoded hyaluronate lyase enzymes have no similarity to the chromosomally encoded lyases. Bacteriophage hyaluronate lyases from *S. pyogenes* show polymorphisms resulting at least in part from intragenic recombinational events (Marciel, Kapur, & Musser, 1997). One major area of variation between the phage lyases is the presence or absence of the Gly-X-Y motif (Hynes & Ferretti, 1989; Hynes, Hancock, & Ferretti, 1995). This repeating motif has been suggested to play a role in stabilizing the enzyme structure (Stern & Stern, 1992), while structures of the lyases with and without the repeat motif have recently been resolved (El-Safory, Lee, & Lee, 2011; Singh, Malhotra, & Akhtar, 2014). Whether or not the phage-encoded hyaluronate lyases play a direct role in pathogenesis is unclear. Antibodies to the lyases have been detected following infection (Halperin, Ferrieri, Gray, Kaplan, & Wannamaker, 1987), which indicates that these lyases are produced. The phage enzyme may add to the overall virulence of the bacteria through functioning as an additional spreading factor (Hynes, 2004).

Streptococcal inhibitor of complement

Streptococcal inhibitor of complement (SIC) is a 31 kDa protein found in M1 strains of *S. pyogenes* (Akesson, Sjöholm, & Björck, 1996; Frick, Akesson, Rasmussen, Schmidtchen, & Björck, 2003). The *sic* gene and resulting protein are highly polymorphic within the M1 strains examined (Hoe, et al., 2001; Stockbauer, et al., 1998). The protein inhibits complement-mediated lysis by inhibiting the formation and function of the membrane attack

complex (Akesson, Sjöholm, & Björck, 1996; Fernie-King, Seilly, Davies, & Lachmann, 2002; Fernie-King, et al., 2001), as well as the activity of other immune response antibacterial proteins involved in bacterial clearance, including lysozyme, LL-37, defensins (Fernie-King, Seilly, Binks, Sriprakash, & Lachmann, 2007; Frick, Akesson, Rasmussen, Schmidtchen, & Björck, 2003), the chemokine MIG/CXCL9 (Egesten, et al., 2007), and some bacterial antimicrobial products (Minami, et al., 2009). Recently, SIC was shown to interfere with the activation of the contact system of the innate immune system (Frick, et al., 2011); when activated, this system generates antimicrobial peptides (Frick, et al., 2006).

The ability to interfere with the complement and contact systems of the host indicate that in those strains that produce SIC, this protein will enhance both virulence and dissemination of the bacteria (Frick, et al., 2011). In addition, SIC contributes to adherence, colonization, and bacterial survival by altering cellular processes that are critical to efficient streptococcal contact, internalization, and killing (Hoe, et al., 2002; Lukomski, et al., 2000). The variation seen in the SIC gene during an epidemic indicates a rapid selection (Hoe, et al., 1999), and suggests that exposure to the immune response during infection results in changes that will enhance bacterial survival.

In addition to SIC, some closely related variants have been reported; CRS (closely related to SIC) variants have been reported in M57 isolates (Binks, McMillan, & Sriprakash, 2003), and distantly related to SIC (DRS) variants in the *emm*12 and *emm*55 (Binks & Sriprakash, 2004). DRS has also been reported to have similar functions in *S. dysgalactiae* subsp. *equisimilis* (Smyth, et al., 2014).

Secretion of extracellular products by *S. pyogenes*, and particularly toxins, plays a major role in pathogenesis. In many cases, the secretion of these toxins requires factors in addition to the Sec translocation pathway; however, SIC may not require additional factors (Vega & Caparon, 2012). Cationic antimicrobial peptides are able to inhibit the secretion of SpeB and SLO through the ExPortal, while the secretion of SIC, which protects the streptococci from such peptides, was unaffected.

Superoxide dismutase

S. pyogenes produces a metalloprotein, superoxide dismutase (SodA), the function of which is to convert superoxide anions to oxygen and hydrogen peroxide; the hydrogen peroxide can then be detoxified by peroxidases (Grifantini, Toukoki, Colaprico, & Gryllos, 2011). SodA has been found on both streptococcal cell surfaces and in culture supernatants (McMillan, Davies, Good, & Sriprakash, 2004). As *S. pyogenes* does not produce a catalase, SodA plays a vital role in detoxifying the oxidative burst produced by host white cells in response to the detection of these pathogenic bacteria. It has recently been shown that these bacteria also produce a glutathione peroxidase (GpoA), which enhances the organism's ability to survive oxidative stress (Kwinn & Nizet, 2007) during phagocytosis.

Ig binding proteins

S. pyogenes encodes a number of surface-associated, immunoglobulin-binding proteins, including M proteins, M-related proteins, and M-like proteins. These proteins bind the Fc portion of the immunoglobulin molecules: M and M-like proteins bind Fc region IgA, while the M-related proteins bind the Fc portion of IgG (Carlsson, Berggård, Stålhammar-Carlemalm, & Lindahl, 2003; Stenberg, O'Toole, & Lindahl, 1992; Walker, et al., 2014). The role of these surface-bound molecules is unclear, but they most likely play a role in immune system evasion.

A highly conserved secreted immunoglobulin-binding protein, SibA, has been reported to be present in most strains of group A streptococci from a variety of different serotypes (Fagan, Reinscheid, Gottschalk, & Chhatwal, 2001). The SibA product is a 45 kDa protein that binds the Fc and Fab region of IgA, IgG, and IgM (Fagan, Reinscheid, Gottschalk, & Chhatwal, 2001; Walker, et al., 2014). Sequence-wise, it lacks homology to M protein,

although structurally it has the N-terminal alpha-helical secondary structure that is implicated in Ig binding by M protein (Fagan, Reinscheid, Gottschalk, & Chhatwal, 2001).

IdeS/MAC

The immunoglobulin-degrading enzyme of *S. pyogenes*, IdeS, is a secreted cysteine proteinase that specifically cleaves the hinge region of IgG (Akesson, Moritz, Truedsson, Christensson, & von Pawel-Rammingen, 2006; von Pawel-Rammingen, Johansson, & Björck, 2002); this proteinase is also known as Mac-1, Sib35, and MspA (Walker, et al., 2014). The proteinase is not essential for phagocyte resistance or virulence in mice (Okumura, et al., 2013). The proteinase preferentially cleaves Fab-bound IgG while allowing non-specifically bound IgG to remain attached to M protein. In doing so, this may aid the streptococcus in resisting phagocytosis and cytotoxicity through antibody-mediated processes (Su, et al., 2011). Mac-2 is a related IgG endopeptidase that prevents the recognition of IgG bound to *S. pyogenes* by competitively blocking IgG from recognition by Fc receptors on host cells (Agniswamy, Lei, Musser, & Sun, 2004). Given that IdeS/Mac have homologs across group A streptococcal strains, it is possible that these proteinases play some other role in streptococcal survival, and under different conditions or in other strains, the proteinases do contribute to virulence (Okumura, et al., 2013). Like a number of the other virulence factors, IdeS is regulated by CodY; when grown in the presence of blood, its expression is reduced in CodY mutants (Malke & Ferretti, 2007).

Another extracellular anchorless immunoglobulin protein, Sib35, has been reported to be present in all strains of *S. pyogenes* examined, but not in other streptococci (Kawabata, et al., 2002). This protein was 35 kDa in size and different from the extracellular SibA (Kawabata, et al., 2002); Sib35 was found extracellularly, as well as on the surface of the cell. The protein was shown to bind IgG, IgA, and IgM, and had similarity to IdeS, the IgG-degrading enzyme (Okamoto, Tamura, Terao, Hamada, & Kawabata, 2005). In addition to the binding of immunoglobulins, this protein was shown to induce B-cell proliferation, as well as differentiation into immunoglobulin-producing plasma cells (Okamoto, Terao, Tamura, Hamada, & Kawabata, 2008). Patients with streptococcal infections had a higher antibody titer to Sib35 than healthy volunteers, which indicates that the protein is produced during an infection. When used to vaccinate mice, those that received Sib35 were found to have enhanced survival rates, as compared to controls, when challenged with group A streptococci (Okamoto, Tamura, Terao, Hamada, & Kawabata, 2005).

EndoS (endo-β-*N*-acetylglucosaminidase)

EndoS is a large (108 kDa) endoglycosidase Ig-degrading enzyme that removes carbohydrates from immunoglobulin G in a highly specific manner (Trastoy, et al., 2014; Walker, et al., 2014). The activity of the enzyme is such that it only hydrolyzes the glycan moiety on native, but not denatured, IgG (Collin & Olsén, 2001a; Collin & Olsén, 2001b). This enzyme enhances survival by reducing binding of IgG to the Fc receptors and impairing complement activation (Collin, et al., 2002). The crystal structure of EndoS has recently been determined (Trastoy, et al., 2014), which will allow for its investigation as a potential therapeutic agent. EndoS aids *S. pyogenes* in evading the host response; understanding the structure of the protein aids in determining potential inhibitors of the activity that can be used to inhibit activity, and therefore potentially improve clinical outcomes. One possible novel use of EndoS is as a treatment in the reduction of the proinflammatory properties of immune complexes in systemic lupus erythematosus patients (Lood, et al., 2012).

A variant of EndoS has been reported to be found exclusively in serotype M49 strains; this is referred to as $EndoS_2$ (Sjögren, et al., 2013). The *ndo*S gene from M1 and *ndo*S2 gene from M49 are 53% identical, and the proteins are 37% identical (Sjögren, et al., 2013). $EndoS_2$ activity differs from EndoS activity by hydrolysis of N-linked glycans on native IgG heavy chains and α_1-acid glycoproteins (Sjögren, et al., 2013); EndoS is specific for the native form of IgG (Collin & Olsén, 2001a). The hydrolysis of two immune system components by $EndoS_2$ suggests a possible role in host immunomodulation and pathogenesis during M49 serotype infections.

In mutagenesis studies in which the EndoS gene was knocked out in an M1T1 isolate, no difference in bacterial survival was seen with immune cell-killing assays or in a systemic mouse model of infection (Sjögren, Okumura, Collin, Nizet, & Hollands, 2011). However, an increased resistance to killing by neutrophils and monocytes *in vitro* was observed. If EndoS was introduced and expressed in an M49 isolate of group A streptococci, there was an observed increase in virulence in a mouse infection model (Sjögren, Okumura, Collin, Nizet, & Hollands, 2011). These results suggest that in the virulent M1T1 serotype, EndoS has minimal impact on pathogenicity; however, in certain strains, high levels of expression or local accumulation may contribute to virulence.

Soluble M protein

M protein is a surface protein of group A streptococci, although it can be released through the action of bacterial- or host-derived proteases (Oehmcke, Shannon, Mörgelin, & Herwald, 2010; Shannon, et al., 2007), and can influence streptococcal virulence in that extracellular state. The M protein released from the bacteria into circulation can contribute to the systemic activation of the coagulation cascade during the infectious process (Oehmcke, Shannon, Mörgelin, & Herwald, 2010; Shannon, et al., 2007). Platelets can be activated by soluble M protein to form complexes with neutrophils and monocytes, which results in the activation of these cells, and can evoke additional inflammatory responses in doing so (Oehmcke, Shannon, Mörgelin, & Herwald, 2010; Shannon, et al., 2007). Binding fibrinogen to soluble M1 results in aggregates that are capable of activating neutrophil β2 integrins (Macheboeuf, et al., 2011). This triggers a release of heparin-binding protein, an inflammatory mediator that induces vascular leakage (Reglinski & Sriskandan, 2014). Soluble M protein has been shown to be an inducer of neutrophils, monocytes, and a T-cell activator (Herwald, et al., 2004; Påhlman, et al., 2006; Påhlman, et al., 2008) being as potent as other streptococcal superantigens, which suggests that soluble M1 protein is a novel superantigen (Påhlman, et al., 2008).

The coagulation pathway can be initiated by both intrinsic and extrinsic pathways, with activation of the intrinsic pathway occurring on the cell surface and the extrinsic pathway being activated through the interaction with soluble M protein (Oehmcke, et al., 2012; Walker, et al., 2014). With the coagulation system and early immune response to a streptococcal infection being tightly linked, any dysregulation will contribute to the invasive pathogenesis of the organism (Walker, et al., 2014).

Perspectives and Future Directions

Group A streptococci are successful pathogens capable of causing significant morbidity, as well as mortality. The diverse arsenal of virulence factors they produce to establish colonization and overcome host defense mechanisms are an important contribution to the disease process. Many of these virulence factors are extracellular and cell bound, as well as those discussed here that are released into the external environment. In addition to their role in virulence, many of the extracellular products produced by group A streptococci, such as proteinases, hyaluronate lyases, DNases, NADase, esterases, and others, as well as extracellular enzymes that are not considered virulence factors (such as amylase) can also function as digestive enzymes that produce nutrients from the host tissue for assimilation by the infecting bacteria. Other extracellular proteins, in particular those that function as virulence factors (streptolysins and pyrogenic exotoxins, among others) act as agents of host tissue damage and provide the opportunity for the digestive enzymes mentioned to obtain essential growth nutrients for the infecting bacteria.

Information on the physical properties of the virulence factors, the regulation, and the role of the released extracellular products is part of the equation that will lead to an understanding of the relationship that exists between the bacteria and its host. With the advent of modern molecular technologies, vast quantities of data and information have been generated, but there is also a need to interpret the meaning of this data. Recent research has moved beyond looking at a single virulence factor in isolation; now it will be necessary to take a broader approach, as these virulence factors do not work in isolation and are not independently regulated. In many cases,

regulatory factors control expression of more than one virulence factor; sometimes in the same manner, sometimes inversely, with one factor being up-regulated while another is down regulated. What this says about virulence factors and their role in the disease process still needs to be determined. There is also a need to examine the role of multiple virulence factors *in vivo*; what happens in culture media may not be indicative of what actually occurs during an infection.

Even though *S. pyogenes* has been studied for over 130 years, there are still many unanswered questions related to pathogenicity and virulence. Infections caused by *S. pyogenes* have widespread effects, both personally to the affected and economically to their communities. The overall control and prevention of streptococcal infections needs to be an area of emphasis for future research in order to reduce morbidity and mortality. Vaccines would be one answer, and understanding the role of the extracellular products in the disease process may provide new ways for looking at the development of such vaccines.

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Streptococcal Superantigens: Biological properties and potential role in disease

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Abstract

Superantigens (SAgs) are a family of highly potent mitogens that share the ability to trigger excessive stimulation of human and other mammalian T lymphocytes. This leads to a massive release of T cell mediators and pro-inflammatory cytokines contributing to diseases such as toxic shock syndrome. In contrast to conventional peptides, SAgs bind as unprocessed molecules to major histocompatibility (MHC) class II molecules outside the peptide-binding groove and sequentially to the variable β-chain of the T cell receptor (TcRVβ). Currently, eleven *Streptococcus pyogenes* SAgs are described in the literature. Together with the SAgs produced by *Staphylococcus aureus*, they build a larger family of structurally related, heat-stable exotoxins.

This chapter provides a comprehensive overview of the discovery, biological function, and disease-associations of these remarkable proteins.

Introduction

Superantigens (SAgs) are a family of highly mitogenic exotoxins that are produced by a small number of bacterial species and some viruses (Fraser & Proft, 2008; Proft, Schrage, & Fraser, 2005) (Fraser & Proft, 2008; Proft, Schrage, & Fraser, 2005). The most common bacterial genus that produces SAgs is *Streptococcus spp*. and includes *Streptococcus pyogenes* (group A streptococcus), *S. dysgalactiae* (group C Streptococcus) and *S. equi* (group G streptococcus) (Proft, Schrage, & Fraser, 2005; Commons, Smeesters, Proft, Fraser, Robins-Browne, & Curtis, 2014; Proft & Fraser, 2003). A variety of SAgs are also found in *Staphylococcus aureus* and coagulase negative staphylococci, which together with the streptococcal SAgs, build a family of structurally related low molecular weight exotoxins, with secretion dependent on a cleavable signal peptide sequence (Fraser & Proft, 2008; Proft & Fraser, 2003; Baker & Acharya, 2004). Other, structurally non-related SAgs are produced by *Mycoplasma arthritidis* (Proft, Schrage, & Fraser, 2005; Rink & Kirchner, 1992) and *Yersinia pseudotuberculosis* (Proft, Schrage, & Fraser, 2005; Donadini & Fields, 2007).

A hallmark of SAgs is their ability to simultaneously bind to major histocompatibility complex (MHC) class II on antigen presenting cells and the T cell receptor on T cells (Fraser & Proft, 2008; Proft & Fraser, 2003; Proft & Fraser, 2007). In contrast to conventional peptide antigens, SAg binding is not restricted by polymorphic determinants of MHC class II molecules and occurs outside the peptide-binding groove. Furthermore, SAgs bind to the variable region of the TcRVβ chain, resulting in extensive heterogeneity in T cell clonal activation (Figure 1). The number of different TcR β-chains in the human T cell repertoire is restricted to less than 50 with only about 25 major Vβ types. Since SAgs generally bind more than one specific Vβ region, up to 25% of an individual's T cell population can be activated, which is in sharp contrast to 1 in 10^5-10^6 naïve T cells that are stimulated in response to conventional peptide antigens. Consequently, each SAg is associated with a characteristic TcRVβ 'fingerprint' that is independent from MHC class II polymorphism (Table 1). For example, streptococcal pyrogenic exotoxin (SPE)-C triggers the activation and expansion of T cells carrying Vβ2.1, Vβ3.2,

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Vβ12.5 and Vβ15.1 with a strong preference for Vβ2.1, whereas streptococcal mitogenic exotoxin (SMEZ) shows specificity for Vβ2.1, Vβ4.1, Vβ7.3 and Vβ8.1 regions with a preference for Vβ4.1 and Vβ8.1 (Table 1). Due to the immense potency to stimulate human, and to a certain degree, other mammalian CD4 and CD8 T cells, the term 'superantigen' was introduced by Philippa Marrack and John Kappler in 1989 (White, et al., 1989). In response to the oligoclonal activation of T cells and antigen presenting cells massive amounts of proinflammatory cytokines, such as interleukin 1 beta (IL-1β), tumor necrosis factor alpha (TNF-α), and T cell mediators, such as IL-2 are released. This 'cytokine storm' can lead to fever and shock. With half-maximum responses between 0.02 and 50 pg/ml for human T cells, SAgs are the most potent T cell mitogens ever discovered.

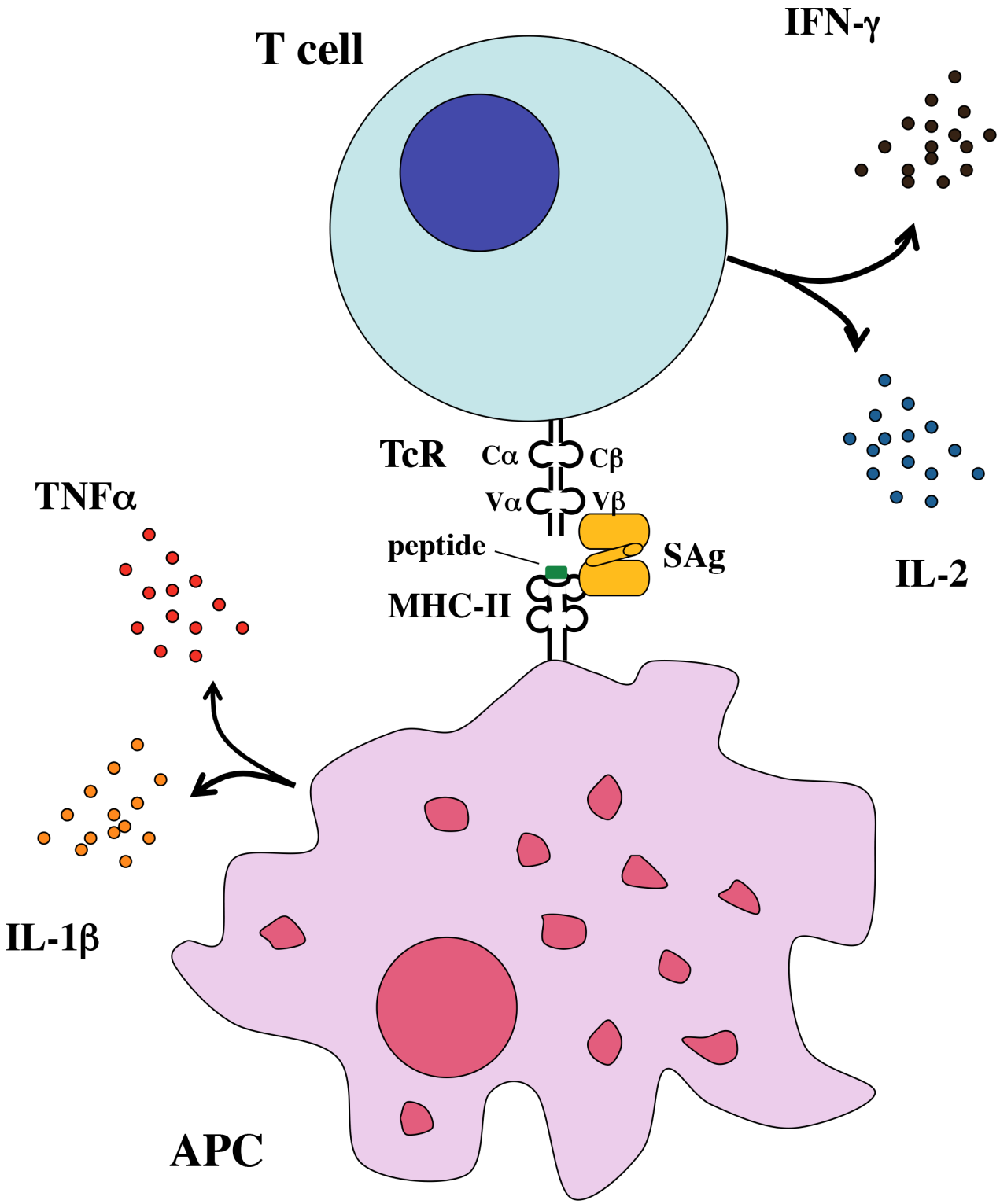

Figure 1. Model of T cell activation by a conventional peptide antigen (Ag) and superantigen (SAg). APC, antigen-presenting cell; MHC II, major histocompatibility class II molecule; TcR, T cell receptor; TNF-α, tumor necrosis factor alpha; IFN-γ, interferon gamma; IL, interleukin.

Table 1. Functional properties of *Streptococcus pyogenes* superantigens

SAg	MW [kDa]	orthologues	alleles	Crystal structure	Zinc binding	MHC II binding α/β chain	Human TcRVβ specificity[f]	P_{50} (h) [pg/ml]	Reference
SPE-A	26.0	Yes[a]	6	+	-	+/-	2.1, 12.2, 14.1, 15.1		(Kim & Watson, 1970; Imanishi, Igarashi, & Uchiyama, 1990; Papagerogiou, et al., 1999; Hartwg, Gerlach, & Fleischer, 1994; Sundberg, et al., 2002)
SPE-C	24.4	Yes[a,b]	2	+	+	-/+	**2.1**, 3.2, 12.5, 15.1	0.1	(Kim & Watson, 1970; Leonard, Lee, Jenkins, & Schlievert, 1991; Roussel, Anderson, Baker, Fraser, & Baker, 1997; Li, Tiedemann, Moffatt, & Fraser, 1997; Sundberg, et al., 2002)
SPE-G	24.6	Yes[a,b]	6	-	+	?/+	**2.1**, 4.1, 6.9, 9.1, 12.3	2	(Proft, Moffatt, Berkahn, & Fraser, 1999)
SPE-H	23.6	Yes[a,c]	2	+	+	-/+	2.1, **7.3,** 9.1, 23.1	50	(Proft, Moffatt, Berkahn, & Fraser, 1999; Arcus, et al., 2000)
SPE-I	26.0	Yes[c]	2	+	+	?/+	6.9, 9.1, **18.1**, 22	0.1	(Proft, Arcus, Handley, Baker, & Fraser, 2001; Brouillard, et al., 2007)
SPE-J	24.6	No	3	+	+	-/+	**2.1**	0.1	(Proft, Arcus, Handley, Baker, & Fraser, 2001; McCormick, Pragman, Stolpa, Leung, & Schlievert, 2001; Baker, et al., 2004)
SPE-K	27.4	Yes[a,c,d]	1	-	+	?/+	**1.1**, 5.1, 23.1	1	(Beres, et al., 2002; Ikebe, et al., 2002; Proft, Webb, Handley, & Fraser, 2003a)
SPE-L	26.2	Yes[a]	3	-	+	?/+	**1.1**, 5.1, 23.1	10	(Proft, Webb, Handley, & Fraser, 2003a; Smoot, et al., 2002a)
SPE-M	25.3	Yes[a,b]	4	+[e]	+	?	**1.1**, 5.1, 23.1		(Smoot, et al., 2002a)
SSA	26.9	No	3	-	-	?	1.1, 3, 15		(Mollick, et al., 1993)
SMEZ1	24.3	No	56	-	+	?/+	2.1, 4.1, 7.3, **8.1**	0.08	(Kamezawa, et al., 1997)
SMEZ2	24.1	No		+	+	?/+	4.1, **8.1**	0.02	(Proft, Moffatt, Berkahn, & Fraser, 1999; Arcus, et al., 2000)

[a] Streptococcus dysgalactiae subsp. equisimilis, [b]Streptococcus dysgalactiae subsp. dysgalactiae, [c]Streptococcus equi subsp. equi, [d]Streptococcus equi subsp. zooepidemicus, [e]Streptococcus dysgalactiae orthologue SPE-M6, [f]principle TcRVβs are in bold and underlined.

The discovery of Group A streptococcal superantigens

Over the last nine decades, eleven SAgs were discovered in *Streptococcus pyogenes* (Figure 2). It all started in 1924 when Dick and colleagues identified a toxin in culture filtrates from hemolytic streptococci isolated from patients with scarlet fever. This toxin was initially named 'scarlet fever toxin' (Dick & Dick, 1983). A second toxin was identified in 1934 and named toxin B (Hooker & Follensby, 1934) followed by the discovery of toxin C from a scarlet fever associated serotype M18 culture filtrate in 1960 (Watson, 1960). The three toxins were immunologically different, but shared several different biological activities, in particular the ability to induce fever when injected into rabbits (pyrogenicity) and the enhancement of susceptibility to endotoxic shock. Based on the strong pyrogenic effect, which was believed to be the primary characteristic of the toxins, Kim and Watson designated the toxins streptococcal pyrogenic exotoxins (SPE) A, B and C (Kim & Watson, 1970). During the 1980s, the toxin genes were cloned and recombinant proteins were produced in *Escherichia coli* and *Bacillus subtilis*, which allowed for a more careful study of the toxin functions in the absence of any contaminating proteins. It was found that SPE-A was identical to Blastogen A, a previously identified T cell mitogen (Schlievert & Gray, 1989), and it was able to activate murine T cells in a MHC class II-dependent and TcRVβ-specific mode (Imanishi, Igarashi, & Uchiyama, 1990). Similarly, the function as a SAg was also established for SPE-C when Leonard and co-workers showed MHC class II and TcRVβ-dependent T cell mitogenicity (Leonard, Lee, Jenkins, & Schlievert, 1991). In contrast, initial findings of SPE-B induced T cell stimulation were later disputed when experiments with recombinant toxin of very high purity could not detect any SAg activity (Gerlach, Reichardt, Fleischer, & Schmidt, 1994). Furthermore, sequencing of the *speB* gene from a serotype M12 *S. pyogenes* strain revealed identity with the gene encoding streptococcal cysteine protease (SCP) (Bohach, Hauser, & Schlievert, 1988).

The identification of SPE-F was reported in 1994 (Norrby-Teglund, Newton, Kotb, Holm, & Norgren, 1994), but it is now believed that the observed mitogenic activity, like in the case of SPE-B, was due to contamination with a powerful SAg. It was later shown that SPE-F is identical with streptococcal DNaseB (Sriskandan, Unnikrishnan, Krausz, & Cohen, 2000).

Musser and colleagues reported in 1993 the discovery of a novel SAg, which they found in the cell culture supernatant of a serotype M3 strain and named streptococcal superantigen (SSA) (Mollick, et al., 1993). Interestingly, SSA showed a higher degree of amino acid similarity to staphylococcal SAgs than to any other streptococcal SAg. Another SAg, called streptococcal mitogenic exotoxin Z (SMEZ), was found in 1997 in the culture supernatant of an M1/T1 *S. pyogenes* strain (Kamezawa, et al., 1997).

SMEZ was the last *S. pyogenes* SAg identified by conventional methods before the start of microbial genomics and the discovery of genes by database mining. The first *S. pyogenes* genome was sequenced from strain SF370, a serotype M1 strain, and raw DNA sequence data was made available on the researchers website at the University of Oklahoma for mining long before completion of the project (Ferretti, et al., 2001). Although the streptococcal (and also staphylococcal) SAgs often share only limited amino acid sequence homologies, they all possess the highly conserved "family signature motifs" Y-G-G-[LIV]-T-X(4)-N (Prosite entry PS00277) and K-X(2)-[LIVF]-X(4)-[LIVF]-D-X(2)-R-X(2)-L-X(5)-[LIV]-Y (PS00278). These motifs were used to mine the SF370 genome database, which resulted in the discovery of four novel *sag* genes, *spe-G, spe-H, spe-I* and *spe-J*. Recombinant forms of the toxins were generated in *E. coli* and functional analysis confirmed their role as SAgs (Proft, Arcus, Handley, Baker, & Fraser, 2001; Proft, Moffatt, Berkahn, & Fraser, 1999). Furthermore, SPE-J was shown to induce fever in rabbits and was lethal in two rabbit models of toxic shock syndrome (McCormick, Pragman, Stolpa, Leung, & Schlievert, 2001). The rapid discovery of novel SAgs by whole genome mining over the following years resulted in an increasingly confusing SAg nomenclature. Analysis of a complete *S. pyogenes* serotype M3 genome in the U. S. resulted in the discovery of a novel *sag* gene that was named *speK* (Beres, et al., 2002). However, this name had already been assigned to an incomplete *sag* gene on the SF370 genome (Ferretti, et al., 2001). In the same year, another group found the same *sag* gene on the genome of a Japanese *S. pyogenes*

serotype M3 strain and named it *speL* (Ikebe, et al., 2002). Shortly after that, this gene was found on a serotype M89 isolate from New Zealand and was also named *speL* (Proft, Webb, Handley, & Fraser, 2003a). It was identified by PCR using specific primers for a previously identified orthologue on a *Streptococcus equi subsp. zooepidemicus* genome. The same strategy also led to the discovery of another novel *sag* gene from a serotype M80 isolate, which was named *speM* (Proft, Webb, Handley, & Fraser, 2003a). At about the same time, two *sag* genes were identified when a serotype M18 isolate genome was completed and named *speL* and *speM* (Smoot, et al., 2002a). *SpeL* is identical to *speM* found on the genome of the M80 isolate, whereas the *speM* gene from the serotype M18 genome had not been reported before. Mitogenic activity of the novel SAgs was confirmed after recombinant proteins were produced in *E. coli* and found to target T cells in a Vβ-specific and MHC class II-dependent mode (Proft, Webb, Handley, & Fraser, 2003a; Smoot, et al., 2002a).

Recently, a novel nomenclature for all streptococcal SAgs was proposed (Commons, et al., 2014). It was suggested to use the name SPE-K for the SAg identified by Beres and co-workers (Beres, et al., 2002), and the names SPE-L and SPE-M for the SAg identified by Smoot and colleagues (Smoot, et al., 2002a). In addition, the names SPE-N, SPE-O and SPE-P were reserved for potential orthologues of the superantigens SzeN, SzeF and SzeP that were recently found in *Streptococcus equi subsp. zooepidemicus* (Paillot, et al., 2010), although these toxins have not been found yet in *S. pyogenes*.

Superantigen orthologues in non-Group A streptococci

Group C Streptococcus (GCS) and Group G Streptococcus (GGS) are commonly regarded as commensals usually found in association with the normal flora of human skin, pharynx and intestine. However, there have been an increasing number of reports implicating GCS and GGS with severe invasive infections, such as necrotizing fasciitis and toxic shock syndrome (Oster & Bisno, 2006). Mitogenic activity in supernatants of clinical GCS and GGS isolates had been reported over several years, but SAgs had not been identified until 2002 when Timoney's group identified two SAgs in *Streptococcus equi*, a bacterium that causes strangles in horses, but can also infect humans.

The *Streptococcus equi* pyrogenic exotoxins H and I (SePE-H, SePE-I) are highly homologous to SPE-H and SPE-I, (>98% amino acid sequence identities) indicating horizontal gene transfer from *S. pyogenes* to *S. equi* or *vice versa* (Artiushin, Timoney, Sheoran, & Muthupalani, 2002). Another two *sag* genes were identified by data mining of the *S. equi* genome at the Sanger Centre and named $speL_{Se}$ and $speM_{Se}$, due to the homology to *speL* and *speM* (recently renamed to *speK* and *speL*, respectively (Commons, et al., 2014)) with 99% and 98.1% nucleotide identities, respectively (Proft *et al.*, 2003b). Two SAgs have been identified from *Streptococcus dysgalactiae* subsp. *equisimilis* called *Streptococcus dysgalactiae*-derived mitogen (SDM) (Miyoshi-Akiyama, et al., 2003) and SPE-G^{dys} (Sachse, et al., 2002). SDM is 99% similar to SPE-M and SPE-G^{dys} is 86% similar to SPE-G.

The recently proposed novel nomenclature for *sag* genes (see above) also included non- *S. pyogenes* SAgs and it was suggested to adapt the names of the *S. pyogenes sag* genes for all orthologues from non- *S. pyogenes sag* genes followed by the allele number (Commons, et al., 2014). For example, *spe-*G^{dys} would be named *speG11* and *sdm* would become *speM6*. Based on this new nomenclature, a search at the National Center for Biotechnology Information Nucleotide (NCBI) database has found *sag* genes in *S. dysgalactiae* subsp. *equisimilis* (3 *speA* alleles, 1 *speC* allele, 16 *speG* alleles, 1 *speH* allele, 3 *speK* alleles, 1 *speL* allele and 1 *speM* allele), S. *dysgalactiae* subsp. *dysgalactiae* (1 *speC* allele, 6 *speG* alleles and 3 *speM* alleles), *S. equi* subsp. *equi* (2 *speH* alleles, 1 *speI* allele and 1 *speK* allele) and *S. equi* subsp. *zooepidemicus* (1 *speK* allele). Interestingly, no orthologues of *ssa*, *smez* or *speJ* were found on any non- *S. pyogenes* genomes. In case of *smez* and *speJ* this is most likely due to the fact that these genes are not associated with mobile DNA elements preventing them from horizontal gene transfer.

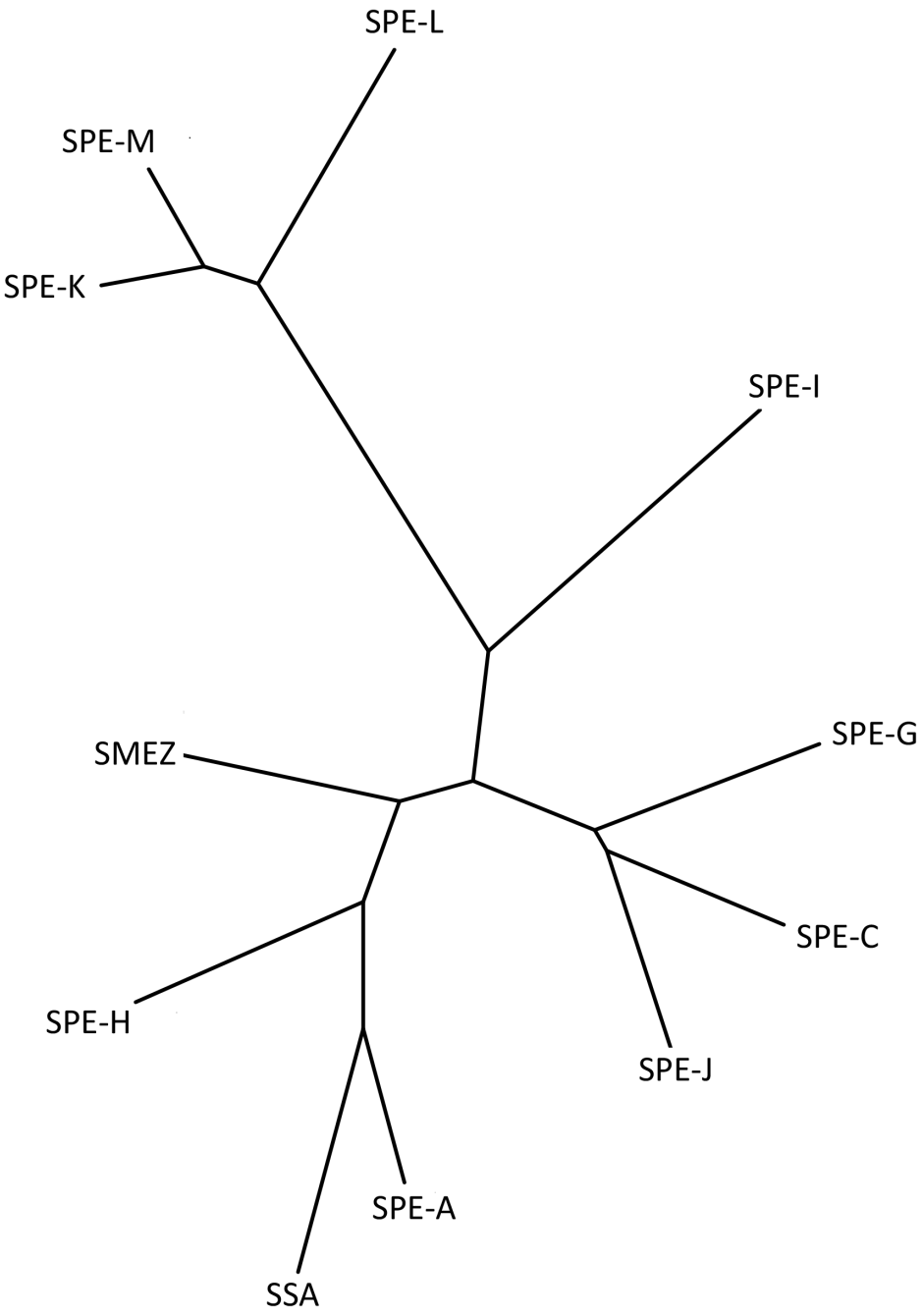

Figure 2. Phylogenetic tree of Group A streptococcal SAgs. The tree was created using ClustalW and is based on primary amino acid sequence homologies of the mature proteins.

Allele diversity and frequency of *sag* genes

In general, *S. pyogenes sag* genes are well conserved and show only minor allelic variation, often not more than just a few nucleic acid differences. The exception is *smez,* of which more than 50 different alleles have been listed in the gene databases. The diversity ranges from single nucleic acid differences to changes in 36 positions (=5%) between *smez-1* and *smez-2*. In addition, several *smez* genes contain nonsense mutations resulting in the expression of truncated and inactive forms of these toxins (Proft, et al., 2000; Turner, et al., 2012). In a recent study, the National Center for Biotechnology Information Nucleotide (NCBI) database was searched for all known streptococcal *sag* gene variants, including genes from non- *S. pyogenes*, and revealed a total of 145 unique alleles belonging to 14 groups. After excluding protein duplicates and truncated variants, a total of 91 unique SAg sequences were identified (Commons, et al., 2014). Currently known SAg variants in *S. pyogenes* are: six SPE-A, three SPE-C, six SPE-G, two SPE-H, two SPE-I, three SPE-J, one SPE-K, three SPE-L, four SPE-M, three SSA, and 56 SMEZ.

S. pyogenes sag genes are generally associated with bacteriophages, with the exception of *speG, speJ* and *smez*, which are chromosomally encoded. However, the *speJ* gene appears to be located on an instable genomic region and is absent in a number of *S. pyogenes* isolates from diverse linages (Friães, Pinto, Silva-Costa, Ramirez, & Melo-Cristino, 2013; Meisal, et al., 2010). It has been suggested that *speJ* has been acquired from a temperate phage that was later lost from the genome of descending *S. pyogenes* lineages (McMillan, et al., 2007). A recent comprehensive profiling of *sag* genes from 480 clinical *S. pyogenes* isolates by multiplex PCR revealed the following distribution: *speA*, 32.1%; *speC*, 51.5%; *speG*, 86.9%; *speH*, 17.1%; *speI*, 15.2%; *speJ*, 32.7%; *speK*, 24.6%; *speL*, 9.2%; *speM*, 9.2%; *ssa*, 35.4%; *smez*, 96% (Friães, Pinto, Silva-Costa, Ramirez, & Melo-Cristino, 2013). The frequencies of individual *sag* genes were generally in agreement with results from previous epidemiological studies (Proft, Webb, Handley, & Fraser, 2003a; Maripuu, Eriksson, & Norgren, 2008; Michaelsen, Andreasson, Langerud, & Caugant, 2011). The *speL* and *speM* genes were detected in only a small fraction of isolates, but were always found together suggesting a stable genetic linkage. Similarly, *speH* and *speI* are relatively rare and were found in association, as expected from their tandem location on a prophage (Commons, et al., 2008), but were also found independently in some isolates from different lineages supporting the idea that *speI* is occasionally lost during phage integration (Proft, Webb, Handley, & Fraser, 2003a; Friães, Pinto, Silva-Costa, Ramirez, & Melo-Cristino, 2013; Maripuu, Eriksson, & Norgren, 2008; Michaelsen, Andreasson, Langerud, & Caugant, 2011).

The origin of *sag* genes is still not entirely clear. The homology between *sag* genes in different streptococcal species, and also in *Staphylococcus aureus*, together with their mainly bacteriophage location suggests horizontal transfer between species. Direct evidence for horizontal gene transfer of a *sag* gene between *S. pyogenes* strains and also between streptococcal species has been provided by Vojtek and co-workers, who showed lysogenic conversion of several *S. pyogenes* M-serotypes and *Streptococcus dysgalactiae* subsp. *equisimilis* clinical isolates with *S. pyogenes* M12-derived prophage phi149 carrying the *ssa* gene (Vojtek, et al., 2008). A recent study has shown that the flanking regions of *speG* in *S. pyogenes* and in *Streptococcus dysgalactiae* subsp. *equisimilis* are conserved suggesting that both species descended from a common ancestor that carried an ancestral *speG* gene (Okumura, et al., 2012).

Regulation of *S. pyogenes* superantigen production

Streptococcus pyogenes SAgs are generally secreted in only small amounts, but little is known about the regulation of these SAgs. In growth medium, gene expression is the highest in the late logarithmic and early stationary phase (Unnikrishnan, Cohen, & Sriskandan, 1999). The production of SMEZ, a potent SAg, is so small that it can only be detected reliably using biological assays involving the detection of T cell mitogenicity (Proft, Sriskandan, Yang, & Fraser, 2003b). There are several lines of evidence showing significant upregulation of *S. pyogenes* SAgs after infection and host factors appear to play a role in this process. SPE-A expression increased

after a diffusion chamber containing *S. pyogenes* was implanted subcutaneously into BALB/c mice. The increase was detected 7 days post-infection and was still high after 21 in vitro passages suggesting a stable switch of the *speA* gene (Kazmi, et al., 2001). The expression of SPE-C could be increased when a *speC*-carrying strain was co-cultured with human pharyngeal cells (Broudy, Pancholi, & Fischetti, 2001). In-vivo up-regulation of SAgs was also shown at the transcription level. In a genome-wide DNA microarray analysis, it was demonstrated that growth of a serotype M1 strain in human blood, compared to growth in growth medium, resulted in significant increase *in speA, speG, speJ* and *smez* transcripts (Graham, et al., 2005). Using the same methodology, *sag* transcription was analyzed during infection of cynomolgus macaques. The *speA, speJ* and *smez* genes were highly expressed in distinct phases of disease. Importantly, *smez* expression was 24-times higher than *speA*, despite the fact that SMEZ is about 10-times more potent in T cell stimulation compared to any other SAg. Furthermore, *smez* expression correlated with peak levels of C-reactive protein (an important inflammation marker) and was the most dominant acute-phase-correlated pro-inflammatory gene. However, there was no correlation of *smez* expression with pharyngitis or tonsillitis suggesting that SMEZ might play an important role in invasive *S. pyogenes* disease (Virtaneva, et al., 2005).

The human factors responsible for SAg upregulation are largely unknown. A study by Kansal *et al.* has shown that expression of SPE-A can be induced by human transferrin and lactoferrin. However, this was not because of a direct effect of these proteins, but rather due to their iron-scavenging activities, as iron deprivation also resulted in increased SPE-A expression, probably due to stress signals (Kansal, Aziz, & Kotb, 2005).

S. pyogenes SAg levels can also be regulated at the protein level. It was shown that SPE-B, a multifunctional cysteine protease is able to degrade SMEZ, whereas SPE-A and SPE-G were more resistant and SPE-J was completely resistant (Nooh, et al., 2006). Interestingly, SPE-B expression is significantly decreased in hypervirulent *S. pyogenes* strains that carry mutations in the two-component CovRS regulator, which suggests that SPE-B might have a role as a global regulator of SAg function through proteolysis (Walker, et al., 2007).

Superantigen protein structure

To date, the protein structures of six *S. pyogenes* SAgs have been solved by X-ray crystallography. These include SPE-A (Papageorgiou, et al., 1999), SPE-C (Roussel, Anderson, Baker, Fraser, & Baker, 1997), SPE-H (Arcus, et al., 2000), SPE-I (Brouillard, et al., 2007), SPE-J (Baker, et al., 2004) and SMEZ-2 (Arcus, et al., 2000). In addition, the protein structure of the *Streptococcus dysgalactiae*-derived mitogen (SDM) has been determined (Saarinen, Kato, Uchiyama, Miyoshi-Akiyama, & Papageorgiou, 2007). SDM shares 92% amino acid identity with SPE-M and has recently been renamed to SPE-M allele 6 (SPE-M6) (Commons, et al., 2014). All protein structures show a conserved two-domain architecture and the presence of a long, solvent-accessible α-helix that spans the center of the SAg molecule, a feature that is shared with the staphylococcal SAgs. The N-terminal domain is a mixed β-barrel with Greek key topology called an oligonucleotide/oligosaccharide binding (OB) fold, which consists of 8 superfamilies, including the 'bacterial enterotoxin' superfamily comprising the 'SAg toxin N-terminal domain' family and the 'bacterial AB_5 toxin' family. Members of the 'SAg toxin N-terminal domain' family also include the staphylococcal SAgs and the superantigen-like toxins (SSLs), which lack mitogenic activity (Arcus, 2002).

The larger C-terminal domain is a β-grasp fold and consists of a twisted β-sheet that is capped by the central α4-helix that packs against a four-strand antiparallel twisted sheet. SAgs are extremely stable proteins that resist denaturing by heat and acid and this is achieved by close packing of the N- and C-terminal domains. The structure is further stabilized by a section of the N-terminus that extends over the top of the C-terminal domain. Notably, the most conserved section of all streptococcal and staphylococcal SAgs, as well as the SSLs, is the region that builds the interface between the α4-helix and the inner side of the N-terminal OB-fold domain.

Molecular interactions of superantigens with host receptor molecules

A hallmark of SAgs is their ability to simultaneously bind to MHC class II molecules on antigen presenting cells and the Vβ-region of the T cell receptor on T cells (Figure 1). A variation in TcR –binding has been shown for the staphylococcal enterotoxin (SE)-H and the *Mycoplasma arthritidis* mitogen (MAM), which both recognize the variable region of the TcR α-chain (Wang, et al., 2007; Petersson, Pettersson, Skartved, Walse, & Forsberg, 2003). However, interaction with TcRVα has not been shown for any of the *S. pyogenes* SAgs. More recently, CD28 has been identified as an additional and essential receptor for cytokine production by both streptococcal and staphylococcal SAgs (Arad, et al., 2011; Kaempfer, Arad, Levy, Hillman, Nasie, & Rotfogel, 2013; Ramachandran, et al., 2013).

MHC class II binding

SAgs have developed a variety of ways for attachment to MHC class II, probably driven by a need to optimize the efficiency of individual SAgs. A very strong and stable binding of SAgs to MHC class II is a prerequisite for the extraordinary amplification in T cell signaling, as less SAg molecules are required for stimulation. This is supported by the fact that about four to five orders of magnitude less SAg molecules are required to stimulate human PBLs compared to mouse PBLs based on a slightly higher affinity towards human MHC class II. Further evidence was provided from experiments with transgenic mice expressing human MHC class II, which are significantly more sensitive to T cell stimulations compared to non-transgenic littermates (Nooh, El-Gengehi, Kansal, David, & Kotb, 2007; Sriskandan, et al., 2001).

In general, streptococcal SAgs bind to MHC class II either via the invariant α-chain or the polymorphic β-chain. The staphylococcal SAgs toxic shock syndrome toxin-1 (TSST-1) and staphylococcal enterotoxin (SE)-B are the prototype SAgs for binding to the MHC II α-chain. Co-crystallization studies with these SAgs bound to HLA-DR have revealed an exposed hydrophobic loop region within the N-terminal β-barrel domain that binds to a hydrophobic groove located in the distal region of the DR α1-domain with binding affinities of 10^{-5} M (Jardetzky, et al., 1994; Kim, Urban, Strominger, & Wiley, 1994). This region on the MHC class II molecule has been referred to as the 'generic' or 'low-affinity' binding site for SAgs. Only two of the eleven *S. pyogenes* SAgs, SPE-A and SSA, use this binding mode (Figure 3A). SPE-A competes with SEB for binding to HLA-DR molecules suggesting common recognition sites for MHC class II. However, the binding sites appear to be non-identical, as SPE-A shows higher affinity towards HLA-DQ compared to HLA-DR and HLA-DP, whereas SEB preferentially binds to HLA-DR (Hartwig, Gerlach, & Fleischer, 1994). The other nine *S. pyogenes* SAgs all bind to the MHC class II β-chain via a single, highly conserved histidine residue (His81) in an otherwise highly polymorphic MHC class II molecule. This interaction is based on the formation of a tetravalent zinc complex that includes three residues within the C-terminal domain of the SAgs, also known as the zinc-binding site, in addition to the His81 of MHC class II β-chain. The relative binding affinity of this interaction is about 100-times higher than the generic low affinity site (10^{-7} M) and has been referred to as the 'high-affinity' binding site. SPE-C was the first streptococcal SAg for which this binding mode was shown. SPE-C-binding to the MHC class II β-chain can be completely abolished by adding EDTA and can be restored by excess of Zn^{2+} over EDTA (Li, Tiedemann, Moffatt, & Fraser, 1997). Structural analysis of SPE-C revealed residues His167, His201 and Asp203 as the zinc-binding residues (Roussel, Anderson, Baker, Fraser, & Baker, 1997). The complete zinc coordinated binding was later confirmed by structural analysis of SPE-C in complex with HLA-DR2a bearing a peptide derived from myelin basic protein (Figure 3B). Interestingly, the co-crystal structure also revealed extensive interaction of SPE-C with the bound peptide (Li, et al., 2001). Structural analysis of SPE-H, SPE-J and SMEZ-2, and computer-generated models of SPE-G, SPE-I, SPE-K, SPE-L and SPE-M showed the conserved zinc-binding site in the C-terminal domain, but absence of a generic MHC class II α-chain binding region. This was confirmed in biochemical assays when removal of Zn^{2+} by EDTA completely abolished MHC class II binding.

Interestingly, Scatchard plot analysis of SPE-G, SPE-H, SMEZ and SMEZ-2 revealed a range of different binding affinities (from nanomolar to micromolar) towards MHC class II for each of the toxins. Based on the fact that the generic low-affinity binding site is absent in these toxins and the observation of the extensive SPE-C - peptide interaction, it was suggested that some SAgs might have a more restricted MHC class II repertoire defined by the bound peptide antigen (Proft, Moffatt, Berkahn, & Fraser, 1999). Fernandez and colleagues have shown that despite the important role of the zinc complex in MHC class II binding, about 25% of the contacts are made to the antigenic peptide. However, the interactions are mainly with the peptide backbone atoms rather than the side-chain atoms. Furthermore, SAgs interact with the MHC class II-bound peptides at their conformationally conserved N-terminal regions, minimizing sequence-specific interactions with peptide residues to enhance cross-reactivity (Fernández, Guan, Swaminathan, Malchiodi, & Mariuzza, 2006).

Several SAgs are capable of forming dimers in solution. For example, SPE-C forms a homodimer using a secondary zinc-binding site, which is located within the N-terminal domain. Consequently, the dimer interface is located opposite the high-affinity HLA-DR β-chain binding site and dimer formation might result in DRβ - SPE-C - SPE-C - DRβ complexes (Roussel, Anderson, Baker, Fraser, & Baker, 1997; Li, Tiedemann, Moffatt, & Fraser, 1997). However, zinc-binding and dimerization of SPE-C are not essential for T cell stimulation (Swietnicki, Barnie, Dyas, & Ulrich, 2003). The biological function of SPE-C dimerization is unknown, but one might speculate that MHC class II crosslinking leads to increased expression of co-stimulatory molecules, such as B7, and cell adhesion molecules on antigen presenting cells. It has previously been shown that cross-linking of MHC class II by staphylococcal enterotoxin A (SEA) is necessary for inflammatory cytokine expression (Mehindate, et al., 1995). Dimer formation has also been demonstrated for SPE-J and SSA (Baker, et al., 2004; De Marzí, et al., 2004). However, in both cases the formation of homodimers would prevent the toxins from binding to the TcR. An alternative function, apart from T cell activation has been suggested, but this has never been confirmed.

TcR binding

SAgs bind to TcR molecules primarily by engaging with the variable region of the β-chain (Vβ-domain). This results in an oligoclonal stimulation of a defined T cell repertoire and the potential activation of >20% of all T cells. The first two co-crystal structures of a streptococcal SAg bound to a TcR β-chain were published in 2002 and showed SPE-A in complex with murine TcRVβ8.2 and SPE-C bound to human TcRVβ2.1 (Sundberg, et al., 2002) (Figure 4A). Considering the structural homology between SPE-A and SEB it was not surprising that the SPE-A - mVβ8.2 complex showed strong similarity to the previously solved SEB - mVβ8.2 structure (Li, et al., 1998a). Residues from the complementarity-determining region 2 (CDR2), framework region 2 (FR2) and, to a lesser extent, hypervariable region 4 and FR3 play a role in the interaction of mTcRVβ8.2 with SAgs. Binding to the CDR2 loop appears to be a requirement for all streptococcal and staphylococcal SAgs, whereas binding to other Vβ domains seems to be responsible for Vβ-specificity (Sundberg, Deng, & Mariuzza, 2007). However, there are also some differences in TcR binding of SPE-A compared to SEB. In addition to intermolecular interaction with CDR2, FR3 and hypervariable region 4, SPE-A also binds to the CDR1 loop of the mVβ8 TcR. In addition, there are several hydrogen bonds between SPE-A and mVβ8.2 that involve side chain atoms, whereas the SEB - mVβ8.2 complex shows exclusively main chain contacts.

SPE-C displays a much higher specificity towards T cells targeting mainly TcRVβ2.1 compared to e.g. SPE-A, which binds to Vβ2.1, Vβ12.2, Vβ14.1 and Vβ15.1. This can be explained by a significantly larger buried surface area and the involvement of all Vβ hypervariable loops, including CDR1, CDR2, CDR3, and HV4 (Li, Llera, & Mariuzza, 1998). In addition, residues on the CDR1 and CDR2 loops are involved in extensive intermolecular contacts. Another variation in TcRVβ-binding has been suggested for SPE-I (Figure 4B), which possesses a unique extension (α3-β8 loop) (Brouillard, et al., 2007). A similar extension has been found in the staphylococcal toxins SEI and SEK and the crystal structure of SEK bound to human TcRVβ5.1 has revealed that

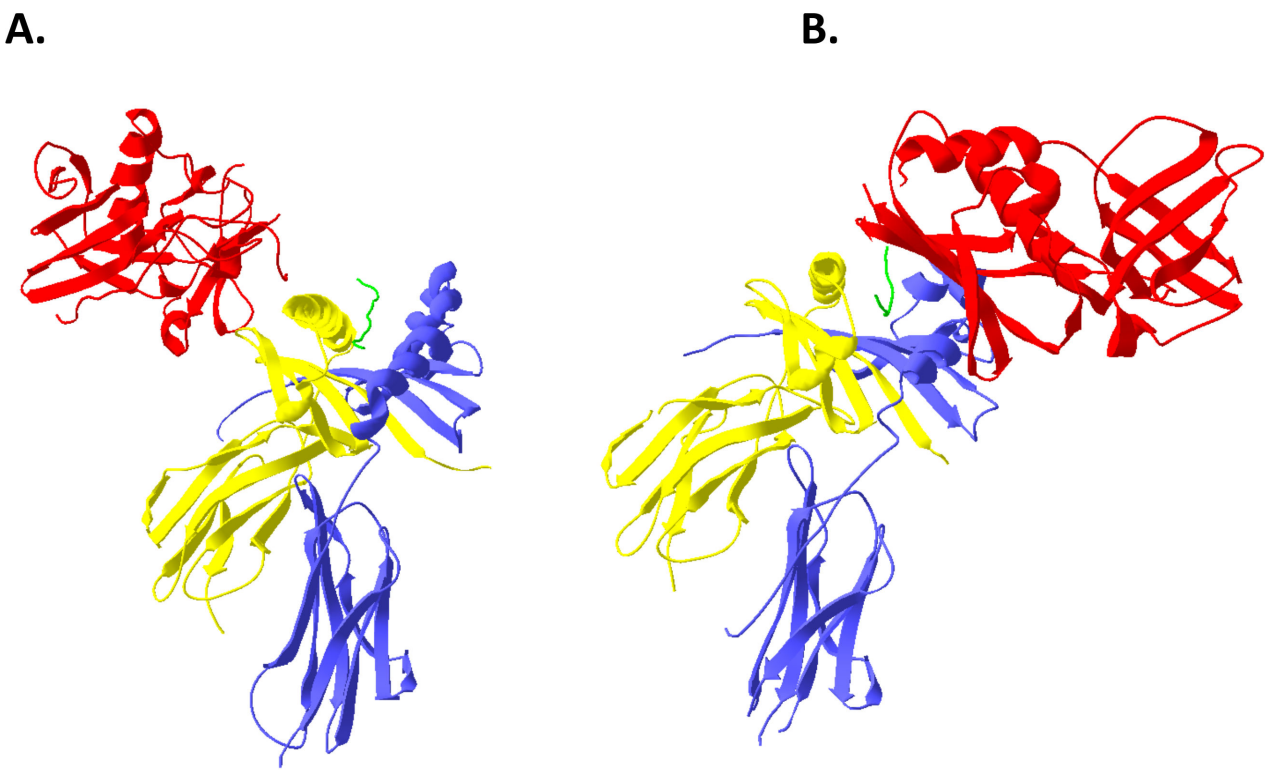

Figure 3. Protein structures of SAg bound to human MHC class II.
A. Structural model of the SPE-A – DR complex in which the SPE-A structure (1B1Z) was superimposed onto the SEB – DR1 structure (1SEB). SPE-A (red) binds to the α-chain of MHC class II (yellow) via the generic "low-affinity binding site" using an exposed hydrophobic loop region within the N-terminal β-barrel domain that binds to a hydrophobic groove located in the distal region of the DR α1-domain.
B. Crystal structure of the SPE-C – DR2 complex (1HQR). SPE-C (red) binds to the polymorphic MHC class II β-chain (blue) with the formation of a tetravalent zinc complex that includes three residues within the C-terminal domain of SPE-C, also known as the zinc-binding site, in addition to the conserved His81 of MHC class II β-chain ("high-affinity binding site"). SPE-C also forms contacts with the bound peptide antigen (green).

residues within the α3-β8 loop make intermolecular contacts with the apical loop of framework region 4 (FR4) (Günther, et al., 2007).

Four categories of SAg - TcR interactions have been proposed: a) highly promiscuous T cell binders, including SEB, that bind to TcRVβ in a simple conformation-dependent mode and only interact with a single CDR2 loop (CDR2); b) moderately promiscuous molecules, including SPE-A, that have direct side chain/side chain contacts in addition to the conformation dependence; c) highly selective T cell activators, like SPE-C, that bind to TcRVβ with the highest degree of structural dissimilarity, and the usage of all three CDR loops (Sundberg, Li, & Mariuzza, 2002) and d) SAgs, like SPE-I, containing the α3-β8 loop and extending the TcRVβ domain binding site into the FR4 region.

Recently, the first ternary complex of a SAg with MHC class II and the TcR was solved. The protein structure of SEB in complex with HLA-DR1 and TcRVα22/Vβ19 confirmed that the SAg adopts a wedge-like position when binding to the TcRVβ-chain, allowing for an interaction between the Vα chain and MHC class II. This binding

mode also circumvents contact between TcR and the presented peptide allowing the SAg to trigger a peptide-independent activation of T cells (Rödström, Elbing, & Lindkvist-Petersson, 2014).

CD28 binding

Over two decades it was believed that the simultaneous interaction of SAg with MHC class II and the TcR was not only necessary, but also sufficient to induce a strong mitogenic activity and the production of large amounts of pro-inflammatory cytokines. This classical view has recently been challenged when it was shown that SAgs can also bind to CD28 (Arad, et al., 2011). CD28 is the general co-stimulatory receptor, which is constitutively expressed on T cells and interacts with B7 molecules (CD80 and CD86) (Riley & June, 2005). It was previously shown that small synthetic peptides mimicking a region that is highly conserved among SAgs (β-strand/hinge/α-helix domain) were strong inhibitors of staphylococcal enterotoxin B (SEB), staphylococcal toxic-shock syndrome toxin-1 (TSST-1), and SPE-A and were protective in mice against a lethal challenge with those SAgs. Notably, the peptide region was neither involved in MHC class II binding, nor in binding to TcR. In contrast, synthetic peptides of regions known to interact with MHC class II or TcR failed to reduce a cytokine response (Arad, Levy, Hillman, & Kaempfer, 2000). It was later shown that the synthetic peptide successfully competed with a monoclonal anti-CD28 antibody for binding to CD28 without directly binding to the antibody. This suggested an interaction of the peptide with CD28. Direct binding of the peptide and of staphylococcal enterotoxin B to CD28 with micromolar affinity was demonstrated by surface plasmon-resonance analysis and this interaction is essential for the induction of pro-inflammatory cytokine genes (Arad, et al., 2011). A structural model was suggested that shows a possible binding interface between the N-terminal 118-residue region of the extracellular domain of CD28 (1yjd) with a freely accessible β-strand/hinge/α-helix domain of staphylococcal enterotoxin C3 (SEC3) in complex with MHC class II α-chain and mTcRVβ8.2 (1jck) (Arad, et al., 2011). More recently, it was demonstrated that a CD28 mimetic peptide protects mice from a lethal challenge with SPE-A, as well as from a lethal *S. pyogenes* infection in a mouse necrotizing soft tissue infection model providing further evidence for the importance of a SAg-CD28 interaction in SAg-mediated disease (Ramachandran, et al., 2013).

It should be mentioned that all the research described above was carried out with SAgs that bind to the MHC class II α-chain. However, nine of the eleven SAgs produced by *S. pyogenes* bind to the MHC class II β-chain via the zinc-binding site in the C-terminal domain and this binding mode would sterically hinder the suggested interaction with CD28. Therefore, it is currently unclear, if the activity of those SAgs is CD28-independent, or if there is another binding site for CD28.

Consequences of SAg binding to host receptors

Engagement of SAg with its receptors results in rapid release of TNF-α and TNF-β, followed sequentially by IL-2, IL-6, IL-1 and IFN-γ. Animal studies with mice have shown a dramatic increase of TNF-α within the first hour of SAg exposure and T cells within the spleen were found to be the major source for the early release of TNF-α (Faulkner, Cooper, Fantino, Altmann, & Sriskandan, 2005). A comparative study with SPE-A and SMEZ showed that the cytokine-inducing capacity of SMEZ was approximately 10-fold higher than observed with SPE-A (Müller-Alouf, et al., 2001). Furthermore, disruption of the *smez* gene in an M89 strain completely abolished cytokine production of a *S. pyogenes* culture supernatant in vitro (Unnikrishnan, et al., 2002).

Early studies have shown that a combination of sub-lethal doses of SAg and LPS can act synergistically to cause shock in rodents, although only when D-galactosamine was used as a sensitizing agent (Bohach, Fasdt, Nelson, & Schlievert, 1990). Similarly, primary human monocytes that were pre-exposed to SAgs for 3 hours showed highly exaggerated TNF-α responses after exposure to LPS. It has been suggested that this synergy results from enhanced pattern recognition of LPS and this is based on the observation that SAg signaling increases expression of toll-like receptor 4 (TLR4), the pattern recognition receptor for LPS (Hopkins, et al., 2005).

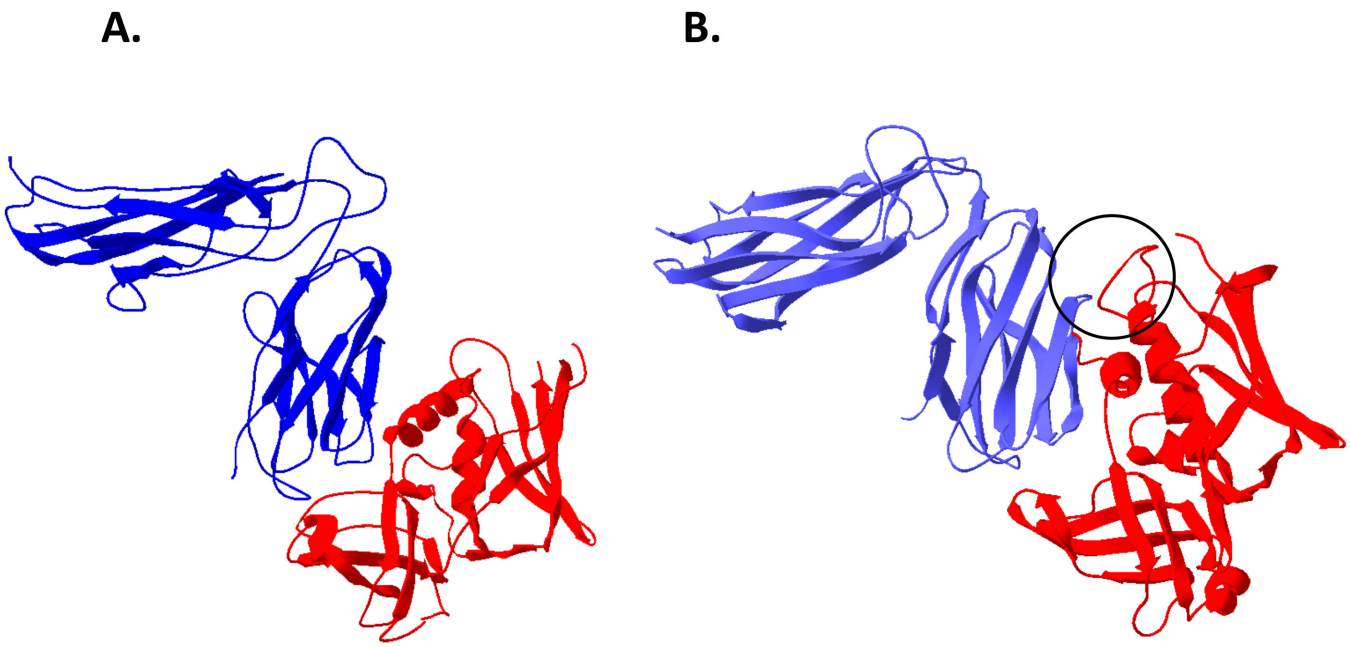

Figure 4. Protein structures of SAgs bound to the T cell receptor.
A. Crystal structure of SPE-C bound to the human TcRVβ2 chain (1KTK). SPE-C (red) shows high specificity for hVβ2.1 (blue) due to extensive interactions involving all hypervariable loops (CDR1, CDR2, CDR3, and HV4).
B. Structural model of SPE-I in complex with hTcRVβ5.1 in which the SPE-I structure (2ICI) was superimposed onto the SEK – hTcRVβ5.1 structure (2NTS). Like SEK, SPE-I (red) has a unique α3-β8 loop that forms intermolecular contacts with the apical loop of framework region 4 (FR4) of the TcRVβ chain (blue) (shown in circle).

Furthermore, several SAgs, including the streptococcal SAgs SPE-A and SMEZ, were able to up-regulate TLR2 on the surface of primary human monocytes. This was dependent on SAg-binding to MHC class II, but did not involve signaling by ligation to TLR2. TLR2 up-regulation was associated with an increase in the pro-inflammatory response to TLR2 ligands, but only at high ligand concentration (Hopkins, et al., 2008).

In contrast to the classical AB family toxins, SAgs are believed to remain extracellular and function by signaling inside the host cell. However, a recent study by Ganem *et al.* has demonstrated an uptake of SAgs by mouse dendritic cells (DCs) without triggering DC maturation. This was followed by SAg recycling to the cell membrane of DCs and the SAg-loaded DCs were capable of triggering a strong lymphocyte proliferation. The authors suggested that intracellular trafficking of SAgs might increase the local concentrations of SAgs and promote their encounter with MHC class II on APCs and the TcR on T cells in lymph nodes (Ganem, et al., 2013).

Streptococcus pyogenes superantigens and disease

SAgs have been implicated in a range of *S. pyogenes* diseases, including invasive infections such as necrotizing fasciitis and streptococcal toxic shock syndrome (STSS), Kawasaki disease, psoriasis and acute rheumatic fever. The potential involvement of SAgs in these diseases has been demonstrated mainly by epidemiological studies, clinical studies and animal infections models. In addition, several studies have shown specific skewing of the TcRVβ-repertoire in stimulated T cells consistent with SAg activity. However, direct evidence for the involvement of SAgs in *S. pyogenes* disease remains inconclusive.

Invasive *Streptococcus pyogenes* disease

Epidemiological studies

The predominant strains isolated from patients with STSS belong to serotype M1 and M3 which both frequently produce SPE-A and SPE-C (Talkington, et al., 1993; Yu & Ferretti, 1989). This association was also found in several other epidemiological studies. The *speA* gene was found in a majority (40-90%) of *S. pyogenes* isolates from the USA associated with invasive disease and STSS, but only in a minority (15-20%) of isolates from non-invasive diseases (Hauser, Stevens, Kaplan, & Schlievert, 1991). Cleary and colleagues reported *speA*-carrying isolates in 90% of 17 isolates causing sepsis, but in only 54% of 37 isolates that caused non-invasive disease (Cleary, et al., 1992). A high frequency of *speA* (80%) was found in STSS isolates collected in Australia (Carapetis, Robins-Browne, Martin, Shelby-James, & Hogg, 1995) and of 53 STSS isolates from Europe and Chile, 64% carried the *speA* gene and 28% carried *speC* (Reichardt, Müller-Alouf, Alouf, & Köhler, 1992). Vlaminckx *et al.* analyzed 170 *S. pyogenes* isolates that caused specific manifestations of invasive disease in The Netherlands between 1992 and 1996. They found a strong correlation of a M1 clone carrying *speA* and *smez* with toxic shock-like syndrome. Furthermore, *S. pyogenes* isolates carrying the *speC* gene were found predominantly in patients with invasive disease not accompanied with streptococcal toxic shock syndrome. The authors also established associations of *speA* with meningitis, *speH* with arthritis and *speC* with puerperal sepsis (Vlaminckx, et al., 2003). In a more recent study conducted in Norway, *speA* was identified in 41% of 22 invasive isolates, but only in 11% of 101 non-invasive isolates (Kittang, Skrede, Langeland, Haanshuus, & Mylvaganam, 2011). Interestingly, a worldwide shift in *speA* alleles has occurred over the past 80 years. Contemporary M1 and M3 strains almost exclusively harbor *speA2* and *speA3*, respectively, and these alleles have been associated with the re-emergence of invasive infections with more virulent *S. pyogenes* strains. A more recent study that analyzed the genome sequences from 3,615 M1/*emm1* strains from different locations between 1920 and 2013 suggests that acquisition of the *speA* gene was an important step in the evolution of a hypervirulent M1/*emm1* strain. It appears that an early M1/*emm1* strain acquired a plasmid carrying the *speA1* allele, which subsequently evolved into the *speA2* allele. Acquisition of a large chromosomal region carrying genes for additional virulence factors (Streptolysin O and NAD^+-glycohydrolase) was the final molecular event preceding the emergence of the hypervirulent M1/*emm1* strain in the 1980s (Nasser, et al., 2014).

However, there are also reports that showed no significant difference in the frequency of *speA* between invasive and non-invasive *S. pyogenes* isolate (Descheemaeker, Van Loock, Hauchecorne, Vandamme, & Goossens, 2000; Haukness, et al., 2002; Hsueh, et al., 1998; Mylvaganam, Bjorvatn, & Osland, 2000). For example, Haukness and co-workers compared the genetic heterogeneity of 63 community pediatric pharyngeal isolates with 17 contemporaneous invasive pediatric isolates and found that more pharyngeal (71%) than invasive isolates (35%) were positive for both *speA* and *speC* (Haukness, et al., 2002).

An association with invasive disease was also reported for other SAgs. An invasive M3/T3 strain emerged during the 1990' in Japan and 100% of 18 isolates carried the phage-encoded *speK* gene (formerly *speL*). In contrast, none of the 10 non-invasive isolates collected before 1992 harbored the *speK* gene (Ikebe, et al., 2002). In another Japanese study with isolates collected between 1994 and 1999, *ssa* was detected in 76% of 17 invasive isolates, but

only in 37% of 299 non-invasive isolates (Murakami, et al., 2002). A recent study compared a collection of 160 isolates recovered from normally sterile sites with 320 isolates associated with pharyngitis in Portugal and observed an association of *speJ* with invasive *S. pyogenes* isolates (Friães, Pinto, Silva-Costa, Ramirez, & Melo-Cristino, 2012).

An association of the *speM* gene with invasive disease was suggested after a study with *S. pyogenes* isolates collected in Germany between 1997 and 2003 showed that *speM* was more commonly found in invasive disease isolates compared to non-invasive isolates (Lintges, et al., 2010).

The *smez* gene is chromosomally encoded and found in almost all *S. pyogenes* isolates. Therefore, there is no association of this toxin gene with invasive disease. However, *smez* is the most variable of all *sag* genes and there are more than 50 *smez* alleles listed in the NCBI database. Furthermore, *smez* alleles are in linkage equilibrium with *S. pyogenes* M-serotypes and there are significant differences in mitogenic potencies between SMEZ variants (Proft, et al., 2000). However, no studies have analyzed a possible correlation of certain *smez* alleles with invasive disease. Notably, it has recently been discovered that the STSS-associated *emm3* strain carries a *smez* variant with a 13-bp deletion that causes a frame-shift and consequently disrupts SAg activity (Turner, et al., 2012).

Clinical Studies

SPE-A was detected in the sera of two patients with STSS using immunoassays. The presence correlated with elevated levels of TNF-α, providing evidence of SPE-A-induced T cell activation (Sriskandan, Moyes, & Cohen, 1996). Strong mitogenic activities were found in the serum of two patients with STSS, one of whom died. PCR-analysis of the infecting *S. pyogenes* isolates identified the presence of several *sag* genes, including *speA*, *speC, speG, speJ* and *smez.* Using a T cell proliferation assay with recombinant protein standards, the mitogenic activity in the serum could be wholly attributed to *smez*, with a small contribution of *speJ* in one case, and the concentration of the circulating SAg was approximately 100 pg/ml. Furthermore, analysis of the convalescent serum from the surviving patient showed sero-conversion to SMEZ, providing further evidence for the involvement of SMEZ in STSS (Proft, Sriskandan, Yang, & Fraser, 2003b). It has been suggested that the lack of neutralizing antibodies against SAgs might be a risk factor in invasive disease and supportive evidence was provided by several other studies. Eriksson and co-workers showed that sera from STSS patients did not neutralize SPE-A-induced lymphocyte mitogenicity and neutralization was low in patients with bacteremia compared with serum levels from uncomplicated erysipelas (Eriksson, Andersson, Holm, & Norgren, 1999). A study by Basma *et al.* found significantly higher plasma levels of neutralizing anti-SPE-A antibodies in patients with severe and non-severe invasive *S. pyogenes* disease compared to age- and geographically matched healthy donors (Basma, et al., 1999). In a case study of a patient with STSS from New Zealand, an *emm118* strain was isolated from the patient and the major mitogenic toxin produced by this isolate was identified as SMEZ-34, which is closely related to the highly potent SMEZ-2 variant. No neutralizing anti-SMEZ-34 antibodies could be detected in the acute serum, but were found in convalescent serum (Yang, et al., 2005).

Animal Infection Models

A baboon model of *S. pyogenes* bacteremia that mimics human STSS was used to demonstrate the in-vivo effect of SAgs. Intravenous infusion of a SPE-A-expressing M3 strain led to profound hypertension leukopenia, metabolic acidosis, renal impairment, thrombocytopenia and disseminated coagulopathy within 3 hours (Stevens, et al., 1996). In another study, a murine model of bacteremia and *S. pyogenes* muscle infection was used to investigate the role of SPE-A. Surprisingly, infection with a *speA* deletion mutant failed to attenuate virulence, but instead resulted in increased bacteremia and a reduction of neutrophils at the site of infection (Sriskandan, et al., 1996b). It was suggested that the reduced binding affinity of SAgs to murine MHC class II molecules might be reason for the unexpected result. Indeed, the use of HLA-DQ transgenic mice rendered the animals susceptible to SPE-A and resulted in massive cytokine production and lethal shock (Sriskandan, et al., 2001). The

same HLA-DQ transgenic mouse model was also used to assess the role of SMEZ in disease. Intraperitoneal infection of the animals with a M89 strain expressing the SMEZ-13 variant resulted in significantly increased cytokine production. In contrast, infection with an isogenic M89 *smez* deletion mutant failed to elicit a response, despite the fact that this *S. pyogenes* isolate also carried other *sag* genes suggesting an important role for SMEZ in invasive disease (Unnikrishnan, et al., 2002). The in vivo role of SAgs was also shown in rabbit infection models with SPE-A (Schlievert, Assimacopoulos, & Cleary, 1996) and SPE-J, which induced fevers and was lethal in two models of STSS (McCormick, Pragman, Stolpa, Leung, & Schlievert, 2001).

Studies based on changing T cell repertoires

Stimulation with SAgs leads to an initial TcRVβ-restricted proliferation of T cells followed by the loss of the particular T cell subsets due to anergy leaving a kind of 'fingerprint'. Michie and colleagues collected two *S. pyogenes* isolates from two patients with STSS which both produced a mitogen specific for the Vβ2 T lymphocyte subset in vitro. Lymphocytes collected from both patients during the acute phase demonstrated a marked reduction in circulating 'naive' and helper T cells expressing Vβ2, and an increase of CD8 T cells expressing Vβ2 (Michie, Scott, Cheesbrough, Beverley, & Pasvol, 1994). Another study compared the TcRVβ repertoire in T cells from variety of disease patients and found a consistent pattern of depletion of Vβ1, Vβ5.1, and Vβ12 in patients with severe *S. pyogenes* infections, but not in patients with non-severe infections or patients with severe no *S. pyogenes* infections (Watanabe-Ohnishi, et al., 1995). Yet another study reported the expansion of TcRVβ2 T cells from two patients with STSS reflecting the production of SPE-C (Thomas, et al., 2009).

Genetic background of the host

The results from several studies suggest that the genetic background of the host, in particular HLA polymorphism, might play an important role in invasive disease susceptibility. SPE-A was shown to stimulate higher proliferation responses when presented by HLA-DQ, compared to HLA-DR1, HLA-DR4, or HLA-DR5 alleles, whereas SPE-C was preferentially presented by HLA-DR4 (Norrby-Teglund, Nepom, & Kotb, 2002). Moreover, patients with the HLA-DRB1*1501/DQB1*0602 haplotype showed significantly reduced responses to streptococcal SAgs and were less likely to develop severe systemic disease compared to individuals with risk or neutral haplotypes (Kotb, et al., 2002). Llewelyn and colleagues reported a stronger affinity of SPE-A for HLA-DQA1*01 compared to HLA-DQA1*03/05, which also resulted in quantitative and qualitative differences in T cell proliferation, cytokine production, and TcRVβ-specific changes in the T cell repertoire (Llewelyn, et al., 2004). In contrast, a study using HLA-DQ transgenic mice found that HLA-DQ6 and HLA-DQ8 elicited comparable in vitro and in vivo immune response to SPE-A, SPE-C and SMEZ (Rajagopalan, et al., 2008).

A more recent study showed that HLA alleles not only influenced the severity of SAg-mediated disease, but also effected the polarization of the cytokine response. In contrast to the high-risk alleles HLA-DR14/DR7/DQ5, HLA-DR15/DQ6 alleles strongly protected against severe invasive *S. pyogenes* disease and elicited significantly higher amounts of anti-inflammatory cytokines, such as IL-10, compared to pro-inflammatory cytokines, like IFN-γ (Nooh, Nookala, Kansal, & Kotb, 2011).

Kawasaki Disease

Kawasaki disease (KD) is an acute multisystem vasculitis of unknown etiology that affects mostly young children leading to coronary artery damage (Takahashi, Oharaseki, & Yokouchi, 2014). Streptococcal SAgs have been proposed as etiological agents in the pathogenesis of KD. Multiple studies reported the selective expansion of T cells bearing TcRVβ2 pointing to a possible involvement of SAgs in the disease, in particular SPE-C and SPE-J which both preferentially stimulate the TcRVβ2 T cell subset (Abe, et al., 1992; Konishi, et al., 1997). Yoshioka *et al.* reported polyclonal expansion of TcRVβ2- and TcRVβ6-bearing T cells and elevated plasma levels of IL-1β, IL-2, IL-6, IL-8, IL-10, IFN-γ, and TNF-α in the acute phase of KD. Moreover, anti-SPE-C antibody levels were significantly higher in acute and convalescent serum from KD patients compared to age-matched controls

(Yoshioka, et al., 1999). High levels of anti-SPE-A IgM antibodies were also found in KD patients and increased with the clinical weeks reaching 43% of KD subjects at the fourth week (Matsubara, et al., 2006).

PCR-analysis of *speA, speC, speG*, and *speJ*, in stool specimen obtained from 60 patients with KD and 62 age-matched children showed higher prevalence of *sag* genes in KD patients compared to controls (Suenaga, Suzuki, Shibuta, Takeuchi, & Yoshikawa, 2009). Two studies that investigated the T cell repertoire in KD patients also provided evidence for a role of SAgs in KD. TcRVβ restricted CD4 and/or CD8 activation was observed in eight of 11 (72%) of the KD patients, a finding not observed in healthy controls. Moreover, 81% children with KD had evidence of either TcRVβ skewing (particularly CD4 Vβ2 and Vβ5.1) and/or TcRVβ restricted activation (Brogan, Shah, Clarke, Dillon, & Klein, 2008). Nagata and colleagues identified 18 strains of Gram-positive cocci from the upper gastrointestinal tract from patients with KD that had superantigenic properties and which induced the expansion of TcRVβ2 T cells in vitro (Nagata, et al., 2009).

However, other investigations have failed to show any evidence for SAg involvement in KD. In particular, data from several serological studies showed no significant difference in the prevalence of SAg antibodies between KD patients and control subjects (Gupta-Malhotra, Viteri-Jackson, Thomas, & Zabriskie, 2004; Morita, Imada, Igarashi, & Yutsudo, 1997; Nomura, Yoshinaga, Masuda, Takei, & Miyata, 2002). Furthermore, IgM transcripts expressed by the B cells in the peripheral blood of KD patients in the acute phase of the disease clearly showed an oligoclonal expansion, suggesting that KD is caused not by stimulation of a SAg, but rather by a conventional antigen (Lee, Shin, Kim, & Park, 2009).

Psoriasis

Psoriasis is a chronic inflammatory multi organ disease with well-characterized pathology occurring in the skin and often the joints (Raychaudhuri, Maverakis, & Raychaudhuri, 2014). It has been reported that a particular form of psoriasis, guttate psoriasis, is triggered by *S. pyogenes* throat infections in 2/3 patients (Nahary, et al., 2008). The causes of psoriasis are not fully understood, but several lines of evidence point to an involvement of SAgs in the disease mechanism. Some studies have demonstrated a TcRVβ-restricted T cell stimulation in psoriasis patients. Leung and co-workers have shown T cell expansion consistent with SAg activity in skin biopsies from two patients with psoriasis, but not in peripheral blood. Skin biopsies from 10 out of 10 patients with acute guttate psoriasis, but not skin biopsies from 12 patients with acute atopic dermatitis or inflammatory skin lesions induced in normal subjects with sodium lauryl sulfate, demonstrated selective accumulation of TcRVβ2 T cells, which occurred in both the CD4+ and the CD8+ T cell subsets. Moreover, the TcR showed extensive junctional region diversity suggesting SAg-induced stimulation of T cells (Leung, et al., 1995). Other studies demonstrated an increase of TcRVβ2 and Vβ5.1 T cells in the skin of patients with guttate and chronic plaque psoriasis compared with peripheral blood (Lewis, et al., 1993) and an increase of TcRVβ2 and Vβ17 cutaneous T cells in patients with guttate psoriasis, but not in control patients (Davison, Allen, Mallon, & Barker, 2001). On the other hand, since 1994, at least 14 studies reported by nine independent groups have indicated that chronic psoriasis lesions are infiltrated by oligoclonal T cells suggesting stimulation by conventional antigens rather than SAgs (reviewed by (Valdimarsson, Thorleifsdottir, Sigurdardottir, Gudjonsson, & Johnston, 2009).

Acute Rheumatic Fever

Acute rheumatic fever (ARF) is a post-streptococcal autoimmune disease. Multiple episodes can result in rheumatic heart disease (RHD), which is the leading cause of preventable pediatric heart disease. It mainly occurs in school age children and young adults after pharyngeal infection with *S. pyogenes* (Carapetis, Steer, Mulholland, & Weber, 2005). Cross-reactive immune responses to cardiac tissue and joints are responsible for inflammation in the host and it has been suggested that SAgs might stimulate the reactive T cells. There is a correlation between M18 isolates associated with ARF in the USA and *speL* and *speM*. These *sag* genes were

found in all M18/emm18 isolates collected over a 69-year period (Smoot, et al., 2002b). Antibodies against SPE-L and SPE-M were more common in convalescent sera from ARF patients compared to pharyngitis patients (Smoot, et al., 2002a). However, serum antibodies against SAgs did not predict the susceptibility of Aboriginal Australians (Yang, et al., 2006).

Therapeutic Interventions

Intravenous immunoglobulin (IVIG) therapy

Pooled human intravenous immunoglobulin (IVIG) is increasingly used in cases of severe invasive *S. pyogenes* disease to neutralize the activity of SAgs. Several studies have shown that the lack of protective antibodies against SAgs is a risk factor for toxic shock syndrome (Eriksson, Andersson, Holm, & Norgren, 1999; Basma, et al., 1999; Norrby-Teglund, Low, & Kotb, 2007). IVIG were used with bacterial culture supernatants and showed good neutralization properties, in particular against streptococcal SAgs (Darenberg, Söderquist, Normark, & Norrby-Teglund, 2004). The clinical efficacy of IVIG in STSS was documented in several case reports, two observational cohort studies, one case-control study and one multicenter placebo-controlled trial (Norrby-Teglund, Low, & Kotb, 2007). However, definitive clinical trial data are still lacking. Several factors need to be considered in the use of IVIG as adjunctive STSS therapy. SAgs appear to have a very fast turnover rate in the patient's blood and might therefore only be beneficial if applied very early after the onset of the disease. Secondly, not much is known about SAg expression during infection. It has been shown in animal infection models that SAgs are up-regulated significantly during disease (Kazmi, et al., 2001; Virtaneva, et al., 2005). Finally, the efficacy of IVIG to neutralize streptococcal SAgs was shown to vary between different preparations of IVIG (Schrage, Duan, Yang, Fraser, & Proft, 2006).

Peptide antagonists

Kaempfer and colleagues synthesized several short peptides derived from various SEB domains and found a dodecapeptide that weakly antagonized SEB activity. A modified version of this peptide was shown to be a more powerful antagonist that inhibited the activity of SEB and TSST-1 in a mouse infection model (Arad, Levy, Hillman, & Kaempfer, 2000). It was originally unclear how this peptide, which is highly conserved in both streptococcal and staphylococcal SAgs would work, as the SEB domain from where the peptide is derived is not involved in either MHC class II or TcR binding. More recently, it was shown that the peptide binds to the co-stimulatory receptor CD28 and this interaction is essential for the induction of pro-inflammatory cytokine genes (Arad, et al., 2011). More recently, it was demonstrated that the CD28 mimetic peptide AB103 protects mice from a lethal challenge with SPE-A, as well as from a lethal *S. pyogenes* infection in a mouse necrotizing soft tissue infection model (Ramachandran, et al., 2013).

Receptor mimics

A bispecific receptor mimic that targets both the MHC class II and the TcR binding site of SAgs was designed by Lehnert and co-workers. This construct consists of a HLA-DR1α1 subunit that is connected to the variable TcR β-chain via a peptide linker. The authors generated several different receptor mimics, each one specific against a particular SAg. For example, human TcRVβ3 was used for a SEB-specific molecule, human TcRVβ2 was used for a TSST-1-specific chimera, and an analogue of murine TcRVβ8.2 was used for SEC3-specific chimera (Lehnert, et al., 2001). In a cell proliferation assay, 20-times excess of the TcRVβ8.2 chimera over SEC3 showed 40% inhibition.

The efficacy of TcR antagonist was later improved by using yeast display libraries of random and site-directed human TcRVβ8 mutants to screen for improved domain stability and increased SAg binding (Buonpane, et al., 2007). A panel of six soluble, high-affinity TcRVβ mutants have been engineered that bind to one of six key staphylococcal and streptococcal SAgs (SEA, SEB, SEC3, TSST1, SPE-A, and SPE-C), at the same epitope as the

wild type receptors. Affinities were in the picomolar to nanomolar range representing 1000 to 3,000,000-fold increases, compared to wild-type (Sharma, Wang, & Kranz, 2014; Wang, Mattis, Sundberg, Schlievert, & Kranz, 2010).

Toxoid vaccines of SAgs

Toxoids of two streptococcal SAgs were generated by Schlievert and colleagues. Double-, triple- and hexa-amino acid mutants of SPE-A targeting MHC class II and TcR binding sites lacked SAg activity, were non-lethal in two rabbit models of STSS and stimulated protective antibody responses (Roggiani, et al., 2000). Similarly, the SPE-C Y15A/N38D double mutant and the SPE-C Y15A/H35A/N38D triple mutant were non-mitogenic, non-lethal in rabbit models of STSS and protected vaccinated animals from challenge with wild-type SPE-C (McCormick, et al., 2000).

SAgs as vaccine conjugates

By triggering MHC class II signals without engaging with the TcR, SAgs might be excellent vaccine adjuvants of the innate immune response due to their priming effects on antigen presenting cells (Hopkins, et al., 2005). In a recent study, the TcR-binding site of SMEZ-2 was mutated by converting three amino acid residues, W75L, K182Q, and D42C. The cysteine at position 42 was introduced to allow for easy coupling with desired peptides. The T cell proliferation response of the mutant (SMEZ-2-M1) was $>10^5$-fold lower compared to wild-type and cytokine production in response to the mutant was undetectable. Vaccination of mice with ovalbumin conjugated to SMEZ-2-M1 resulted in anti-ovalbumin IgG titers being 1,000-10,000-fold higher than in mice immunized with unconjugated ovalbumin (Radcliff, et al., 2012). Conjugating antigens to SMEZ-2-M1 also increased the efficiency for cross-presentation. When co-injected with an adjuvant, the SMEZ-2-M1 conjugates also elicited potent T cell responses with antitumor activity (Dickgreber, et al., 2009). More recently, it was demonstrated that dendritic cells pulsed with the nucleocapsid of hepatitis B virus conjugated to SMEZ-2-M1 (M1:HBcAgs) stimulated virus-specific CD(8+) T cells more effectively than dendritic cells pulsed with native virus capsid, which also suggests that SMEZ-2-M1 conjugates increase cross-presentation by APCs (McIntosh, et al., 2014).

In another study, SMEZ-2-M1 conjugated with myelin oligodendrocyte glycoprotein 35-55 peptide suppressed the development of experimental autoimmune encephalomyelitis (EAE) in mice via antigen-specific suppression of T cell responses and re-establishing of suppressor function of Ly6G(-)CD11b(+) blood monocytes (Slaney, Toker, Fraser, Harper, & Bäckström, 2013). These studies suggest a potential use of SMEZ-2-M1 as antigen carrier for vaccination, anti-tumor therapy and treatment of autoimmune diseases.

What are SAgs doing for the bacteria?

After more than two decades of intensive research, the question of why SAgs are important for the bacteria remains largely unanswered. There are currently 11 SAgs found in *S. pyogenes* and many of them have orthologues in other streptococci, all of them sharing a common protein fold and the same target receptors on host cells, MHC class II and TcR. The evolutionary advantage of SAg production seems therefore eminent and is supported by the fact that SAg-producing streptococci only infect hosts with adaptive immunity. Furthermore, most SAgs show allelic variation, in particular SMEZ with >50 variants, that results in antigenic rather than functional differences. This confirms that SAg evolution is mainly driven by host immunity. It is almost certain that the role of SAgs is not to induce systemic lethal shock in the host. Significant antibody responses to bacterial SAgs are commonly found in healthy adults, indicating that SAg exposure must occur during either non-severe infections or asymptomatic colonization (Basma, et al., 1999). A possible advantage of SAg production might involve the corruption of the host immune response. SAgs interfere with the adaptive immune system resulting in profound Th1 type responses with non-specific T cell proliferation and massive release of type 1 cytokines, such as IL-2, IFN-γ and TNF-α. This might suppress local inflammation at the site of infection, although there is

no evidence that SAgs directly enhance colonization. By promoting a Th1 type response, SAgs might also suppress a type 2 response and prevent the production of high-affinity cytotoxic antibodies. Another possible mechanism of how SAgs corrupt the immune system might be their ability to induce T cell anergy, a non-responsive state that results from the systemic stimulation of T cells by SAgs. Anergic T cells are unable to produce IL-2 and therefore SAg stimulation might lead to local IL-2 deficiency, which could limit the expansion of antigen-specific T cells (Lavoie, Thibodeau, Erard, & Sékaly, 1999; Miller, Ragheb, & Schwartz, 1999; O'Hehir & Lamb, 1990). More recently, Llewelyn and colleagues suggested that SAgs are able to induce a regulatory T cell phenotype restricted only by the Vβ specificity of the toxin or toxins produced. They showed that stimulation of PBMCs with SPE-K (previously SPE-K/L) resulted in the rise of CD4(+) CD25(+) T regulatory T cells (Tregs) from CD4(+) CD25(-) T cells. This was Vβ-specific and required APCs. Furthermore, the Tregs expressed the anti-inflammatory cytokine IL-10 at lower SAg concentrations than was required to trigger IFN-γ production (Taylor & Llewelyn, 2010). It was later shown that SAgs are also potent inducers of human regulatory CD8(+) T cells, which were able to suppress the proliferation of CD4(+) CD25(-) T cells in response to anti-CD3 stimulation in a cell contact dependent mode. SAg induced stimulation of Tregs might therefore be a feature of acute bacterial infections contributing to immune evasion by the microbe and disease pathogenesis (Taylor, Cross, & Llewelyn, 2012). In a recent study, Kasper *et al*. have shown that acute *S. pyogenes* infection in the nasopharynx of mice is dependent upon both bacterial SAgs and host MHC class II molecules (Kasper, et al., 2014). *S. pyogenes* was rapidly cleared from the nasal cavity of wild-type C57BL/6 (B6) mice, but infection was enhanced up to ~10,000-fold in B6 mice that express the human MHC class II molecule HLA-DQ8. This infection phenotype was dependent on the production of SPE-A since an *speA*$^-$ isogenic strain showed markedly reduced infection in the noses of the B6–DQ8 transgenic mouse. Moreover, pre-vaccination with an MHC class II binding mutant toxoid of SPE-A inhibited infection. This is the first study to show that survival of *S. pyogenes* in a common niche is indeed enhanced by the production of a SAg, and gives some credence to the long held notion that SAgs are indeed virulence as well as pathogenicity factors.

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Streptococcus pyogenes Biofilm

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Introduction to Biofilms

Bacterial infections can occur in almost every part of the human body, which indicates that bacteria have adapted to survive in physiologically distinct anatomical locations. To facilitate this process, an organism must express the proper growth and virulence factors at the appropriate time, endure a potentially harsh surrounding chemical environment, and thwart a host's immune defenses. Several bacterial species use structures referred to as biofilms to combat these hazards.

A bacterial biofilm is defined as a sessile community of organisms encased in a matrix of extrapolymeric substances and attached to a substratum, interface, or to each other. Biofilms tend to exhibit an altered phenotype with respect to growth rate and gene transcription (Donlan & Costerton, 2002). The development of biofilms can be described by a five-stage process (Sauer, Camper, Ehrlich, Costerton, & Davies, 2002; Stoodley, Sauer, Davies, & Costerton, 2002). Briefly, Stage 1 consists of planktonic cells transiently adhering to a surface. At this stage, only small amounts of extrapolymeric components are associated with the attached cells and many cells are still capable of independent movement (O'Toole & Kolter, 1998). During Stage 2, cells begin to produce larger amounts of extracellular polymer, which leads to a more stable attachment. Stages 3 and 4 involve the establishment and maturation of biofilm architecture. Cell clusters interspersed with water channels form three-dimensional structures that are widely recognized today as microcolonies, and cells within these microcolonies begin to alter their physiology. Stage 5 is associated with the dispersal of individual cells or pockets of cells from the biofilm structure. These cells are free to disseminate, recolonize, and repeat the cycle of biofilm development. In *Streptococcus pyogenes*, a mature biofilm is known to consist of proteins, DNA, and a polysaccharide-containing material known as glycocalyx (Doern, et al., 2009; Akiyama, Morizane, Yamasaki, Oono, & Iwatsuki, 2003; Cho & Caparon, 2005). (Figure 1)

Biofilms are responsible for a large medical burden throughout the world. According to the US National Institute of Health, biofilms account for over 80% of microbial infections in the human body (Davies, 2003). An estimated 17 million new biofilm infections arise annually in the United States, which result in as many as 550,000 fatalities each year (Worthington, Richards, & Melander, 2012) and cause an ever-growing economic burden, due to chronic infections and longer hospital stays. Biofilms pose a significant health risk because they are inherently tolerant to host defenses and are up to a thousand times more resistant to conventional antibiotics (Rasmussen & Givskov, 2006). Additionally, biofilms formed within medical devices such as prosthetic heart valves, intrauterine devices, central venous catheters, and urinary catheters can be very difficult to eliminate. Their removal requires the use of aggressive antibiotic therapies, surgical debridement, and removal of the infected device (Donlan & Costerton, 2002; Stewart & Costerton, 2001). Biofilm-residing bacteria, including *S. pyogenes,* are able to persist on both biotic and abiotic surfaces (including soft toys, books, cribs, and other hard surfaces) for extended periods of time. This results in an increased chance of exposure from contact with surfaces that were previously disregarded as a source of transmission (Marks, Reddinger, & Hakansson, 2014a).

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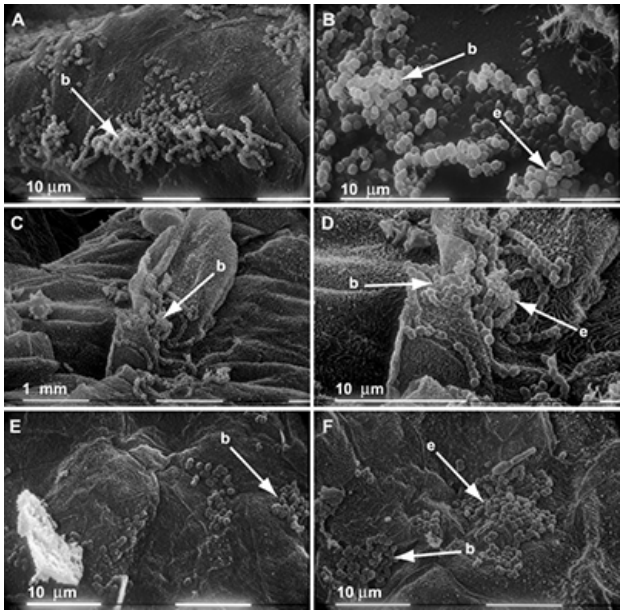

Figure 1. Scanning electron microscopy showing chains of adherent cocci organized into biofilms attached to the surface of pig skin epithelium (A and B) and to the surface of a tonsil removed due to recurrent S. pyogenes **infection (C and D) or adenotonsillar hypertrophy (E and F)**. Reprinted from (Roberts A. L., et al., 2012) with permission.

Streptococcus pyogenes Biofilms in Human and Murine Disease

Streptococcus pyogenes (group A streptococcus) is a Gram-positive pathogen that is responsible for a wide variety of human disease. Diseases range from relatively mild clinical illnesses such as pharyngitis, cellulitis, and impetigo, to life-threatening conditions, such as puerperal sepsis, myositis, toxic shock syndrome, and necrotizing fasciitis (flesh-eating disease). *S. pyogenes* infections can also lead to post-infectious sequelae, such as acute rheumatic fever, rheumatic heart disease, and acute post-streptococcal glomerulonephritis (Bisno, Brito, & Collins, 2003; Cunningham, 2000). *S. pyogenes* has been shown to form biofilms both *in vitro* and *in vivo*. Although biofilm production and regulation have been well studied in organisms such as *Pseudomonas aeruginosa* and *Staphylococcus* species, much less is known about *S. pyogenes* biofilms and their contributions to human disease.

Some of the first images of *S. pyogenes* biofilm associated with mild human disease were obtained by Akiyama et al. from infected impetigo skin lesions (Akiyama, Morizane, Yamasaki, Oono, & Iwatsuki, 2003). Using confocal laser scanning microscopy (CLSM), *S. pyogenes* microcolonies were visualized in infected human tissue, which indicates that *S. pyogenes* residing in a biofilm state may influence impetigo disease pathogenesis. A similar phenomenon was observed in a murine model of skin infection. Murine skin irritated and inflamed by the addition of croton oil resulted in the formation of an *S. pyogenes* microcolony at the site of infection. This occurred even when mice were orally treated with the antimicrobial agent cefdinir (CFDN). *S. pyogenes* microcolony formation in the presence of CFDN suggests that biofilm formation affords some level of protection as compared to their planktonic cells, an observation noted from research on other biofilm-producing organisms.

Recent literature suggests that *S. pyogenes* biofilm formation may be important for maintaining an asymptomatic carriage state in the human tonsil (Roberts, et al., 2012). With tonsils excised from patients suffering from adenotonsillar hypertrophy (ATH) or recurrent *S. pyogenes* pharyngitis, Roberts et al. were able to use immunofluorescence microscopy to visualize *S. pyogenes* microcolonies within tonsillar reticulated crypts (Roberts, et al., 2012). Three-dimensional bacterial communities arranged in biofilm-like structures were also visible in scanning electron microscopy (SEM) images. Because up to one third of pharyngitis cases don't

respond to antimicrobial treatment (Kuhn, et al., 2001; Macris, et al., 1998), the presence of *S. pyogenes* biofilm in tonsillar crypts may demonstrate one mechanism for the increased survival of this microorganism during antimicrobial therapies. Roberts et al. (Roberts, Connolly, Doern, Holder, & Reid, 2010) studied *S. pyogenes* biofilm pathogenesis in a chinchilla middle-ear model of otitis media. Hemotoxylin and Eosin (H&E) and Gram stains were used to analyze macroscopic structures removed from the middle ear cavity of chinchillas that had been infected with *S. pyogenes* by intrabullar injection. *S. pyogenes* microcolonies were observed with both staining techniques, indicating their ability to colonize the middle ear cavity. SEM analysis also revealed the presence of three-dimensional biofilms in the removed macroscopic structures (Roberts, Connolly, Doern, Holder, & Reid, 2010).

In work designed to mimic periapical periodontitis, Takemura et al. (Takemura, et al., 2004) analyzed the ability of *S. pyogenes* to form biofilms on gutta percha points, the material used in tooth cavity repair and root canal procedures. In dental practice, *S. pyogenes* is routinely isolated from infected root canal (Le Goff, Bunetel, Mouton, & Bonnaure-Mallet, 1997). With the use of SEM, *S. pyogenes* biofilms were seen on the surface of infected gutta percha points, which demonstrates their ability to colonize a clinically relevant abiotic surface.

The contribution of *S. pyogenes* biofilm to cellulitis was explored by Connolly et al. using a murine model of soft tissue infection (Connolly, Roberts, Holder, & Reid, 2011a). Following subcutaneous infection with *S. pyogenes*, excised sections of infected murine tissue were processed for the presence of microcolony formation. Gram staining of the excised sections, as well as immunofluorescence microscopy, revealed microcolony formation within the tissue.

Neely et al. (Neely, Pfeifer, & Caparon, 2002) used zebrafish to study *S. pyogenes* pathogenesis associated with myositis. In H&E stained and immunofluorescent images of infected zebrafish muscular tissue, *S. pyogenes* was present in large, densely packed clusters of bacteria. There was also a marked absence of inflammatory cell infiltration at the site of tissue damage. This disease pathology was also previously seen in a baboon model of fatal intramuscular streptococcal infection (Taylor, et al., 1999).

S. pyogenes biofilms may also play a role in the severity and progression of life-threatening human disease. Hidalgo-Grass et al. (Hidalgo-Grass, et al., 2004) used H&E staining to visualize *S. pyogenes* cell clusters in surgically debrided tissue sections from two patients suffering from necrotizing fasciitis (flesh-eating disease) and myonecrosis. Tissue sections were characterized by large amounts of *S. pyogenes* cells and a lack of neutrophil infiltration.

Notably, data suggests that biofilm-residing *S. pyogenes* colonizing nasal-associated lymphoid tissue (NALT) in mice are naturally transformable, as compared to planktonic bacteria that are typically non-competent. The extent to which this contributes to *S. pyogenes* genetic variation *in vivo* is unknown (Marks, Mashburn-Warren, Federle, & Hakansson, 2014b).

The *S. pyogenes* Cell Surface and Biofilm Formation

M Proteins

M proteins are well characterized virulence factors and are the major determinants for *S. pyogenes* serotyping, with over 200 serotypes currently categorized (Cunningham, 2000; Cole, Barnett, Nizet, & Walker, 2011). The primary M-protein used for serotyping is encoded by the *emm* gene, which is found within the *mga* regulon (Courtney, et al., 2009). This family includes the M protein (Emm), M-related protein (Mrp), and an M-like protein (Enn). Some *S. pyogenes* serotypes can encode just the *emm* gene, while others possess a combination of *emm, enn* and *mrp* (Bessen, Izzo, McCabe, & Sotir, 1997; Bessen, Sotir, Readdy, & Hollingshead, 1996; Kalia & Bessen, 2004). This family of cell surface proteins has been shown to play a role in *S. pyogenes* biofilm formation.

Lembke et al. were interested in characterizing biofilms produced by clinically relevant serotypes of *S. pyogenes*. These authors studied one isolate from nine different clinically relevant *S. pyogenes* serotypes (M1, M2, M3, M6, M12, M14, M18, M28, and M49) to assess primary adhesion to varying surfaces. These surfaces included uncoated and coated plastic surfaces with fibronectin, fibrinogen, collagen types I and IV, and laminin. Variation was observed among the different serotypes, with M2, M6, M14, and M18 adhering to the different host matrix protein-coated surfaces in measurable amounts. Serotype strains M1, M12, M28, and M49 had little primary adherence to both uncoated or matrix protein-coated surfaces (Lembke, et al., 2006). Lembke et al. suggested that the strains tested from serotypes M1, M12, M28, and M49 are unable to form potential biofilms. Serotype strains M2 and M6, and to a lesser extent those from M14 and M3, were able to transition from planktonic to sessile cells on fibronectin- and fibrinogen-coated surfaces. Adhesion was only supported on collagen I, collagen IV, and lamin for M2 after 72 hours of incubation. The M18 serotype isolate only adhered to collagen type I- or IV-coated surfaces. Since M6 and M14 strains could adhere to any of the matrix protein-coated surfaces, as well as the uncoated plastic surface, the authors suggested that these isolates qualified as potential biofilm builders. The biofilm formation behavior was investigated between different isolates of the serotypes M1, M3, and M6, and revealed heterogeneity amongst the strains. Lembke et al. suggested that biofilm formation is a trait of the individual *S. pyogenes* isolate, rather than of the defined *S. pyogenes* serotypes (Lembke, et al., 2006).

These isolates were further studied to determine if they were able to form typical biofilm architectures. Safrinin staining was used to visualize the biofilm architecture of *S. pyogenes* isolates after 72 hours of incubation on plastic coverslips coated with their preferred substrates. Serotypes M6 and M14 were able to adhere to all surfaces, including uncoated polystyrene, whereas serotype M2 bound to fibronectin, fibrinogen, lamin, and collagen types I and IV. Binding was only seen for serotype M2 on lamin and collagen types I and IV after 72 hours. Serotype M18 bound exclusively to collagen types I and IV. The strains from serotypes M1, M12, and M49 were unable to form dense layers on any substrate tested, which is consistent with their inability to gain measurable amounts of primary adherence on these substrates. Scanning electron microscopy (SEM) was performed to confirm that the structures seen using light microscopy were in fact biofilm-like structures. A *S. pyogenes* strain from serotype M49 displayed few bacterial chains on the fibronectin-coated surface, but no multilayered meshwork after 72 hours of incubation. The M6 isolate grown on plastic-coated surfaces and the M18 isolate grown on collagen type IV-coated surfaces both formed three-dimensional, multilayered, dense biofilms. Increased resolution revealed the presence of a multilayer structure, which consisted of "secondary colonizers" adhering to the apical surface of the primary attached layer. Although typical biofilm structures could be revealed through SEM, no extrapolymeric substance (EPS) was detected for the biofilm-positive *S. pyogenes* serotype strains under the conditions tested (Lembke, et al., 2006).

Confocal laser scanning microscopy (CLSM) was then used to determine the depth of biofilm. The *S. pyogenes* isolates from serotypes M2, M6, and M18 were transformed with a plasmid that constitutively expressed *egfp* (enhanced green fluorescent protein gene), which created strains TM2, TM6, and TM18, respectively. Three-dimensional structures of 72-hour biofilms of TM2, TM6, and TM18 had thicknesses measuring 13.3, 13.6, and 28 microns, respectively. Additionally, Lembke et al. calculated that these biofilms contained 13–46 cell layers by using the assumptions that the *S. pyogenes* cell diameter ranges from 0.6 to 1 microns. The state of the biofilm was assessed over extended incubations of 72, 96, and 120 hours. At 72 hours, M18 reached maximum attachment. After 96 hours, serotype strains M2 and M6 reached peak biofilm formation, but longer incubations resulted in decreased attachment. 120 hours of incubation of serotype strain M2 resulted in partial disintegration of the biofilm, as well as significantly shorter chains (Lembke, et al., 2006).

Lembke et al. suggested that morphological changes occur in *S. pyogenes* biofilms during long incubation times. *S. pyogenes* strains from serotypes M2 and M18 were analyzed under continuous flow conditions, and were found to form biofilms within this chamber system. Using SEM, the M18 isolate was found to form biofilms; however, these were not as dense as those observed in static conditions. These biofilms were also oriented in the direction of the medium flow and had no EPS material present. The M2 isolate had a more compact biofilm

structure under flow conditions, as compared to the M18 strain, and also had chains connected by threadlike structures of unknown chemical composition (Lembke, et al., 2006).

A later study by Thenmozhi et al. was interested in characterizing biofilms of different clinical *S. pyogenes* M serotypes (Thenmozhi, Balaji, Kumar, Rao, & Pandian, 2011). Eleven serotypes were grown in Todd Hewitt broth (THB) for 24, 48, 72, and 96 hour intervals to observe biofilm formation. Crystal violet staining was used to quantitate the biofilms and CLSM was used to calculate surface area and thickness. Five of the 11 serotypes tested in this study did not form any biofilms and were classified as non-biofilm formers (M49, M63, M88.3, M122, and st2147). Six of the serotypes (M56, M65, M74, M89, M100, and st38) formed substantial biofilms, comparable to the positive control (*Streptococcus mutans* UA159, a known biofilm former). These strains formed significant biofilms at 48 hours with gradual reduction at later time intervals. However, serotype strain M65 showed significant biofilm formation at 96 hours, as compared to the other time points. Overall, variation was observed among the biofilm formers at the various time points. Serotype M56 alone produced copious amount of biofilms at all the time points (Thenmozhi, Balaji, Kumar, Rao, & Pandian, 2011).

Biofilm-forming serotypes showed maximum surface area coverage at 48 hours, except for st38. Serotype strain M56 formed dense biofilms, as compared to the other serotypes, and had a maximum of 35% surface area coverage. Thickness between serotypes was independent of the time intervals. Serotype strains M56 and M100, which formed the largest biofilms, had thicknesses measuring 8.2 to 6.8 microns, respectively (Thenmozhi, Balaji, Kumar, Rao, & Pandian, 2011).

Oliver-Kozup et al. were interested in studying the biofilm formation of strains from multiple serotypes of *S. pyogenes* (M1: MGAS5005, M3: MGAS315, M28: MGAS6143, and M41: MGAS6183) (Oliver-Kozup, et al., 2011). Each of these strains was assessed for its ability to form biofilms using the crystal violet staining method, followed by CLSM and SEM. After 24 hours, M41 and M28 serotypes produced the largest biomass, as compared to M1 and M3, as assessed by crystal violet staining. Serotype M3 did not produce a substantial biomass, as compared to the other serotypes. Oliver-Kozup et al. suggested that these findings indicate a variation among *S. pyogenes* strains in their ability to form biofilms *in vitro* (Oliver-Kozup, et al., 2011).

CLSM was performed to visualize biofilm formation of three M3 serotype strains (MGAS315, MGAS2079, and MGAS158). The CLSM results confirmed what was seen in the crystal violet assay: a deficiency in the ability to form any appreciable biofilm structure. M41, M28, and M1 serotypes were capable of forming appreciable biofilms. After 24 hours, the average thickness was found to differ among all three serotypes, in a manner similar to the crystal violet data. Serotype M41 produced the thickest biofilms at 15 microns with M28 and M1 producing thinner biofilms at 12 microns and 9 microns, respectively. Additionally, biofilm cross-sections revealed architectural differences among the three serotypes. The thickest biofilms (M41 and M28), were less dense but had more elevated supracellular assembly. Serotype M1, the thinnest biofilm, was made of densely-packed cells that formed continuous layers. Field emission scanning electron microscopy (FESEM) was used to gain more insight on the architecture of the thinnest and thickest biofilms (M1 and M41). The M41 serotype biofilm had a more diverse architecture, with depressions and crypts, as compared to serotype M1. Additionally, the M41 cells had a studded cell surface morphology with protrusions that linked both adjacent cells and chains. Alternatively, M1 biofilms lacked pronounced surface characteristics and had a more smooth appearance with a rich bacterial-associated extracellular matrix (BAEM). BAEM was not observed in the biofilm of the M41 serotype. To further compare the production of BAEM between the biofilms of the M1 and M41 strains, green-fluorescent protein (GFP) expressing strains were created. TRITC-concanavalin A (ConA), a fluorescently-labeled lectin that binds to the extracellular polysaccharides in biofilms (Maeyama, Mizunoe, Anderson, Tanaka, & Matsuda, 2004), was used to stain serotype M1 and M41 biofilms grown for 24 hours on glass coverslips. Fluorescent microscopy was performed to visualize the BAEM material. The results showed that the M1 strain had a dense and closely associated matrix. Oliver-Kozup et al. suggested that the stability of *S. pyogenes* biofilms

differs among serotype strains and that more BAEM production does not necessarily pre-determine a larger biofilm mass (Oliver-Kozup, et al., 2011).

M Protein Family; Direct role of M protein in biofilm formation

A study by Cho and Caparon investigated whether virulence factors, such as M protein, were crucial for biofilm formation; they observed biofilm formation of the wild-type M14 serotype strain HSC5 (with a mutagenic plasmid integrated at an intergenic locus) and HSC5 strain with an insertional disruption of the *emm* gene. Using a microtiter plate assay, biofilms were grown in a peptide rich, but carbohydrate poor C medium at 23°C for 24, 48, 72, and 96 hours (Cho & Caparon, 2005). The HSC5 strain with a disruption in the *emm* gene was unable to form biofilms at any of the time intervals. Because this assay was used to detect the initial cell-surface interactions required for biofilm formation, Cho and Caparon suggest that this data indicates that M protein influences the initial stage in biofilm development. Additionally, growth in flow chambers was assessed under the same conditions using the wild-type and disrupted *emm* gene strain of HSC5. Similar to the microtiter assay, a disruption in the *emm* gene resulted in the inability to form biofilms under flow conditions. Cho and Caparon indicate that based on this data, M protein is important for initial cell-surface interactions in biofilm development (Cho & Caparon, 2005).

To test the dependence of surface proteins on biofilm formation Courtney et al added trypsin to *S. pyogenes* growth media (Courtney, et al., 2009). Trypsin cleaves surface proteins, but does not affect the growth of *S. pyogenes*. All strains tested were unable to form biofilms. Therefore, Courtney et al. suggest that *S. pyogenes* biofilm formation is dependent on streptococcal surface proteins. To determine the role of the various members of the M protein family (Emm, Mrp, Enn, and Spa) on biofilm formation M protein family mutants were compared to their specific wild-type for their ability to form biofilms. In serotypes with only one *emm* gene that encodes M protein (M1, M5, M6, and M24), inactivation resulted in a deficiency in biofilm formation. Serotypes with multiple genes that encode M proteins were shown to have varying effects when individual genes were inactivated. Inactivation of *emm2* in an M2 strain had a minor effect on biofilm formation, with the inactivation of *mrp2* having no significant effect. *mrp4* in an M4 strain resulted in a 96% reduction in biofilm formation relative to wild-type, and inactivation of *emm4* and *enn4* had no effect. Reductions of 30% and 50% were observed with inactivation of *emm49* and *mrp49,* respectively in serotype strain M49. In serotype M18, inactivation of Spa (streptococcal protective antigen surface protein) resulted in a decrease of biofilm formation by 42%, as compared to wild-type M18 (Courtney, et al., 2009).

M Protein-LTA Interactions

A later study by Courtney et al. sought to determine which members of the M protein family are involved in *S. pyogenes* biofilm formation. More specifically, researchers were interested in whether these M protein family members were anchoring lipoteichoic acid (LTA) in a manner that contributes to hydrophobicity. LTA has been known to contribute to the hydrophobicity of Gram-positive bacteria (Doyle & Rosenberg, 1990; Miörner, Johansson, & Kronvall, 1983; Fedtke, et al., 2007). Hydrophobicity of several *S. pyogenes* serotypes is dependent on the expression of surface proteins that form complexes with LTA, such that the ester linked fatty acids of LTA are exposed on the *S. pyogenes* surface (Courtney, et al., 2009; Ofek, Whitnack, & Beachey, 1983).

Biofilm formation was studied using mutant strains from serotypes M1, M2, M4, M5, M6, M18, M24, and M49, which were constructed through allelic replacement of specific genes that encode the M protein(s). The addition of glucose to tryptic soy broth (TSB) had a minor effect on planktonic growth, but had a three-fold increase on biofilm formation. However, Todd-Hewitt broth plus yeast (THY) was the best medium for growth (as well as for biofilm formation), and was chosen as the standard for the remaining studies in this area. Variation in biofilm formation was observed among all serotypes. Serotypes M2 and M6 had the highest degree of biofilm formation, while serotype M49 had the least degree of biofilm formation (Courtney, et al., 2009).

Courtney et al. showed that M proteins are involved in biofilm formation, hydrophobicity, and adhesion to hexadecane. A competitive inhibition enzyme-linked immunosorbent assay (ELISA) revealed that M proteins play a role in the amount of protein-bound LTA. To determine if the levels of M protein could have an effect on hydrophobicity and biofilm formation, Courtney et al. constructed a recombinant strain that overexpresses *emm1*, with a two-fold increase in Emm1 production in the M1 serotype. Results indicated that increased production of Emm1 increases the amount of protein-bound LTA, hydrophobicity, and biofilm formation, as compared to the wild-type strain and *emm1* inactivated strain. Courtney et al. suggested that the formation of complexes between M proteins and LTA directly contributed to both hydrophobicity and biofilm formation in most *S. pyogenes* serotypes; however, in some serotypes, a direct link could not be demonstrated. These authors also suggested that the absence of a direct link might be attributed to some serotypes that possess multiple M protein family members and that the inactivation of a single member is not sufficient to alter these functions (Courtney, et al., 2009).

Pili

Pili are long filamentous structures found on the surface of several bacterial species, including *S. pyogenes* (Mora, et al., 2005). *S. pyogenes* pilus-associated proteins are encoded on a pathogenicity island named the fibronectin-binding, collagen-binding, T-antigen (FCT) region (Bessen & Kalia, 2002). This region contains genes that code for pilus structural subunits and for the sortase enzymes required for pilus assembly (Bessen & Kalia, 2002; Manetti, et al., 2007). In specific *S. pyogenes* strains, multiple components of pili assembly and structure have been identified for their role in biofilm formation. These include *srtA,* which encodes a housekeeping sortase, the T shaft protein (*tee* gene), and Ancillary Protein 1 (AP1) (Becherelli, et al., 2012; Nakata, et al., 2011; Kratovac, Manoharan, Luo, Lizano, & Bessen, 2007). This locus is highly variable, with 9 FCT variants having been identified (Kratovac, Manoharan, Luo, Lizano, & Bessen, 2007; Falugi, et al., 2008). The role of pili in the *S. pyogenes* biofilm life cycle is becoming ever more appreciated (Manetti, et al., 2007; Becherelli, et al., 2012; Köller, et al., 2010; Manetti, et al., 2010; Nakata, et al., 2009; Kimura, et al., 2012).

Evaluation of 24-hour biofilms grown in the peptide-rich, but carbohydrate-poor C medium C-medium by Manetti et al. demonstrated the importance of pili to the maturation of the biofilm structure (Manetti, et al., 2007). A wild-type *S. pyogenes* serotype M1 strain SF370 (FCT type 2) was five to six times more efficient at generating biofilms on polystyrene than isogenic pilus backbone and sortase C1 mutants. Confocal microscopy of *S. pyogenes* growing on polylysine-coated coverslips revealed that after 72 hours, strain SF370 was able to form biofilms with an average thickness of 10.8um while the two pili mutants were unable to form a significant multilayered biofilm structure. Manetti and colleagues concluded that one of the main roles of pili is to allow *S. pyogenes* to switch from planktonic to biofilm growth.

Nakata et al. investigated the effects of pilus-associated protein deletions on biofilm formation using *S. pyogenes* serotype M49 strain 591 (FCT type 3) (Nakata, et al., 2009). Various FCT-3 component deletion strains (*cpa* operon and single component mutants: Δ*cpa*, Δ*fctA*, Δ*fctB*, Δ*srtC2,* and Δ*lepA*), as well Δ*prtF2* and a strain lacking *srtA*, were tested for their abilities to form static biofilms in C-medium on polystyrene plates at 28°C and 37°C. Results indicated that strains lacking *srtA* produced significantly less biofilm, regardless of the temperature used. All other mutants produced wild-type levels of biofilm under both conditions, which led Nakata and colleagues to suggest that although *srtA* is required for biofilm formation of strain 591, *srtC2* and other components of the *cpa* operon do not play a significant role.

Koller et al. evaluated the relationship between FCT type and biofilm formation (Köller, et al., 2010). 183 *S. pyogenes* isolates (representing pharyngitis, skin infection, and invasive disease) were obtained from University Hospital in Rostock, Germany, from 2001–2006 and were subjected to 24-hour static biofilm assays in C-medium and brain-heart infusion (BHI). Crystal violet analysis of biofilm mass revealed that FCT type 1 strains formed robust biofilms in both culture media; FCT type 2, 5, and 6 formed robust biofilms in BHI broth, but weak biofilms in C-medium; FCT type 9 strains formed weak biofilms in either culture media; FCT type 3 and 4

strains exhibited heterogeneous biofilm growth within their FCT groups in both media. Koller and colleagues concluded that FCT typing represents an additional method for characterization of *S. pyogenes* and that biofilm growth in defined media may represent a novel epidemiological marker (Köller, et al., 2010).

Manetti et al. established a link between acidic environmental conditions and pilus-mediated *S. pyogenes* biofilm formation (Manetti, et al., 2010). Using 44 clinical isolates obtained from University Hospital in Rostock, Germany, from 2001–2006, static biofilm assays were carried out in C-medium or C-medium supplemented with 30mM glucose. FCT type 1 strains formed robust biofilms in both culture media; FCT type 2, 3, 5, 6 and a subset of type 4 strains exhibited increased biofilm formation in supplemented media; and FCT type 9 and a subset of type 4 strains failed to form biofilms in either condition. Manetti and colleagues reasoned that a pH decrease in the media due to sugar metabolism was the cause of the glucose-mediated biofilm formation results. They addressed this possibility by performing 12-hour biofilm assays in unbuffered C-medium at either pH 6.4 or 7.5. Results indicated that FCT type 1 strains formed biofilms at both pH levels; FCT type 2, 3, 5, 6, and a subset of type 4 strains showed increased biofilm when grown in media with lower starting pH; FCT type 9 and a subset of type 4 strains were poor biofilm formers in both culture conditions. Manetti and colleagues also demonstrated that pH-dependent biofilm formation was directly associated with differential pilus expression, which led them to conclude that most *S. pyogenes* FCT type strains sense environmental pH as a signal to build pili on their surface, and that this process may lead to biofilm formation (Manetti, et al., 2010).

The role of the FCT type 1 pili in *S. pyogenes* biofilm formation was investigated by Kimura et al. using pilus-associated gene deletions (Kimura, et al., 2012). Pilus-associated deletions (Δ*tee6*, Δ*fctX*, Δ*srtA*, Δ*srtB*) were created in a *S. pyogenes* serotype M6 strain TW3558 and the effects on static 24-hour biofilm formation in C-medium were examined. Crystal violet analysis revealed that deletion of pilus-associated proteins resulted in decreased biofilm formation, as compared to wild-type levels. These results were confirmed with confocal microscopic analysis. To determine if the pilus mutant phenotype was present in other M6 strains, *tee6* deletions were made in strains SE1303, S43, SE1387, and 97A-85 (Kawabata, et al., 1999; Murakami, et al., 2002). Static biofilm analysis demonstrated that *tee6* deletions in strains SE1303, S43, and SE1387 yielded reduced biofilm formation. Therefore, Kimura and colleagues concluded that pili composed of T6 shaft protein are crucial for the biofilm formation process in M6 strains (Kimura, et al., 2012).

Becherelli et al. evaluated how ancillary protein 1 of FCT type 1 pili mediates *S. pyogenes* biofilm formation (Becherelli, et al., 2012). *S. pyogenes* serotype M6 strain HRO-27_M6 (obtained from University Hospital in Rostock, Germany) and its isogenic Δ*ap1_M6* mutant were subjected to 10-hour static biofilm assays in C-medium on polystyrene. Deletion of ancillary protein 1 resulted in reduced biofilm formation when compared to wild-type levels. Additionally, incubation of wild-type cells with polyclonal antibodies raised against rAP1_M6 abolished biofilm formation. The data obtained lead Becherelli and colleagues to hypothesize that AP1 pilus components mediate tissue colonization through the formation of large cell-adhering microcolonies (Becherelli, et al., 2012).

It should be noted that several reports have examined the role of *S. pyogenes* pili in host cell adherence (Abbot, et al., 2007; Crotty Alexander, et al., 2010; Edwards, et al., 2008; Smith, et al., 2010). Because these reports did not specifically investigate biofilm formation, their details have been omitted from this review.

Hyaluronic acid capsule

The hyaluronic acid capsule is a major virulence determinant of *S. pyogenes* (Cunningham, 2000). It is required for resistance to phagocytosis (Wessels & Bronze, 1994) and is an important adherence factor in the pharynx because of its ability to bind CD44 on host epithelial cells (Schrager, Albertí, Cywes, Dougherty, & Wessels, 1998). The capsule is composed of a polymer of hyaluronic acid that contains repeating units of glucuronic acid and N-acetylglucosamine (Stoolmiller & Dorfman, 1969). Synthesis of the polymer involves gene products of the *has* operon (Dougherty & van de Rijn, 1992) and *csrR*. CsrR is a regulator in the two-component system CsrRS

(also known as CovRS) and has been shown to be a negative regulator of the capsule synthesis process (Levin & Wessels, 1998). The role of the hyaluronic acid capsule in *S. pyogenes* biofilm formation is still not fully understood.

Cho et al. investigated the importance of the *S. pyogenes* capsule on biofilm maturation by evaluating the role of the *hasA* gene. The *hasA* gene (coding for hyaluronate synthase, the first gene in the *has* operon) was deleted in serotype M14 strain HSC5, and both static and continuous flow biofilm analyses were conducted. Static biofilm experiments with C-medium at 23°C revealed that a strain that lacked a hyaluronic acid capsule was capable of attaching to a polystyrene substrate. However, continuous flow biofilm analysis revealed that adherent HSC5Δ*hasA* cells remained as an unorganized layer, rather than forming the typical three-dimensional biofilm structure. These results led Cho and colleagues to conclude that the *hasA* mutant was fully competent for adherence to the substrates tested, but was unable to progress through the subsequent stages of biofilm maturation. Notably, the *covR* mutant, which overproduces capsules, was also incapable of forming biofilms under the conditions tested (Heath, DiRita, Barg, & Engleberg, 1999).

Sugareva et al. also examined the effects capsule synthesis has on *S. pyogenes* biofilm formation (Sugareva, et al., 2010). Static biofilm formation on noncoated, fibronectin-coated, or human collagen type 1-coated plastic coverslips in BHI media supplemented with glucose was evaluated for a panel of strains that represented serotypes M2, M6, M18, and M49. Deletion of *covS* (a sensor kinase involved in regulation of hyaluronic acid synthesis) in these clinical isolates revealed that effects on biofilm formation appear to be both serotype- and strain-specific. As examples, *covS* deletions in serotype M18 strains resulted in decreased biofilm formation, whereas two of the serotype M6 strains examined exhibited increased biofilm formation, as compared to wild-type levels. It should be noted that in all strains tested, the amount of capsule detected in *covS* mutants was increased in comparison with the wild-type parental strain, which indicates that capsular effects on *S. pyogenes* biofilm formation may be strain-specific.

AspA

Zhang et al. sequenced the genome of serotype M28 *S. pyogenes* strain MGAS6180 (Green, et al., 2005) and identified and characterized M28_Spy1325. M28_Spy1325 is denoted as AspA, for A Streptococcus surface protein A. AspA is a member of the antigen I/II (AgI/II)-family of polypeptides (Brady, et al., 2010; Zhang, Green, Sitkiewicz, Lefebvre, & Musser, 2006), which are important cell surface-anchored molecules produced by oral streptococci (Jenkinson & Demuth, 1997). AgI/II proteins are structurally complex, multifunctional adhesins that bind human salivary glycoproteins and assist in colonization of the oropharynx (Jenkinson & Demuth, 1997; Jakubovics, Strömberg, van Dolleweerd, Kelly, & Jenkinson, 2005). Furthermore, AspA has been shown to play a role in mediating adherence and biofilm formation in group A streptococci (Maddocks, et al., 2011).

Maddocks et al. used two serotype M28 strains, MGAS6180 and H360, to study the role of AspA in biofilm formation on a salivary pellicle. Allelic replacement was used to delete the entire coding region of the *aspA* gene and insert a spectinomycin antibiotic cassette (*aad9*) in these strains. The Δ*aspA* mutants of both strains showed identical colony morphology, growth rates in minimal C medium, and adherence levels to immobilized salivary agglutinin glycoprotein (gp-340), as compared to their respective parent strains. When Maddocks et al. grew biofilms on salivary pellicle-coated cover slips, their results indicated a significant difference in biofilm formation between the Δ*aspA* mutants and their wild-type strains. After 24 hours of incubation, wild-type strains MGAS6180 and H360 formed biofilms approximately 25μm thick that consisted of densely packed cells with a structure that covered almost the entire underlying salivary pellicle. Both Δ*aspA* mutants from each strain showed reduced biofilm thickness, visibly revealing much of the salivary pellicle substratum. MGAS6180 Δ*aspA* had a 60% reduction in biomass, while H360 Δ*aspA* had a more severe defect in biofilm formation, with a greater than 80% reduction when compared to the H360 parent strain. Δ*aspA* mutants complemented *in trans* had restored biofilm formation, but cells were more loosely packed and the architecture appeared more

disorganized, as compared to the respective wild-type strains. Maddocks et al. suggested that in these two particular M28 strains, AspA production is essential for biofilm formation (Maddocks, et al., 2011).

In order to define the specificity of AspA in biofilm formation, Maddocks et al. studied the ability of wild-type and Δ*aspA* mutants to form biofilms on various substrata, including gp-340, 10% saliva-coated polystrene, and polystrene surfaces. Biofilm formation of the wild-type strains MGAS6180 and H360 was similar among the three surfaces. However Δ*aspA* mutants for both strains showed a reduction in biofilm biomass by 60% when grown on gp-340, and a 50% reduction when grown on 10% saliva-coated polystyrene. There was no significant decrease in biomass when grown on polystyrene-only surfaces between mutant and wild-type strains. When Δ*aspA* mutants were complemented *in trans* and grown on these various substrata, they showed biofilm biomasses relative to wild-type levels. Maddocks et al. suggested that AspA has a specificity to the salivary glycoprotein substratum (Maddocks, et al., 2011).

Maddocks et al. further confirmed a role for AspA in promoting adherence and biofilm formation by expressing AspA in *Lactococcus lactis* strain MG1363. *L. lactis* wild-type and AspA-expressing strain were grown on salivary pellicle-coated coverslips for 24 hours. *L. lactis* expressing AspA produced a densely packed biofilm 26μm thick, which was three-fold higher in biomass, as compared to the *L. lactis* wild-type strain that produced a sparse biofilm (Maddocks, et al., 2011).

Scl1

The extracellular protein Streptococcal collagen-like protein 1 (Scl1) is encoded by the *scl1* gene and has been found in every *S. pyogenes* strain investigated (Lukomski, et al., 2000; Rasmussen, Edén, & Björck, 2000). The *scl1* gene is positively regulated by Mga (Almengor & McIver, 2004; Almengor, Walters, & McIver, 2006), and it is upregulated during biofilm formation and development (Cho & Caparon, 2005). Scl1 participates in *S. pyogenes* adherence to host epithelial cells and contributes to its virulence (Lukomski, et al., 2000). The role of Scl1 in the biofilm life cycle has also been evaluated.

Oliver-Kozup et al. demonstrated the importance of Scl1 to biofilm formation for a set of pathogenically diverse *S. pyogenes* strains (Oliver-Kozup, et al., 2011). Deletions of *scl1* were created in MGAS6183 (M41), MGAS5005 (M1), MGAS6143 (M28), and static 24-hour biofilm assays in THY media on polystyrene were performed. Crystal violet analysis indicated that isogenic Δ*scl1* mutants had a substantially decreased average biofilm thickness, as compared to wild-type levels. Notably, MGAS315, a serotype M3 strain that possesses a naturally truncated *scl1* allele, also failed to form a robust biofilm under the conditions tested. To further demonstrate the role of Scl1 in biofilm formation, Scl1 (and specifically the serotype M41 allele) was expressed in *Lactococcus lactis* MG1363. Expressing Scl1.41 on the surface was sufficient to confer increased biofilm formation capability on *L. lactis* when compared to the wild-type strain. Oliver-Kozup and colleagues concluded that Scl1 is a significant determinant for *S. pyogenes* biofilm formation (Oliver-Kozup, et al., 2011).

Intracellular Proteins and Gene Expression

Altered Gene Expression in Biofilms

During the development of a biofilm, bacterial gene products are often up- or down-regulated in order to allow for the change from a planktonic to sessile state. For *S. pyogenes*, it has been demonstrated that the expression of a large number of specific genes are altered in this transition. Virulence factors such as the cysteine protease (SpeB), mitogenic factor (mf), and immunogenic secreted protein (isp) are upregulated in the biofilm state, as compared to the planktonic state. The virulence factor streptokinase (Ska) on the other hand, is downregulated in the biofilm state. It has been reported that genome wide, approximately 212 genes, or 14% are upregulated in the biofilm, and 203 genes or 13% are downregulated. Of these genes that display a changed gene expression profile, the majority of their roles involve one of the following categories: energy production and conversion,

carbohydrate transport and metabolism, secondary metabolites biosynthesis, transport and catabolism, lipid transport and metabolism, and nucleotide transport and metabolism. This biofilm expression profile is altered further in the context of an in vivo infection. Further changes in gene expression have been observed in biofilms extracted from infected zebrafish muscle, which indicates that the host environment also plays a major role in the expression profile of biofilm-residing bacteria (Cho & Caparon, 2005).

Mga

The multiple gene activator of *S. pyogenes*, Mga, is responsible for the regulation of numerous virulence factors and regulates the expression of approximately 10% of the genome (Hause & McIver, 2012). Some of these virulence factors include M protein and M-like proteins (Hause & McIver, 2012). Mga has been shown to have a role in colonization and infection of the upper respiratory tract and deeper soft tissue in various animal models (Limbago, McIver, Penumalli, Weinrick, & Scott, 2001; Hondorp & McIver, 2007; Yung, McIver, Scott, & Hollingshead, 1999; Virtaneva, et al., 2005; Graham, et al., 2006). Mga may also play a role in biofilm formation, due to its role in autoaggregation (Cho & Caparon, 2005). The process of aggregation is thought to be one precursor to the formation of microcolonies and biofilms. Autoaggregation assays performed by Luo et al. reveal the importance of Mga in the initial stages of *S. pyogenes* biofilm formation. The WT Alab49 strain of *S. pyogenes* shows steady increases in autoaggregation, as demonstrated by a 75% decrease in turbidity over time. The mutant Δmga of the same strain shows low levels of autoaggregation, with a decrease in turbidity of only 5%. These data indicate that Mga-related genes are essential for autoaggregation and biofilm formation for strain Alab49 (Luo, Lizano, Banik, Zhang, & Bessen, 2008).

CodY

The transcription factor CodY can be found in *S. pyogenes*, as well as in other low G + C Gram positive bacteria. CodY is a global regulator that controls the transcription of a variety of genes, such as exoproteins, and is important in the response to amino acid depletion and nutritional stresses. When intracellular branched amino acids are plentiful, they bind to CodY, which results in an increased affinity for DNA binding and the subsequent repression of gene expression. When these amino acids are depleted, DNA affinity decreases, which allows for transcription. CodY has been shown to play a role in *S. pyogenes* biofilms under nutrient-depletion conditions. A Δ*codY* mutant shows decreased static biofilms, as compared to its parent strain, when grown in CDM. No difference is observed in nutrient-rich media such as THY, which indicates that CodY has a minor effect on *S. pyogenes* biofilms under specific environmental conditions (McDowell, Callegari, Malke, & Chaussee, 2012).

CovR/CsrR

CovR is a transcriptional regulator involved in the regulation of capsule production, as well as 15% of the *S. pyogenes* genome (Graham, et al., 2002). A *covR* mutant that contains capsule overexpression is unable to form a biofilm, as compared to its wild-type strain. This indicates that while capsule is known to be required for biofilm formation in certain strains, a CovR-regulated product other than capsule may also be involved in biofilm formation (Cho & Caparon, 2005).

Quorum Sensing in Biofilms

Quorum sensing is the release of low molecular weight compounds between intra- and inter-species that results in a change in bacterial gene expression. This event depends on density and is used as a means of intercellular communication (Chang, LaSarre, Jimenez, Aggarwal, & Federle, 2011; Davies, et al., 1998), Currently, the *S. pyogenes* quorum sensing pathway is known to consist of the *sil* pathway, found in less than twenty percent of isolates (Belotserkovsky, et al., 2009; Eran, et al., 2007), and a highly conserved *rgg-shp* regulated pathway (Chang, LaSarre, Jimenez, Aggarwal, & Federle, 2011). Both of these pathways allow *S. pyogenes* to sense extracellular signaling peptides and respond to these cues by genome-wide changes in gene expression (Chang,

LaSarre, Jimenez, Aggarwal, & Federle, 2011; Ibrahim, et al., 2007a; Fontaine, et al., 2010; Mashburn-Warren, Morrison, & Federle, 2010). Quorum sensing has been known to contribute to biofilm formation in several clinically relevant bacterial species, such as *Pseudomonas aeruginosa* (de Kievit, 2009) and *Staphylococcus aureus* (Kong, Vuong, & Otto, 2006), but its mechanisms in *S. pyogenes* have yet to be elucidated (Davies, et al., 1998).

Rgg-SHP quorum sensing pathway

The Rgg-SHP quorum sensing pathway is highly conserved among *S. pyogenes* strains (Chang, LaSarre, Jimenez, Aggarwal, & Federle, 2011). The Rgg-SHP pathway consists of the Rgg (regulator gene of glucosyltransferase (Sulavik, Tardif, & Clewell, 1992))-family of cytoplasmic receptors for intercellular signaling peptides in Streptococcal species (Ibrahim, et al., 2007a; Fontaine, et al., 2010; Mashburn-Warren, Morrison, & Federle, 2010). In *S. pyogenes*, four *rgg*-like genes have been identified: *comR, ropB, rgg2*, and *rgg3* (Chang, LaSarre, Jimenez, Aggarwal, & Federle, 2011). Rgg2 and Rgg3 have antagonist activities, in which Rgg2 is an activator and Rgg3 is a repressor of gene expression (Chang, LaSarre, Jimenez, Aggarwal, & Federle, 2011). Additionally, *rgg2* and *rgg3* are adjacent to two small open reading frames that encode short hydrophobic peptides (SHPs) (Ibrahim, et al., 2007b). These two adjacent *shp* genes are important for positively regulating their own expression and inducing the expression of their neighboring genes, *rgg2* and *rgg3* (Chang, LaSarre, Jimenez, Aggarwal, & Federle, 2011). Furthermore, this Rgg-SHP pathway has been shown to be involved in *S. pyogenes* biofilm formation (Chang, LaSarre, Jimenez, Aggarwal, & Federle, 2011).

Chang et al. studied the Rgg-SHP system to determine its role in *S. pyogenes* biofilm development. Deletion strains were made in the wild-type NZ131, an M49 strain, for both *rgg2* and *rgg3*. Crystal violet assays revealed the deletion of *rgg3* had a three-fold increase in biofilm mass, as compared to the wild-type strain. The deletion of *rgg2* resulted in a low production of biofilm, similar to the wild-type strain. Δ*rgg2Δ rgg3* displayed low biofilm production, similar to that of the wild-type and the single deletion of *rgg2* (Chang, LaSarre, Jimenez, Aggarwal, & Federle, 2011).

ropB is an *rgg*-like gene that encodes RopB, a positive regulator of *speB* transcription. SpeB is a *S. pyogenes* cysteine protease (Lyon, Gibson, & Caparon, 1998). Using the NZ131 parent strain, a deletion in the *ropB* gene was constructed to create a strain incapable of producing SpeB. The Δ*ropB* strain had enhanced biofilm formation, as compared to the wild-type strain. The double mutant Δ*ropB*Δ*rgg3* resulted in an additive effect on biofilm production. However, the double mutant Δ*ropB*Δ*rgg2* resulted in a decrease in biofilm formation, as compared to the single Δ*ropB* mutant, which eliminated the enhanced biofilm effects of the *ropB* deletion (Chang, LaSarre, Jimenez, Aggarwal, & Federle, 2011).

sSHP3-C8 is a synthetic peptide that contains the 8-C terminal amino acids of SHP3. The addition of exogenous sSHP3-C8 at a concentration of 50 nM caused a two-fold increase in biofilm production in the wild-type NZ131 strain. The addition of sSHP3-C8 to the Δ*rgg3* mutant did not further increase its biofilm formation. In the Δ*rgg2* and Δ*oppD* mutants (Opp: oligopeptide permease, D is a subunit of the transporter), biofilm formation was not stimulated upon sSHP3-C8 addition. When sSHP3-C8 was added to the Δ*ropB* mutant strain, there was a six-fold increase in biofilm formation, as compared to the wild-type. Chang et al. suggested that this observation can be attributed to the cells being more receptive to sSHP3-C8, that Δ*ropB* strain biofilms are more stabilized, or that the lack of SpeB protease in the Δ*ropB* strain allows for sSHP3-C8 to be more stable. Overall, Chang et al. suggest that there is a role for the Rgg2/3 pathway and its RopB counterpart in regulating the development of *S. pyogenes* biofilms (Chang, LaSarre, Jimenez, Aggarwal, & Federle, 2011).

Since *shp* genes can regulate their own expression and the expression of *rgg*2/3 genes, studies were performed to determine if surrounding genes contributed to the changes in biofilm formation. The proximal regions of the *shp* genes were deleted and these mutants revealed that proximal genes did not affect biofilm production. Therefore, Chang et al. suggest that genes near the *rgg*2/3-*shp* open reading frames are not likely to be involved in the biofilm process (Chang, LaSarre, Jimenez, Aggarwal, & Federle, 2011).

SilC

SilC (*silC*) is an important signaling peptide of the Sil (streptococcal invasion locus) quorum-sensing pathway in *S. pyogenes* (Hidalgo-Grass, et al., 2004; Eran, et al., 2007; Hidalgo-Grass, et al., 2002). It is known that *silC* is required for the virulence of the M14 strain JS95 during invasive soft-tissue infection (Hidalgo-Grass, et al., 2004; Hidalgo-Grass, et al., 2002). Additionally, *silC* has been investigated for its potential role in biofilm formation of strains from several different *S. pyogenes* serotypes (Lembke, et al., 2006; Thenmozhi, Balaji, Kumar, Rao, & Pandian, 2011).

Lembke et al. studied the potential role of the SilC signaling peptide in the biofilm development of *S. pyogenes* strains from serotypes M14 and M18. M14 and M18 *sil*C-deficient mutants were assessed for their ability to form biofilms, relative to their respective wild-type strains. Strain M14 *sil*C had been shown to have a reduced adherence to fibronectin, fibrinogen, and plastic, as compared to the parent strain; however, adherence was not statistically significant. An M18 *sil*C-deficient mutant strain had significantly reduced adherence to collagen type I and IV substrates, as compared to its wild-type strain. The M14 *sil*C-deficient mutant had a biofilm structure that was more cleft than its wild-type strain. The M18 *sil*C-deficient mutant biofilm displayed a patchy, thin biofilm, as compared to the thick and solid wild-type biofilm (Lembke, et al., 2006).

Thenmozhi et al. screened a library of strains, including biofilm formers and non-biofilm formers, for the presence of the *silC* gene (signaling molecule). Thenmozhi et al. studied eleven *S. pyogenes* strains from different M serotypes (M49, M56, M63, M65, M74, M88.3, M89, M100, M122, st38, and st2147). Crystal violet staining was used to quantitate biofilms and categorize serotypes, according to those that were biofilm formers (M56, M65, M74, M89, M100 and st38), and those that were non-biofilm formers (M49, M63, M88.3, M122, and st2147). The *silC* gene was found in the genomes of some biofilm formers (M100, M74, and st38) and a single non-biofilm former (M122). The most proficient biofilm former observed in this study, M56, did not possess the *silC* gene. From their results, Thenmozhi et al. suggest that *S. pyogenes* strains from different serotypes have the ability to form biofilms, regardless of whether the *silC* gene is present (Thenmozhi, Balaji, Kumar, Rao, & Pandian, 2011).

Environmental conditions and biofilm formation

Nutrients

Baldassarri et al. evaluated the ability of *S. pyogenes* clinical isolates to form biofilms, and investigated 289 clinical strains. The effect of temperature on biofilm formation was assessed for a set of 50 randomly chosen strains from the total of 289. Temperature appeared to have no effect on *S. pyogenes* growth, and so the growth conditions of 37°C and a THB medium were chosen for the remainder of the experiments. 18-hour biofilms were assessed by crystal violet to quantitate biofilms and were further examined by SEM to visualize the biofilm architecture. The number of strains capable of forming biofilms increased with the atmospheric conditions: 85% of isolates formed biofilms in unmodified atmosphere, 88% in 5% CO_2, and 91.4% in anaerobiosis. Biofilm production did not vary for single isolates under the different atmospheric conditions. As a whole, there was a significant increase in biofilm formation under conditions of anaerobiosis when compared to conditions of 5% CO_2 (Baldassarri, et al., 2006).

To study the effect of glucose on *S. pyogenes* biofilm formation, Thenmozhi et al. used a biofilm proficient strain, M100 (positive control), and a non-biofilm forming strain, st2147 (negative control). The results showed that st2147 can form biofilms with exogenous glucose supplementation. The addition of glucose (0.5–1.5%) was directly proportional to biofilm formation. Strain M100 produced biofilms profusely at 72 hours with the addition of all concentrations of glucose (0.5%, 1%, 1.5%). For both serotypes, a twofold increase in biofilm formation was observed when grown in media supplemented with 1.5% glucose (Thenmozhi, Balaji, Kumar, Rao, & Pandian, 2011).

The addition of glucose affected the surface area and thickness of the biofilms of both M100 and st2147 strains in different ways. Strain M100 had a decrease in surface area with increasing concentrations of glucose and reached its maximum surface area at 72 hours in both 0.5% and 1% glucose. An increase of glucose to 1.5% resulted in a significant elevation of the biofilm surface area of st2147 to 35% (Thenmozhi, Balaji, Kumar, Rao, & Pandian, 2011).

When the effect on biofilm thickness was evaluated, there appeared to be a significant decrease in thickness with increasing concentrations of glucose. The maximum biofilm thickness for strains M100 and st2147 was reached after 48 hours with varying concentrations of glucose. Strain M100 biofilms reached a maximum thickness of 14 microns in 1% glucose. The addition of 0.5% glucose resulted in a maximum biofilm thickness of 12 microns for strain st2147. Therefore, Thenmozhi et al. suggest that nutrients, such as glucose, play an important role in biofilm development (Thenmozhi, Balaji, Kumar, Rao, & Pandian, 2011).

Biofilm Dispersal

The final stage in the biofilm life cycle involves single cells or groups of cells being released from an established biofilm. This cellular detachment facilitates biological dispersal, bacterial survival, and disease transmission. Dispersal can be achieved in either an active or passive manner. Active dispersal is initiated by the biofilm bacteria themselves, while passive dispersal involves external forces such as fluid shear, predatory grazing, interspecies antimicrobial compounds, and human intervention (Choi & Morgenroth, 2003; Erard, Miyasaki, & Wolinsky, 1989; Kaplan J. B., 2010; Lawrence, Scharf, Packroff, & Neu, 2002; Ymele-Leki & Ross, 2007).

At the cellular level, the methods by which cells release from a biofilm structure fall into three categories: erosion, sloughing, and seeding dispersal. Erosion is defined as the continuous release of single cells or small groups of cells at a low level throughout the course of biofilm development. Sloughing refers to an abrupt detachment of a large portion of biofilm, usually occurring during later stages of the biofilm life cycle (Lappin-Scott & Bass, 2001; Marshall, 1985; Stoodley, et al., 2001; Wilson, Hamilton, Hamilton, Schumann, & Stoodley, 2004). Seeding dispersal, also referred to as "central hollowing," involves the rapid release of single cells or small groups of cells from hollow cavities formed inside the biofilm microcolony structure (Boles, Thoendel, & Singh, 2005; Ma, et al., 2009).

Although biofilm dispersal remains the least studied and understood aspect of biofilm development, a small number of mechanisms associated with biofilm dispersal have been identified. These include active biofilm dispersal, as well as chemical and environmental signals that regulate biofilm dispersal (Kaplan, 2010; Karatan & Watnick, 2009). Organisms can produce extracellular enzymes that degrade biofilm matrix components, as seen with dispersin B of *Aggregatibacter actinomycetemcomitans* (Kaplan, Ragunath, Ramasubbu, & Fine, 2003a) and the surface protein-releasing enzyme (SPRE) of *Streptococcus mutans* (Lee, Li, & Bowden, 1996). Enzymes produced by biofilm cells can also degrade biofilm substrates. *Streptococcus intermedius* hyaluronidase (Pecharki, Petersen, & Scheie, 2008) and the hemagglutinin protease of *Vibrio cholerae* (Finkelstein, Boesman-Finkelstein, Chang, & Häse, 1992) are two examples of such enzymes.

Seeding dispersal has been well characterized in *A. actinomycetemcomitans* (Kaplan, Ragunath, Ramasubbu, & Fine, 2003a; Kaplan, Meyenhofer, & Fine, 2003b; Kaplan & Fine, 2002) and *Pseudomonas aeruginosa* (Sauer, Camper, Ehrlich, Costerton, & Davies, 2002; Boles, Thoendel, & Singh, 2005; Ma, et al., 2009; Pamp & Tolker-Nielsen, 2007; Hunt, Werner, Huang, Hamilton, & Stewart, 2004; Schooling, Charaf, Allison, & Gilbert, 2004). Along with seeding dispersal, *P. aeruginosa* can also produce rhamnolipids, which are extracellular surfactants that decrease the adhesiveness of cell to surface interactions (Soberón-Chávez, Lépine, & Déziel, 2005; Neu, 1996), in order to facilitate its transition to a planktonic state. Other mechanisms include: modulation of fimbrial adherence, as in enteroaggregative *Escherichia coli* (EAEC) (Sheikh, et al., 2002; Velarde, et al., 2007) and enteropathogenic *E. coli* (EPEC) (Cleary, et al., 2004; Knutton, Shaw, Anantha, Donnenberg, & Zorgani, 1999); cell division-mediated dispersal (Allison, Evans, Brown, & Gilbert, 1990; Gilbert, Evans, & Brown, 1993);

and induction of motility, as seen in *E. coli* and *P. aeruginosa* (Purevdorj-Gage, Costerton, & Stoodley, 2005; Jackson, et al., 2002).

S. pyogenes Mechanisms of Biofilm Dispersal

Little is known about the ways in which *S. pyogenes* actively regulates biofilm dispersal. There is increasing evidence that the streptococcal regulator of virulence (Srv) and the streptococcal cysteine protease (SpeB) may play a major role in this process. Srv is a Crp/Fnr-like transcriptional regulator with homology to PrfA of *Listeria monocytogenes* (Reid, Montgomery, & Musser, 2004). The deletion of *srv* results in decreased biofilm formation, coupled with increased levels of SpeB production both *in vitro* and in two different animal models of infection (Doern, et al., 2009; Roberts A. L., Connolly, Doern, Holder, & Reid, 2010; Connolly, Roberts, Holder, & Reid, 2011a; Reid, et al., 2006; Roberts, Holder, & Reid, 2010a; Connolly, Braden, Holder, & Reid, 2011b). The addition of proteinase K is sufficient to inhibit biofilm formation and disrupt established biofilms *in vitro* (Doern, et al., 2009; Connolly, Braden, Holder, & Reid, 2011b), which indicates that *S. pyogenes* biofilm structures contain a protein component. Specific chemical inhibition of SpeB with the protease inhibitor E64 as well as allelic replacement of *speB* are sufficient to restore wild-type levels of biofilm formation in strains that lack Srv *in vitro* and *in vivo* (Doern, et al., 2009; Connolly, Braden, Holder, & Reid, 2011b). Western blot analysis and immunofluorescence microscopy can detect elevated levels of SpeB associated with strains that lack Srv (Doern, et al., 2009; Connolly, Roberts, Holder, & Reid, 2011a). Taken together, these data support a *S. pyogenes* biofilm regulation model whereby Srv acts as a repressor of SpeB, which maintains proteinase levels low enough to allow for biofilm formation (Figure 2). When the proper cues signal *S. pyogenes* to disperse from the biofilm, Srv repression is alleviated, SpeB levels increase, and subsequent degradation of protein components in the biofilm matrix allow biofilm cells to return to a planktonic state.

Due to the presence of DNA and protein in the matrix material of *S. pyogenes* biofilms (Doern, et al., 2009), it is also possible that an unidentified DNase or protease may be involved in the regulation of biofilm dispersal. Notably, the relationship between Srv and SpeB in relation to biofilm dispersal was shown across multiple serotypes (Connolly, Braden, Holder, & Reid, 2011b).

Antibiotic Resistance and Antimicrobial Therapeutics

S. pyogenes biofilms provide protection against some antibiotics, but do not confer complete resistance to some antibiotics, such as penicillin (Baldassarri, et al., 2006; Conley, et al., 2003). Therefore, penicillin is typically the drug of choice for *S. pyogenes* infections, but can be replaced with macrolides (such as erythromycin) for patients with penicillin allergies. The increased use of macrolides in the treatment of *S. pyogenes* infections has resulted in increased resistance, according to the CDC.

Macrolides

Baldassarri et al. studied the relationship between macrolide resistance and biofilm formation among 289 *S. pyogenes* isolates. Using polymerase chain reaction (PCR) analysis, macrolide resistance genes were found in 122 strains. 50 strains contained *erm*(B), 36 strains possessed *mef*(A), 10 strains had *erm*(A) subclass *erm*(TR), and the other strains had a combination of these. Additionally, crystal violet staining used to quantitate the biofilms indicated that macrolide-susceptible *S. pyogenes* isolates produced significantly more biofilm than resistant strains. More specifically, isolates possessing genes that encode macrolide resistance (such as *erm*(B)- and *erm*(A) subclass *erm*(TR)- positive strains) through the methylation of 23S rRNA appeared to negatively affect biofilm production, as compared to susceptible strains and *mef*(A)-positive strains. The *emm6* isolates, the strongest biofilm producers, and three of the strong biofilm-forming *emm77* isolates were susceptible to macrolides. Of the other 15 *emm77* isolates in this study, all but one contained one of the *erm* genes of macrolide resistance. There was no effect on biofilm production for those isolates that were tetracycline-resistant (Baldassarri, et al., 2006).

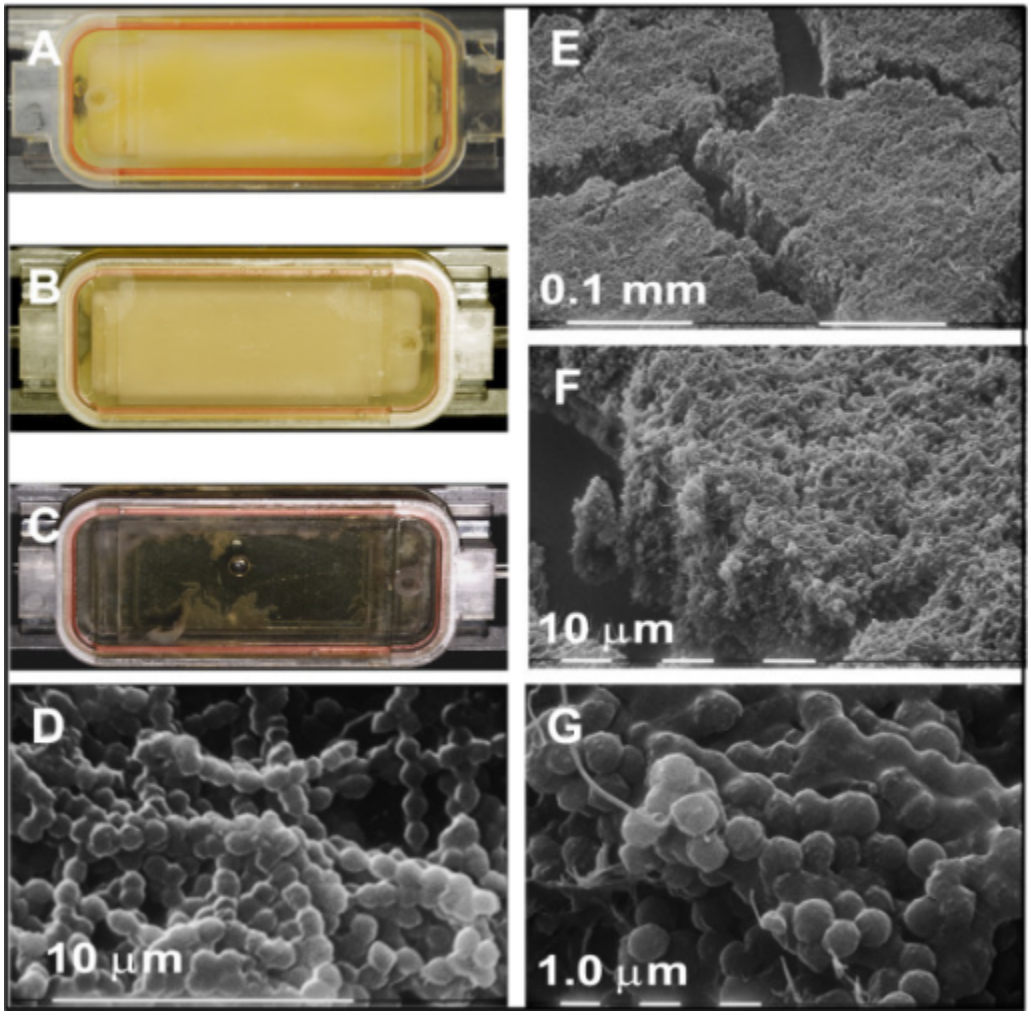

Figure 2. MGAS5005Δ*srv*Δ*speB* biofilm formation under continuous flow conditions. (A-C) Representative flow cell chambers containing 24 h grown cultures under a flow rate of ~ 0.7 mL/min of MGAS5005Δ*srv*Δ*speB*, MGAS5005, and MGAS5005Δ*srv*, respectively. (A and B) Chambers inoculated with (A) MGAS5005Δ*srv*Δ*speB* or (B) MGAS5005 were filled with dense viscous material, indicative of S. pyogenes biofilms. (C) MGAS5005Δ*srv* was unable to form biofilms under flow conditions. Scanning electron microscopy of a 24 h (D) MGAS5005 and (E-G) a MGAS5005Δ*srv*Δ*speB* continuous flow biofilm clearly depicts chains of cocci organized into a 3-dimensional structure encased in a matrix-like material. Reprinted with permission from (Roberts, Holder, & Reid, 2010b).

Additionally, Baldassarri et al. investigated the relationship between the presence of the *prtF1* gene and biofilm formation. The *prtF1* gene has been strongly related to erythromycin resistance (Facinelli, Spinaci, Magi, Giovanetti, & Varaldo, 2001). Of the 76 isolates investigated, 57 were *prtF1*-positive and 19 were *prtF1*-negative. There was a significant increase in biofilm formation of the *prtF1*-negative strains compared to the biofilm formation of the *prtF1*-positive strains. A negative association was also confirmed between *prtF1* and macrolide resistance mediated by 23S rRNA methylation by grouping the isolates based on the presence of these genes (Baldassarri, et al., 2006). Baldassarri et al. suggested that macrolide-susceptible *S. pyogenes* strains may use biofilm to escape antimicrobial treatments and survive within the host (Baldassarri, et al., 2006).

Since they were interested in the relationship between macrolide resistance and biofilm formation, Thenmozhi et al. classified six of the eleven serotypes in this study as biofilm forming M serotypes (M56, M100, M74, M65, st38, M89). Of these six serotypes, M56 was resistant to erythromycin and carried both the *erm*(B) and *mef*(A) genes. The M56 serotype formed the thickest biofilms at about 8μ, as compared to the susceptible isolates of other M-serotype biofilm formers. Of the five serotypes classified as non-biofilm formers (M49, M63, M88.3,

M122, st2147), only M49 was macrolide resistant. Thenmozhi et al. suggest that there is a negative association between having macrolide resistance determinants and having the ability to form biofilms (Thenmozhi, Balaji, Kumar, Rao, & Pandian, 2011).

S. pyogenes exhibits worldwide resistance to the fluoroquinolone family of antimicrobials (Jacobs, 2005). However, sub-lethal concentrations of fluoroquinolones can inhibit *S. pyogenes* biofilm formation in a concentration-dependent manner (Balaji, Thenmozhi, & Pandian, 2013). The metabolic activity of biofilm-residing *S. pyogenes* and biofilm biomass is also decreased after exposure to various derivatives of fluoroquinolone (Shafreen, Srinivasan, Manisankar, & Pandian, 2011).

Summary

There is still much to know about biofilm formation, regulation, dispersal, and its impact on *S. pyogenes* disease. The present evidence suggests that variation will continue to be present among strains, and points to the likelihood that the biofilm changes in nature to suit the environmental conditions and genetic repertoire of the strain in question. Regardless, further examination is likely to reveal regulatory pathways and integral components of biofilm structure that may serve as important therapeutic targets.

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Group A Streptococcal Adherence

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Abstract

Streptococcus pyogenes (group A streptococci) is an exclusive human pathogen that causes a wide range of infections, from acute pharyngitis and impetigo to life-threatening necrotizing fasciitis. The attachment of *S. pyogenes* to the pharyngeal and skin epithelial cell surfaces represents a critical first step in establishing such infections. While the importance of particular surface-exposed adhesins tends to be serotype- or infection-model–specific, adherence to human cells is generally regarded as a two-step process. The first step involves a relatively weak interaction mediated by lipoteichoic acid that allows the bacteria to overcome electrostatic repulsion. This initial interaction is followed by a stronger, more specific, and irreversible binding of a variety of tissue-specific protein or carbohydrate receptors to streptococcal surface molecules. This chapter reviews a number of the well-characterized streptococcal adherence mechanisms and will summarize some of the major advances that have been made in our understanding of this initial stage of infection.

Streptococcus pyogenes (group A streptococci) is the causative agent of various human infections that involve the upper respiratory tract and skin, including acute pharyngitis and impetigo. Therefore, it follows that the pharyngeal mucosa and skin are the primary sites of adherence and colonization by these microorganisms, and the basis for the observed tissue tropism is reviewed elsewhere (Bessen & Lizano, 2010; Bessen, et al., 2011). The attachment of *S. pyogenes* to epithelial surfaces represents a critical first step in establishing infection, and this chapter serves to review a number of the well-characterized streptococcal adherence mechanisms.

A number of streptococcal surface proteins have been implicated in adherence to target tissues. While the importance of particular surface-exposed adhesins tends to be serotype- or infection-model–specific, adherence to human cells is generally regarded as a two-step process. In the most widely accepted model, the first step involves a relatively weak interaction mediated by lipoteichoic acid that allows the bacteria to overcome electrostatic repulsion (Courtney, Hasty, & Dale, 2002). The second phase is characterized by stronger, more specific, and irreversible binding of a variety of tissue-specific protein or carbohydrate receptors to streptococcal surface molecules (Figure 1).

Streptococcal adherence and its role in pathogenesis has been the subject of numerous reviews in the past few years (Courtney, Hasty, & Dale, 2002; Beachey, Giampapa, & Abraham, 1988; Cunningham, 2000; Hasty & Courtney, 1996; Hasty, Ofek, Courtney, & Doyle, 1992; Nobbs, Lamont, & Jenkinson, 2009). The majority of the literature has focused on a small subset of well-known virulence factors, which includes, but is not limited to lipoteichoic acid, M protein, GAPDH, fibronectin-binding proteins (including protein F), pili, protein H, collagen binding proteins, and opacity factor. Recently, however, Rodriguez-Ortega et al. used novel proteomic approaches to determine that there are as many as 60-70 PSORT-predicted, surface-associated proteins of *S. pyogenes*, many of which could play a role in the adherence process (Rodríguez-Ortega, et al., 2006). This chapter will review the role of a number of the major surfaced-exposed molecules in adherence of streptococci to target tissues, and will summarize some of the major advances that have been made in our understanding of this initial stage of infection.

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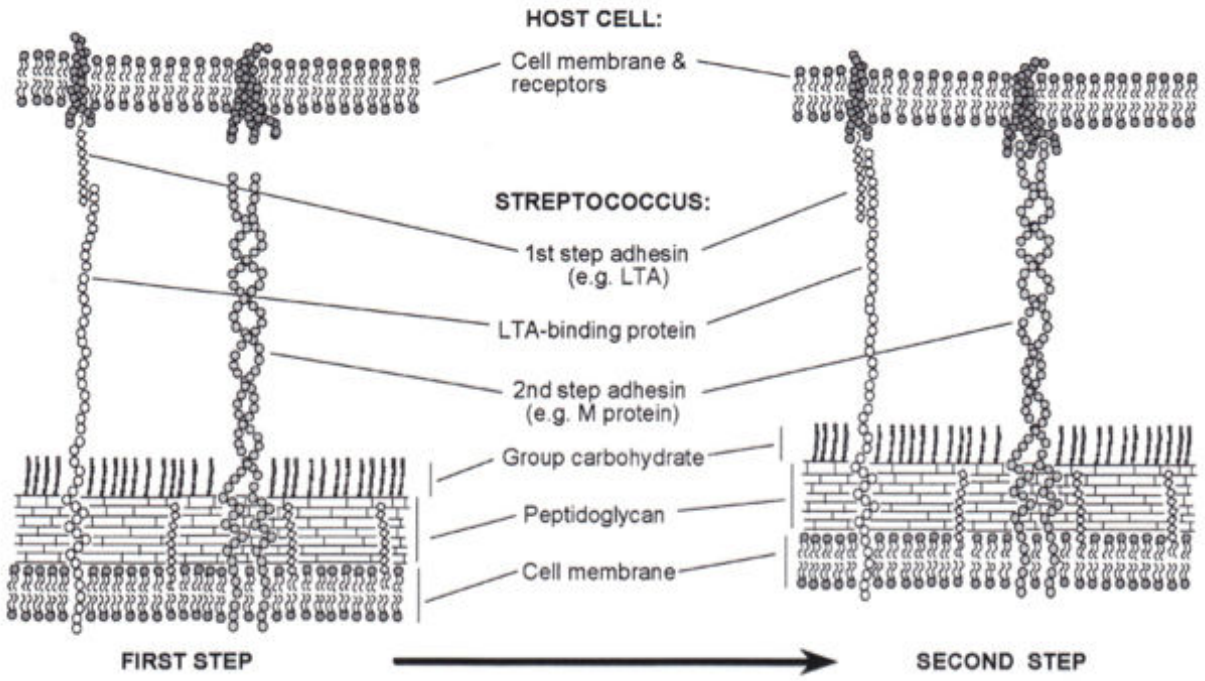

Figure 1. Two-step model for *S. pyogenes* adhesion to host cells. It is proposed that *S. pyogenes* adhesion occurs in two distinct steps. The first step serves to overcome electrostatic repulsion that occurs between the bacteria and host cells. LTA mediates this first step by hydrophobic interactions between its lipid moiety and receptors on host surfaces. The second step leads to high affinity adhesion and relies on other adhesins, including M protein and fibronectin-binding proteins. Figure adapted from Courtney et al. (Courtney, Hasty, & Dale, 2002).

Lipoteichoic Acid

Lipoteichoic acid (LTA) is a major hydrophobic component of the cell surface of Gram-positive organisms that contributes to adhesion and biofilm formation. In addition to conferring a number of physiological properties (such as the regulation of autolytic enzymes and chelation of metals (Neuhaus & Baddiley, 2003)), LTA is vital to numerous Gram-positive bacteria, as mutants lacking this molecule do not replicate (Gründling & Schneewind, 2007; Percy & Gründling, 2014).

In the two-step model of streptococcal adherence, the fatty acid moiety of LTA modulates the initial weak interaction between streptococci and fatty acid-binding domains within host cell membranes (Hasty, Ofek, Courtney, & Doyle, 1992). The surface anchoring of LTA, in an orientation that exposes its fatty acid moieties, results from a complex of LTA with additional streptococcal surface proteins, such as the M protein and other trypsin-sensitive proteins that have yet to be identified (Hasty, Ofek, Courtney, & Doyle, 1992; Ofek, Simpson, & Beachey, 1982). The proper orientation of LTA is a significant contributor to streptococcal surface hydrophobicity and biofilm formation (Courtney, et al., 2009).

While proteinaceous adhesins seem to play an important role in the attachment of a variety of streptococcal serotypes (reviewed below), LTA is important in the adherence of particular strains of *S. pyogenes* to a number of mammalian cell types. For example, adherence of an M5 streptococcal strain to buccal epithelial cells results from the binding of the lipid moiety of LTA to the N-terminal region of fibronectin present on the surface of these human cells (Beachey & Courtney, 1987). These authors later reported the LTA-fibronectin-mediated adherence of the same M5 strain to pharyngeal epithelial cells (Courtney, et al., 1992). Courtney et al. have also described the role of LTA in adherence of an M24 strain to HEp-2 cells (Courtney, Ofek, & Hasty, 1997).

While many strains of *S. pyogenes* are reported to have the capacity to bind to human epithelial cells via an LTA-mediated mechanism, adherence of other strains is mediated by protein adhesins that act either simultaneously with, or subsequently to, LTA to promote more secure attachments to host tissue. As one such example, proteins (rather than LTA) were shown to be involved in the adherence of particular streptococcal strains to HEp-2 cells (Talay, Valentin-Weigand, Jerlström, Timmis, & Chhatwal, 1992; Sela, Marouni, Perry, & Barzilai, 2000). These additional adhesins are discussed below in more detail.

M protein

The M protein, discovered by Rebecca Lancefield over 80 years ago (Lancefield, 1928) is by far one of the most extensively studied surface molecules of *S. pyogenes*, and has been the subject of numerous comprehensive reviews (Le Breton, et al., 2013; Fischetti, 1989; Fischetti, 1991; Fischetti, 2006; Metzgar & Zampolli, 2011; Oehmcke, Shannon, Mörgelin, & Herwald, 2010). The alpha-helical coiled-coil fibrillar molecule extends approximately 60 nm from the cell surface, and is oriented such that the N-terminal region is the most distal from the cell surface and the C-terminal region is anchored to the cell wall. Protective immunity to *S. pyogenes* infection is achieved through antibodies directed against the M protein (Fischetti, 1989; Fischetti, 1991), and its antigenic targets form the basis of the major serological typing scheme (Lancefield, 1928; Fischetti, 1989). More recently, however, this typing scheme has been replaced by nucleotide sequence typing of the 5' end of the *emm* gene (Beall, Facklam, & Thompson, 1996). There are currently over 250 recognized *emm* types that have been identified by this method.

The M protein is considered to be the major virulence factor of *S. pyogenes* because it renders the streptococci resistant to phagocytosis and is involved in adherence to mammalian cells and immunoglobulins (Fischetti, 1989). Beginning in the 1970s, some of the earliest work on the M protein provided evidence that it was involved in adherence to human buccal mucosal cells (Ellen & Gibbons, 1972). Notably, it wasn't until approximately 20 years later that M protein was considered to be a major adhesin of *S. pyogenes*; however, the role of different M proteins in adherence to particular tissues has been the subject of great debate. For example, Courtney et al. detailed the role of various M proteins (serotypes 1, 5, 6, and 24) as adhesins necessary to promote attachment to HEp-2 cells, but found little evidence to suggest that they were involved in binding to buccal epithelial cells or pharyngeal epithelial cells (Courtney, Ofek, & Hasty, 1997). These results were in accordance with a previously published study by Caparon et al. who found that the M6 protein was not involved in the attachment to buccal cells, primary tonsillar cells, or tonsillar organ cultures (Caparon, Stephens, Olsén, & Scott, 1991). However, Caparon et al. did conclude that the M6 protein was important in the formation of microcolonies (or aggregates) on human tonsillar cells, which may confer a selective advantage in the colonization process. Interestingly, the formation of aggregates, as noted in this study, occurs only *after* M-protein independent adhesion (Caparon, Stephens, Olsén, & Scott, 1991).

While an exhaustive review of all reports detailing the adhesin affinities, or lack thereof, for individual M proteins to particular cell types is beyond the scope of this review, here we will summarize some of the major findings in this field. It is worth noting that despite the large body of work, the epitopes with which individual M proteins interact on each contacted eukaryotic cell surface, and the details of the complex processes involved in M-protein-mediated adherence, have yet to be fully elucidated.

In addition to the studies on buccal epithelial cells, the interaction between streptococci and HEp-2 cells has been the subject of many reports. Only a handful of these studies demonstrated that the M protein was directly involved in this interaction; however, quite a few also presented contradictory data to indicate that the M protein was, in fact, not associated with adherence to this cell type. Wang et al. demonstrated that the N-terminal domain of the M6 protein mediated adherence to HEp-2 cells by interacting with fucose-containing oligosaccharide receptors (Wang & Stinson, 1994a; Wang & Stinson, 1994b). The M3 protein was also shown to be directly involved in adherence to HEp-2 cells, and while the receptor was not identified, Eyal et al. were able

to demonstrate that the M3 molecule adhered via a fibronectin-independent mechanism (Eyal, Jadoun, Bitler, Skutelski, & Sela, 2003). As mentioned above, Courtney et al. demonstrated the role of various M proteins in binding to HEp-2 cells, but not buccal or pharyngeal epithelial cells (Courtney, Ofek, & Hasty, 1997); however, it is worth noting that the same genetic background was not used to test the various M proteins in this study. To rule out background genetic influences, the authors published a subsequent study using isogenic mutants in which they demonstrated that the M24 protein is important for adherence to both HEp-2 and buccal epithelial cells (Courtney, Bronze, Dale, & Hasty, 1994).

Of important note, the HEp-2 cell line, once believed to be derived from a laryngeal carcinoma and thus considered an appropriate system to study streptococcal adherence to human throat cells, was subsequently determined (by biochemical and genetic analyses) to have been established via HeLa cell contamination (Chen, 1988; Masters, 2002). Although HeLa cells were propagated from a cervical carcinoma and are therefore not the most relevant model for studying streptococcal pharyngitis or skin infections, we have gained a great deal of knowledge regarding the general pathogenic mechanisms of *S. pyogenes* from the HEp-2 cell line studies.

Given the large body of work detailing streptococcal tropism for particular tissue types, it is reasonable to conclude that adherence studies on pharyngeal, tonsillar, and keratinocyte cell lines are more clinically relevant in delineating the role of M protein in the adherence-mediated onset of throat and skin infections. Next, we summarize a number of the seminal studies on such cell types.

Tylewska et al. assessed the adherence of a variety of different M-type strains and their isogenic M- mutants to clinically-relevant, primary human cells, including pharyngeal, tongue, and buccal epithelial cells (Tylewska, Fischetti, & Gibbons, 1988). They described the remarkable selectivity of streptococcal strains to attach to particular epithelial cell surfaces. In this study, they showed that the parental (M+) strains adhered in greater numbers to all epithelial cells tested, as compared to the appropriate M- mutant. Furthermore, all M+ strains were able to bind to human pharyngeal epithelial cells in higher numbers than to buccal or tongue epithelial cells derived from the same donors.

In work from our laboratory, we used Detroit 562 pharyngeal cells to assess the role of M protein in streptococcal adherence (Ryan, Pancholi, & Fischetti, 2001). Understanding the limits of the conclusions that can be drawn using transformed cell lines, the human pharyngeal epithelial cell line Detroit 562 was chosen for a number of reasons. This cell line is derived from human pharyngeal tissue, which is an appropriate model given that the human throat is the only known reservoir of *S. pyogenes*. Furthermore, this cell line has been reported to more faithfully display the carbohydrate epitopes that are representative of the native (non-transformed) cells, as compared to other pharyngeal cell lines (Barthelson, Mobasseri, Zopf, & Simon, 1998). We demonstrated that the M6 protein, and specifically the N-terminal domain of the molecule, binds to sialic acid-containing receptors on Detroit 562 pharyngeal cells, and this interaction mediates adherence to the pharyngeal cell surface. We also showed that the same M protein-sialic acid interaction is involved in the binding to (salivary) mucin, the major glycoprotein component of mucus gels (Ryan, Pancholi, & Fischetti, 2001).

Frick et al. demonstrated that the M proteins from representative M1, M5, and M6 serotypes were able to bind to Detroit cells through an interaction between the M protein and dermatan sulfate and/or heparin sulfate (Frick, Schmidtchen, & Sjöbring, 2003). More recently, Anderson et al. (Anderson, et al., 2014), used a different phayrngeal cell line, OKP7/tert pharyngeal keratinocytes, and found that the M1 protein from the highly virulent M1T1 clone (Aziz, et al., 2010) was sufficient to promote adherence to this cell line when expressed without other *S. pyogenes* virulence factors (in a *L. lactis* background). Notably, the binding of exogenous fibronectin to the M1 protein greatly limited pharyngeal cell adherence, and the contribution of the M protein to adherence was growth-phase dependent. Taken together, these studies underscore the ability of streptococcal M proteins to exert different effects on host cell interactions depending on the model system in which they are studied.

When considering the complexity of the interactions between M proteins and host cells, it has been shown that the adherence mechanisms used by skin tropic strains may differ from those of throat strains. Okada et al. found that the C-terminal domain of the M6 protein mediates binding to undifferentiated keratinocytes (cutaneous epithelial cells) to a greater degree than differentiated cells, and identified the membrane cofactor protein CD46 as the receptor on these epithelial cells (Okada, Pentland, Falk, & Caparon, 1994; Okada, Liszewski, Atkinson, & Caparon, 1995). Further work by Giannakas et al. mapped the specific M6 protein binding site on CD46 to two particular short consensus repeat (SCR) domains within the CD46 molecule (Giannakis, et al., 2002). Despite these detailed findings, numerous reports have detailed the tropism of M6 throat strains for respiratory epithelial cells more than skin keratinocytes, which calls the clinical relevance of these findings into question (Darmstadt, Mentele, Podbielski, & Rubens, 2000). To address this question, a skin tropic M49 strain was assessed for adherence to epidermal keratinocytes, and Darmstadt et al. reported that the M protein-CD46 adhesin-receptor pair was not involved in this interaction (Darmstadt, Mentele, Podbielski, & Rubens, 2000). To add to these conflicting reports, in a subsequent study, Feito et al. showed that a large array of clinical isolates that represented multiple M serotypes had a high affinity for CD46 (Feito, et al., 2007); however, the M protein itself was only tested from one of the serotypes (M18), and the authors reported that this particular M protein did not bind to CD46.

In an attempt to delineate the general binding characteristics of the M protein, and to determine if the reported discrepancies are due to strain-dependent effects, Berkower et al. (Berkower, Ravins, Moses, & Hanski, 1999) expressed specific M proteins in a single isogenic background so as to alleviate the influence that different genetic backgrounds have on the interpretation of M protein-mediated adherence. Their results indicated that only particular M proteins are able to bind to CD46, which led them to the now widely-accepted conclusion: different streptococcal serotypes are able to recognize different repertoires of receptors on the surfaces of eukaryotic cells.

Epidemiological data have long suggested that *S. pyogenes* exhibits tropism for human tonsils (Osterlund, Popa, Nikkilä, Scheynius, & Engstrand, 1997), and that the persistence of this pathogen in tonsils (following antibiotic treatment or during long-term throat carriage) is the primary source of the organism. To more thoroughly assess the streptococcal interaction with tonsillar tissue, Park et al. described a mouse model using nasal associated lymphoid tissue (NALT), a functional homologue of the human tonsil (Park, Francis, Yu, & Cleary, 2003). The NALT model has been used in a number of studies to assess streptococcal invasion and survivability within tissue (Hyland, et al., 2009; Fan, et al., 2014). We propose that it would be of great interest to further extend the M protein-mediated adherence studies to this infection model.

Pili

Once thought to be exclusively Gram-negative structures, pili were first described a decade ago in *S. pyogenes* (Mora, et al., 2005). These surface-exposed, trypsin-resistant pili are encoded by four genes, which are localized to a genomic region known as FCT (for **F**ibronectin- and **C**ollagen-binding proteins and **T** antigen-encoding loci). To date, comparative genomic techniques have identified nine different FCT islands across different strains and serotypes. Each strain harbors only one FCT island (Falugi, et al., 2008; Bessen & Kalia, 2002).

Structurally, pili encoded by the different islands are quite similar, though they have a few distinct differences, as reviewed previously (Falugi, et al., 2008). Pili are covalent polymers that are composed of a backbone protein (BP) and either one or two ancillary proteins: AP1 (also referred to as Cpa protein), and AP2. The structural proteins are assembled and linked to the cell wall by a series of sortase-mediated transpeptidase reactions (Falugi, et al., 2008).

Recent reports have demonstrated that pili are involved in the formation of biofilms and in the adherence of *S. pyogenes* to human tonsils, keratinocytes, lung and throat epithelial cells, which distinguishes it as a major adhesin of the organism (Abbot, et al., 2007; Crotty Alexander, et al., 2010; Manetti, et al., 2007; Smith, et al.,

2010). In our own laboratory, we performed transcriptome analysis of an M1 isolate (strain SF370) and determined a significant up-regulation of the entire pilus locus during adherence to Detroit 562 pharyngeal cells (Ryan, Kirk, Euler, Schuch, & Fischetti, 2007). We created a series of isogenic knock-out mutants, deficient in either the sortase or AP2 gene in the FCT locus, and observed marked decreases in adherence (66%) of both mutants to pharyngeal cells. When taken together, the data generated from pili studies to date indicate that these appendage-like molecules are important in the colonization of the human throat, tonsils and skin.

Fibronectin binding proteins

Host extracellular matrix proteins are some of the most widely studied receptors for *S. pyogenes* on the surface of eukaryotic cells. These proteins are tightly bound to the surface of eukaryotic cells, and are widely distributed among tissue types. Two such proteins, fibronectin and collagen, present on most, if not all, human cells, are typically located at the basal membrane surface. Although this would seemingly discredit their importance as potential receptors during initial adherence events (Darmstadt, Mentele, Fleckman, & Rubens, 1999), when small breaks in, or disruptions to, the cellular strata occur, these molecules can become delocalized to the apically exposed surface and therefore become available for adherence interactions with *S. pyogenes* (Kreikemeyer, Klenk, & Podbielski, 2004a; Terao, et al., 2001).

Fibronectin is a large glycoprotein that exists in a soluble form in blood and other extracellular fluids, and in an insoluble form on eukaryotic cell surfaces and within the connective tissue matrix. There are approximately 11 defined fibronectin-binding proteins (FBPs) in *S. pyogenes* (Kreikemeyer, Klenk, & Podbielski, 2004a; Terao, et al., 2001; Yamaguchi, Terao, & Kawabata, 2013). The array of encoded proteins depends on the streptococcal serotype, with the most virulent strains possessing numerous FBPs (Terao, et al., 2001; Yamaguchi, Terao, & Kawabata, 2013; Talay, Ehrenfeld, Chhatwal, & Timmis, 1991). Several of the FBPs are not implicated in adherence, but rather promote internalization of streptococci into various human cells (Talay, Ehrenfeld, Chhatwal, & Timmis, 1991; Talay, et al., 2000; Molinari, Talay, Valentin-Weigand, Rohde, & Chhatwal, 1997). Detailed information on this mechanism can be found in the Intracellular Invasion chapter of this publication.

Protein F1 (SfbI)

The best-studied fibronectin-binding proteins are SfbI and its allelic variant, F1 (Hanski & Caparon, 1992; Hanski, Horwitz, & Caparon, 1992; Sela, et al., 1993). Studies support that these proteins are directly involved in streptococcal adherence to a variety of host eukaryotic cells including non-transformed hamster tracheal cells (Hanski & Caparon, 1992; Hanski, Horwitz, & Caparon, 1992), HEp2 cells (Talay, Valentin-Weigand, Jerlström, Timmis, & Chhatwal, 1992), A549 lung cells (Talay, et al., 2000), and HeLa cells (Ozeri, et al., 1996; Ozeri, Rosenshine, Mosher, Fässler, & Hanski, 1998). Talay et al. analyzed the biological role of the two distinct binding domains of protein F1/SfbI and the target site on fibronectin, and determined that the C-terminal repeat region of protein F mediates adherence of an M type 23 strain to fibronectin on A549 and HEp2 cells (Talay, Valentin-Weigand, Jerlström, Timmis, & Chhatwal, 1992; Talay, et al., 2000). Furthermore, they showed that there are two distinct binding domains in the C-terminal region of protein F1/SFb1: the repeat region and the spacer domain. Each region interacts with a different portion of fibronectin and results in two separate, but related outcomes. First, the binding of the repeat region to the 30 kDa fibronectin fragment activates the adjacent spacer domain of F1/SfbI to then bind to the 45 kDa fibronectin fragment. The interplay between these two regions and the fibronectin molecule determines the outcome of the interaction: the repeat region of protein F1/SfbI protein mediates adherence (a necessary step for subsequent invasion), and the spacer domain efficiently triggers the invasion of streptococci into the eukaryotic cell. This is the first example of cooperative binding between a bacterial surface protein and a eukaryotic receptor protein (Talay, et al., 2000; Molinari, Talay, Valentin-Weigand, Rohde, & Chhatwal, 1997).

Protein F2, PFBP and FbaB

Protein F2, initially described by Jaffe et al., is functionally similar to protein F in terms of its fibronectin-binding capacity, even though the two proteins are structurally distinct (Jaffe, Natanson-Yaron, & Caparon, 1996). Kreikemeyer et al. first demonstrated the role of protein F2 in the adherence of *S. pyogenes* to eukaryotic cells and showed that this protein was directly involved in the binding of serotype M49 strains to HEp-2 cells. This property was particularly prominent in strains that lacked the protein F1 gene (Kreikemeyer, Oehmcke, Nakata, Hoffrogge, & Podbielski, 2004b).

A novel fibronectin binding protein, PFBP, first described by Rocha and Fischetti (Rocha & Fischetti, 1997; Rocha & Fischetti, 1999) is homologous to protein F2, but has been shown to be a distinct fibronectin-binding protein. The genes that encode both proteins (*pfbp* and *prtF2*, respectively) are located within the FCT chromosomal region, and the major difference between the two is caused by a frameshift mutation that results in the loss of 105 amino acids at the N-terminus of PFBP. Further comparisons of the regions that encode these particular fibronectin-binding proteins to those encoding an additional FBP named FbaB (encoded by *fbaB*) have provided clues to suggest that *pfbp* and *fbaB* represent two distinct variants of *prtF2* (Ramachandran, et al., 2004; Terao, Kawabata, Nakata, Nakagawa, & Hamada, 2002a). The direct role of PFBP in the adherence process has remained speculative, but FbaB has been shown to be involved in the adherence of particular M3 and M18 toxic-shock associated strains to HEp-2 cells (Terao, Kawabata, Nakata, Nakagawa, & Hamada, 2002a). FbaB protein from serotype M3 mediates adhesion to endothelial, but not epithelial, cells (Amelung, et al., 2011).

Serum Opacity Factor (SOF, SfbII) and *S. pyogenes* fibronectin-binding protein X (SfbX)

Serum opacity factor (SOF), which has served as a marker for serotyping and delineating streptococcal lineages, has dual biological functionality (Kreikemeyer, Talay, & Chhatwal, 1995). The N-terminal domain of the protein exhibits enzymatic activity that results in opalescence of the host serum, while the C-terminal domain has been shown to bind to fibronectin (Kreikemeyer, Talay, & Chhatwal, 1995; Courtney, et al., 1999; Rakonjac, Robbins, & Fischetti, 1995). Downstream of the gene encoding SOF (*sof)* is the *sfbX* gene, which encodes an additional fibronectin-binding protein, known as SfbX, that is co-transcribed with *sof* as a bicistronic message (Jeng, et al., 2003). Without knowledge of SfbX, Courtney et al. demonstrated that SOF contributes to virulence in a mouse intraperitoneal infection (Courtney, et al., 1999). After accounting, however, for the presence of the downstream SfbX protein, Oehmcke et al. provided the first evidence that the C-terminal (fibronectin-binding) domain of SOF from an M2 serotype was directly involved in streptococcal adherence to HEp-2 cells (Oehmcke, Podbielski, & Kreikemeyer, 2004).

M protein as a fibronectin binding protein and protein H

As mentioned, certain streptococcal M proteins adhere to immunoglobulins, serum proteins and extracellular matrix proteins including fibronectin (Fischetti, 1989; Smeesters, McMillan, & Sriprakash, 2010; Cue, Lam, & Cleary, 2001; Cue, Dombek, Lam, & Cleary, 1998; Cue, et al., 2000; Schmidt, Mann, Cooney, & Köhler, 1993). Cue et al. demonstrated that the M protein-fibronectin interaction increased the adherence of a particularly virulent M1 clone to A549 lung cells (Cue, Lam, & Cleary, 2001; Cue, Dombek, Lam, & Cleary, 1998; Cue, et al., 2000). They proposed that fibronectin functions as a molecular bridge between the M1 protein and integrin α5β1 on this cell type (Cue, et al., 2000). It is interesting to note that this clone does not encode the genes for protein F1/SfbI. Other serotypes, such as M3, have been shown to also bind fibronectin, but with the exception of an M6 serotype, no direct link to eukaryotic cell adherence via a fibronectin-M protein mechanism has yet been established (Schmidt, Mann, Cooney, & Köhler, 1993).

Protein H, which is a member of the M protein family, is another surface-exposed fibronectin-binding protein (Fischetti, Streptococcal M protein: molecular design and biological behavior, 1989). Frick et al. demonstrated that protein H binds to domains of fibronectin that are different from those that interact with F1/SfbI (Frick, Crossin, Edelman, & Björck, 1995). In addition, protein H was shown to mediate streptococcal aggregation, which in turn promoted adhesion to Detroit 562 epithelial cells (Frick, Mörgelin, & Björck, 2000), although this was not directly linked to fibronectin binding.

Fbp54

Fbp54 is a streptococcal adhesin that is expressed in the human host environment. In certain serotypes such as M5, Fbp54 can mediate preferential streptococcal binding to certain types of human cells, such as buccal epithelial cells, but not to others, such as HEp-2 cells (Courtney, Dale, & Hasty, 1996). Fbp54 is reportedly present in all serotypes tested thus far (Kreikemeyer, Klenk, & Podbielski, 2004a; Kawabata, et al., 2001), further strengthening the widely accepted notion that the adherence mechanisms of *S. pyogenes* depend not only on the characteristics of the strain, but also on the particular type of host cell and host environment in which the specific interaction is assessed.

Streptococcal hemoprotein receptor (Shr)

As with other surface-exposed adhesins, the contribution of the streptococcal hemoprotein receptor (Shr) to adherence seems to be serotype-specific. For example, Fisher et al. demonstrated the role of this protein in fibronectin-mediated attachment to HEp-2 cells (Fisher, et al., 2008), whereas Dahesh et al. showed no clear role in adherence of an M1T1 strain to HEp-2 epithelial cells and HaCaT keratinocytes cells (Dahesh, Nizet, & Cole, 2012). Further examination of these results is clearly required to understand the role of Shr in adherence of particular strains of *S. pyogenes*.

SDH/GAPDH binds to fibronectin and other eukaryotic receptors

Streptococcal surface dehydrogenase (SDH), also known as glyceraldehyde-3-phosphate dehydrogenase (GAPDH), is a multifunctional glycolytic enzyme that exists as an anchorless protein on the surface of streptococci (Pancholi & Fischetti, 1992; Jin, Song, Boel, Kochar, & Pancholi, 2005). The protein is able to bind numerous proteins from the mammalian host, including plasmin(ogen), laminin and fibrinogen. In terms of adherence to human cells, SDH binds to the urokinase plasminogen activator receptor on Detroit 562 pharyngeal cells (Jin, Song, Boel, Kochar, & Pancholi, 2005).

Given the essential role of a glycolytic enzyme in the viability of streptococci, it was initially difficult to conclusively assess the role of SDH in adherence, as the deletion of a gene essential for metabolism is lethal. This held true until Boel et al. devised a mutation strategy that allowed for a functional GAPDH enzyme to be produced, but not exported to the bacterial surface (Boël, Jin, & Pancholi, 2005). This mutant displayed a marked reduction in adherence to Detroit 562 pharyngeal cells, and while the effect was not directly attributable to a deficiency in fibronectin binding, this study implicated this enzyme in adherence to eukaryotic cells. Furthermore, this mutant strain demonstrated attenuated virulence in a mouse intraperitoneal model of infection (Boël, Jin, & Pancholi, 2005). Microarray analysis determined that the mutant was not able to express numerous adhesins, such as M protein and a collagen-like surface protein, so it is unclear whether the adherence defect is due entirely to the impaired surface expression of SDH or to the down-regulated expression of other known adhesins (Jin, Agarwal, Agarwal, & Pancholi, 2011). Since fibronectin is not only found as part of the ECM, but also in soluble form in plasma and other body fluids, SDH is a likely candidate to interact with fibronectin during the colonization process and/or during blood-borne infections.

Streptococcal collagen-like proteins

Numerous M types encode two collagen-like proteins, Scl1 (SclA) (Lukomski, et al., 2000) and Scl2 (SclB) (Rasmussen & Björck, 2001; Lukomski, et al., 2001), which contain long G-Xaa-Yaa sequences similar to mammalian collagen (Xu, Keene, Bujnicki, Höök, & Lukomski, 2002). Eukaryotic cells are known to bind collagen; thus, upon their discovery, it was reasonable to predict that streptococcal collagen-like proteins may play a role in adherence of *S. pyogenes* to the host. Accordingly, Scl1, which is expressed by virtually all streptococcal strains, has been implicated in adherence to A549 and HEp-2 cells, and this interaction involves binding of the Scl1 protein to integrins α2β1 and α11β1 on these mammalian cells (Caswell, et al., 2008; Caswell, Lukomska, Seo, Höök, & Lukomski, 2007; Chen, et al., 2010; Humtsoe, et al., 2005). Although the role of Scl2 during infection has not been elucidated, the crystal structure was recently solved, which provided clues about its function (Squeglia, Bachert, De Simone, Lukomski, & Berisio, 2014). The data suggest that Scl2 interacts with T cells and cause hyper-activation of the immune response, as is commonly seen during streptococcal toxic shock syndrome infections. Also, due to exposed hydrophobic residues, Scl2 may play a role in adherence to hydrophobic eukaryotic cell surfaces. Further research is currently underway to determine the precise role of this second collagen-like protein in the infection process.

Hyaluronic acid capsule

The hyaluronic acid capsule, which helps streptococci resist complement-mediated phagocytosis, forms the outermost layer of the streptococcal cell (Wessels, Moses, Goldberg, & DiCesare, 1991; Wessels, Goldberg, Moses, & DiCesare, 1994). As a result, numerous studies have investigated its role in the adherence process (Stollerman & Dale, 2008). Some of the earliest studies (Bartelt & Duncan, 1978), together with subsequent reports, suggest that the hyaluronic acid capsule decreases streptococcal adherence by preventing surface-exposed adhesins from attaching to host cell receptors. Conversely, other investigators used a mouse intranasal/intratracheal model of infection to demonstrate a critical role for the capsule in adherence (Wessels, Moses, Goldberg, & DiCesare, 1991; Husmann, Yung, Hollingshead, & Scott, 1997). Wessels et al. suggested that although their studies had found that the capsule was involved in adherence, in terms of its role in the pathogenic program of streptococci, capsular adherence may actually be less important than the anti-phagocytic protection it provides (Wessels, Moses, Goldberg, & DiCesare, 1991).

Schrager et al. investigated some of the apparent discrepancies in the studies on capsule-mediated adherence and determined that encapsulated streptococcal strains are indeed capable of adhering, but that the amount of capsule, together with the type and origin of host cell, influence the magnitude of the difference between encapsulated and those without capsule (Schrager, Albertí, Cywes, Dougherty, & Wessels, 1998). The extent to which the capsule interferes with M protein-mediated adherence (for example) was shown to be both strain and host-cell type dependent. Furthermore, this report and others have identified CD44 on human keratinocytes as the capsule receptor (Cywes, Stamenkovic, & Wessels, 2000).

Despite a large body of work, the debate surrounding the role of capsule in adherence (and in the overall infection process) continues today, with recent genomic studies revealing that some of the clinical isolates from human infections do not even encode the gene necessary for capsule biosynthesis (Flores, Jewell, Fittipaldi, Beres, & Musser, 2012). When taken together, the results of these studies suggest that while the capsule seems to be an important virulence factor in some strains and model systems, streptococci must encode multiple, alternate pathogenic mechanisms.

Laminin binding proteins

Laminins are large, multi-domain glycoproteins that are major components of the extracellular matrix (Martin & Timpl, 1987). Earlier studies showed that *S. pyogenes* has the ability to bind laminin (Switalski, Speziale, Höök,

Wadström, & Timpl, 1984), and evidence was presented to suggest that SpeB, a cysteine protease, was involved in this interaction (Hytönen, Haataja, Gerlach, Podbielski, & Finne, 2001). Terao et al. identified an additional laminin-binding protein, Lbp, which was found in all M type strains of *S. pyogenes* tested in their study (Terao, Kawabada, Kunitomo, Nakagawa, & Hamada, 2002b). This protein has been shown to be involved in adherence to HEp-2 cells, and its homolog in other serotypes, Lsp, is involved in adherence to A549 cells (Elsner, et al., 2002).

FbaA

Additional fibronectin-binding proteins are encoded in a small set of streptococcal genomes, and therefore, their contribution to adherence is likely strain specific. One such protein, FbaA, is proposed to be involved in the adherence of particular M1 strains to HEp-2 cells; however, the *fba* gene has been found in only a handful of serotypes, including 1, 2, 4, 22, 28, and 49 (Terao, et al., 2001). Therefore, while this protein may play a fibronectin-binding role in certain M1 strains, as well as in others that harbor the gene, it is not a universal fibronectin-binding protein (Terao, et al., 2001).

Streptococcal surface enolase (SEN)

Pancholi and Fischetti identified a plasminogen binding protein on the surface of *S. pyogenes* that they determined to be alpha-enolase, a key glycolytic enzyme (Pancholi, Fontan, & Jin, 2003). This protein, termed streptococcal surface enolase (SEN), has been shown to mediate adherence to Detroit 562 pharyngeal cells in the presence of plasminogen (Pancholi & Fischetti, 1998). Interestingly, there are other plasminogen-binding proteins on the surface of streptococci; namely, certain M proteins and GAPDH, although the plasminogen domain to which M protein binds is different from that of SEN and SDH (Walker, McArthur, McKay, & Ranson, 2005).

Transcriptional changes during adherence

The ability of all bacterial pathogens to infect a human host depends (at least partially) upon coordinated regulation of diverse gene sets that are required for survival in host environments. Microarray technology and analyses are now commonly used to reveal such genome-wide transcriptional changes. In our laboratory, we have used custom oligonucleotide microarrays to reveal genome-wide transcriptional responses of streptococci during adherence to human pharyngeal cells (Ryan, Kirk, Euler, Schuch, & Fischetti, 2007). This study, together with the work of others revealed that streptococci sense and respond (on a transcriptional level) to signals and cues in various environments of the human body, which include the surface of cells and tissue (Banks, Lei, & Musser, 2003; Broudy, Pancholi, & Fischetti, 2001; Broudy, Pancholi, & Fischetti, 2002; Shelburne, et al., 2008; Musser & Shelburne, 2009; Livezey, et al., 2011).

S. pyogenes uses elegant genetic switches, including small RNAs, stand-alone response regulators, and intricate networks to control gene expression (Aziz, et al., 2010; McIver, 2009; Kreikemeyer, McIver, & Podbielski, 2003; Le Rhun & Charpentier, 2012; Mangold, et al., 2004). By taking this information into account and expanding on the studies that have explored adhesin-receptor pairs in various strains and model systems, we can greatly advance our understanding of the complex process of streptococcal adherence to human tissues, as well as its ability to rapidly adapt during infection. Given the contradictory nature of the various studies reviewed in this chapter, it is clear that the story of streptococcal binding to host cells is far from straightforward, with the bacteria deploying numerous surface-exposed molecules to adhere to a variety of tissue types. Although different strains appear to use different strategies, ultimately the high numbers of streptococcal infections each year demonstrate that they all accomplish the same initial goal—adherence to human cells.

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Adhesion and invasion of *Streptococcus pyogenes* into host cells and clinical relevance of intracellular streptococci

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Introduction

The heterogeneous genus of Streptococci plays an important role in human disease. Streptococci are estimated to cause 700 million human infections each year worldwide, with an estimated total of 500,000 deaths (Carapetis, McDonald, & Wilson, 2005). Louis Pasteur recognized streptococci as one of the first microorganisms to cause contagious disease in 1879. For family physicians, *Streptococcus pyogenes* has generally been associated with a sore throat (strep throat) and less often associated with complications, like rheumatic fever or glomerulonephritis. Since the late 1980s, a resurgence of severe infections by *S. pyogenes* have been reported, which involve expanding and invasive soft tissue infections, as well as necrotizing fasciitis, and which are often accompanied by streptococcal toxic shock syndrome (STSS) (Reglinski & Sriskandan, 2014). In 1998, a sudden onset of neuropsychiatric illness, pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections (PANDAS) following pharyngitis, was described (Swedo, et al., 1998). During the last decade, it became clear that a related species, *S. dysgalactiae subsp. equisimilis,* can cause many of the same kinds of human infections with similar complications. Over the past 20 years, β-hemolytic species of streptococci were recognized as highly capable intracellular pathogens that are able to efficiently invade human cells in cell culture. (LaPenta, Rubens, Chi, & Cleary, 1994; Greco, et al., 1995; Rohde & Chhatwal, 2013). Evidence indicates that streptococci can survive and persist within human cells and remain impervious to antibiotic treatment and innate immune defenses.

A well-established assumption is that bacterial pathogens must first attain intimate contact with host extracellular matrix proteins (ECM) on host cells in order to establish successful infections. That initial contact with ECM proteins or cells is accomplished by highly specific adhesins (Courtney, Hasty, & Dale, 2002; Jenkinson & Lamont, 1997; Nobbs, Lamont, & Jenkinson, 2009). Adhesins and other macromolecules that trigger the uptake of bacteria or invasion of the host cells are named invasins. One hallmark of streptococci is the expression of a highly variable and extensive repertoire of adhesins and invasins. Those proteins are differentially regulated and expressed in response to signals from the different environments within the human host (Nobbs, Lamont, & Jenkinson, 2009). Streptococci sometimes use mechanisms similar to those of other intracellular bacterial species and viruses to invade host cells. Due to their variable repertoire of adhesins and invasins, streptococci have evolved numerous strategies to be internalised and survive in host cells for their own benefit, namely escaping antibiotic treatment and the host immune system (Cunningham, 2000; Courtney, Hasty, & Dale, 2002; Nitsche-Schmitz, Rohde, & Chhatwal, 2007; Nobbs, Lamont, & Jenkinson, 2009; Rohde & Chhatwal, 2013; Talay, Gram-positive adhesins, 2005). This chapter will focus on the extensive repertoire of adhesins and invasins that are expressed by β-hemolytic streptococci and will examine their molecular interactions with host human host cells, as well as address the clinical and epidemiologic relevance of intracellular streptococci.

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Adhesion of Group A streptococci

The adhesion of streptococci to the extracellular matrix (ECM) is one of the initial and essential steps of streptococcal infections. In order to colonize their host, streptococci must overcome a number of obstacles: competition with other bacterial members of the normal flora, electrostatic and mechanical forces, and physiological responses that can dislodge or physically remove them from host tissues. The strategies for adhesion are multiple, complex and variable (Jenkinson & Lamont, 1997; Nobbs, Lamont, & Jenkinson, 2009). Moreover, the expression of specific adhesins is significantly influenced by their microenvironments (Rohde & Chhatwal, 2013). Therefore, interpretations of experimental data related to adhesion mechanisms are sometimes complicated by the use of different streptococcal strains, experimental settings, and the choice of cell cultures and animals models.

Aside from tight adhesion to specific tissues, it may at times be advantageous for a pathogen to detach from a surface in order reach a more favorable environment. Therefore, adherence should be considered a dynamic process. Due to the fact that most host surfaces are covered with extracellular matrix proteins, such as collagen, fibrinogen, laminin, vitronectin or fibronectin, many streptococcal adhesins specifically bind to these various components of the extracellular matrix (Cremer, Rosloniec, & Kang, 1998; Debelle & Tamburro, 1999; Dempfle & Mosesson, 2003; Pankov & Yamada, 2002; Schvartz, Seger, & Shaltiel, 1999). Once the first step of colonization is established, streptococci may multiply extracellularly to form small colonies and/or develop biofilm-like structures that sequester them from host defenses; however, on mucosal surfaces, adhesion often leads to internalization by host cells.

Cell wall anchored and anchorless adhesins

Recognizing and binding to various ECM proteins is an important precursor for streptococcal colonization of human tissue. The ECM supports cells and tissue, maintains strength and elasticity of the body and is, therefore, ubiquitous. However, the ECM is frequently exposed by trauma and injury, which creates a prime target for streptococcal adhesion. Streptococcal adhesins can be grouped into four different families, depending on their association with their surfaces: i) those that are covalently linked by their C-terminus to the cell wall peptidoglycan through a LPxTz motif; ii) those that are tethered to the bacterial cell membrane through N-terminal modifications of lipids to form lipoproteins; iii) those that are bound to the bacterial surface by non-covalent interactions; or iv) those that are expressed and retained on the surface by an as-yet unknown mechanism. The most prominent adhesins belong to the family of cell wall-anchored proteins that are covalently linked to the peptidoglycan by membrane-associated transpeptidases, called sortase A (Marraffini, Dedent, & Schneewind, 2006).

Hyaluronic acid capsule and lipoteichoic acid

β-hemolytic streptococci are known to produce polysaccharide capsules composed of hyaluronic acid (HA), a glycosaminoglycan that is a linear polymer of alternating monosaccharide-units of N-acetylglucosamine and glucuronic acid. Expression of the HA capsule provides protection against phagocytosis by cells of the immune system. For example, the HA capsule was reported to decrease the association with PMNs, thereby counteracting phagocytosis (Dale, Washburn, Marques, & Wessels, 1996). Wessels et al. were the first to observe that encapsulated strains were better able to colonize the nasopharynx than non-encapsulated strains (Wessels, Moses, Goldberg, & DiCesare, 1991). Further studies revealed that the HA capsule can act as a non-protein adhesin by binding to the hyaluronic acid receptor CD44 on skin keratinocytes and murine epithelial keratinocytes (Cywes, Stamenkovic, & Wessels, 2000; Schrager, Rheinwald, & Wessels, 1996; Schrager, Albertí, Cywes, Dougherty, & Wessels, 1998). Moreover, highly encapsulated strains exhibit the ability to breach epithelial barriers and allow dissemination into deeper soft tissue of the human body. The interaction of HA with CD44 receptors leads to cytoskeletal rearrangements in human epithelial cells. These rearrangements can cause

disruption of intracellular junctions and allow the dissemination of streptococci into deeper, underlying sterile tissue (Cywes & Wessels, 2001).

Chain-like, glycerol phosphate polymers constitute the backbone of lipoteichoic acid (LTA). The polymers are covalently anchored to glycolipids and represent a component of the streptococcal cell wall. LTA is thought to mediate the first-step of adhesion and have little cellular specificity (Beachey & Ofek, 1976; Courtney & Hasty, 1991; Courtney, et al., 1992; Leon & Panos, 1990; Simpson & Beachey, 1983). To establish tighter links to host cells, a second, high-avidity, cellular-specific step must follow (Courtney, et al., 1992; Hasty, Ofek, Courtney, & Doyle, 1992). As a result, the latter is thought to significantly influence tissue tropisms of infections.

Fimbrious structures or pili

It is widely accepted that pili (which are sometimes referred to as fimbriae) are important mediators of Gram-negative pathogen adherence to host cells, but their role in overall pathogenesis is less studied. The expression of fibrils was associated with growth, colonization and survival of oral streptococci in the oral cavity (Handley, Carter, & Fielding, 1984; Handley, et al., 1987). Negative staining with 2% aqueous uranyl acetate is the method of choice for detecting appendages on bacterial surfaces by electron microscopic imaging. By applying this method, fibrils were detected on the cell wall of oral streptococci. Pili are long hair-like extensions on the cell surface, and were first described in Gram-positive Corynebacteria in 1968 and then in streptococci during the 1990s (Wu & Fives-Taylor, 1999; Yanagawa, Otsuki, & Tokui, 1968; Yanagawa & Honda, 1976). Streptococcal pili have recently attracted attention as promising candidates for vaccine development (Gianfaldoni, et al., 2007). All three of the invasive streptococcal pathogens (*S. pyogenes, S. dysgalactiae subsp equisimilis,* and *S. pneumonia*) were shown to have pili on their surface (Barocchi, et al., 2006; Lauer, et al., 2005; Mora, et al., 2005; Rosini, et al., 2006).

Contrary to their fragile appearance, the mechanical strength of pili is achieved by novel chemical modifications. These and other cell surface proteins contain isopeptide and thioester bonds—highly unusual intramolecular covalent linkages between amino acid side chains within the shaft—while the adhesion subunit is located at the pilus tip. These findings were very surprising, since the thioester binding domain was only known to exist in the C3 and C4 complement proteins and in alpha-macroglobulin. Similar thioester domains were identified in cell wall proteins of other Gram-positive pathogens, such as *Clostridium diphtheriae*, *C. perfringens*, and *Bacillus cereus* (Kang, Coulibaly, Clow, Proft, & Baker, 2007; Kang & Baker, 2012; Kang & Baker, 2011; Kang & Baker, 2009; Linke-Winnebeck, et al., 2014; Pointon, et al., 2010). The discovery of thioester bonds in *S. pyogenes* invasin, FbaB, is an interesting finding (Hagan, et al., 2010). Studies have shown that internal isopeptide bonds form spontaneously during the assembly process of streptococcal pili, and that isopeptide bonds are responsible for both the proteolytic and thermal stability, as well as for mechanical resistance of streptococcal pili. Therefore, pili can withstand tensile forces during the first steps of adhesion to the host cells. Although there is little evidence for its involvement in adhesion, the thioester domain was postulated to form covalent interactions with host cells (Walden, Crow, Nelson, & Banfield, 2014).

Genes that encode pilus proteins are located on pathogenicity islands (PI) in the streptococcal genome and are clustered in operons within close proximity to those that encode sortases (Scott & Zähner, 2006). Remarkably, genes that code for the fibronectin-binding, collagen-binding, and T-antigen region (or the FCT-region) are located in the same vicinity on the chromosome of *S. pyogenes.* The Lancefield T-antigen was used for years to serotype *S. pyogenes* (Cunningham, 2000). Mora et al. (Mora, et al., 2005) first showed that a T-antigen is the shaft of pili. On these pili, the adhesin molecule is on the tip, but a third component, the AP1 (ancillary protein 1), was identified that is added at intervals along the shaft of the pilus and that has the capacity to bind collagen (Falugi, et al., 2008). This observation suggests that pili mediate the first contact with the ECM that leads to adhesion to host cells. Cell culture models of infection demonstrate that pili are involved in the adhesion process for a broad range of host epithelial cells, including cells from the nasopharynx, tonsils, lung, cervix, and intestine (Abbot, et al., 2007; Crotty Alexander, et al., 2010). Remarkably, *S. agalactiae's* pili can trigger the paracellular

passage through an epithelial cell barrier and promote uptake by brain microvascular endothelial cells (Maisey, Hensler, Nizet, & Doran, 2007; Pezzicoli, et al., 2008). The so-called minor pili, which are not as long as the other pili, were first identified in both *Corynebacterium* and streptococci. These pili may also be involved in streptococcal tissue tropisms, since they mediate adhesion to human tonsil epithelium and primary keratinocytes, which are some of the prime colonization targets of streptococci (Abbot, et al., 2007). The minor pilin protein, Cpa, of *S. pyogenes* can bind collagen (Kreikemeyer, et al., 2005).

In conclusion, a putative model of cell adhesion through pili was postulated by Telford et al. (Abbot, et al., 2007; Telford, Barocchi, Margarit, Rappuoli, & Grandi, 2006). The first contact with ECM proteins of the host is mediated by extended pili via the AP2-protein (ancillary protein 2) on their tips. This interaction may be a non-covalent, reversible reaction that allows streptococci to find the specific tissue. The next step may involve the interaction of AP1-protein (ancillary protein 2, interdispersed in the pilus backbone or forming branches in the pilus backbone) with collagen (Talay, 2005), which more firmly attaches the bacterium to host cells. Consequently, streptococci come into closer contact with the cellular surface, which allows the bacterial cell surface anchored adhesins to establish intimate contact. Although the current hypothesis of bacterial adherence to host cell receptors favors strong non-covalent interactions, the finding of thioester bonds in pili and other surface proteins (invasins, adhesins) suggests an alternative new hypothesis; namely, the adhesion through a covalent bond to the eukaryotic cell surface. Future studies will test this possibility and further refine the role of pili in adherence of *S. pyogenes* to different tissues.

Anchorless adhesins

Several streptococcal proteins are localized at the cell surface, but lack the LPxTz peptidoglycan anchor motif. In addition, these so-called anchorless proteins or "moonlighting" proteins lack N-terminal signal sequences. How they are exported from the cytoplasm to the cell surface and then remain associated with the Gram-positive cell wall is still a mystery. The anchorless adhesins represent a group of very diverse proteins, both in their structures and in their functions. In addition, they bind to different ligands. Many represent members of the glycolytic cycle, such as triosephosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 3-phosphoglycerate kinase, 3-phosphoglycerate mutase, and alpha-enolase, with a cytoplasmic localization in the bacterial cell. All five of these proteins were found to be associated with the cell wall surface (Kinnby, Booth, & Svensäter, 2008; Pancholi & Fischetti, 1992; Pancholi & Fischetti, 1998). Moonlighting proteins interact with multiple ECM proteins, including fibronectin, fibrinogen, plasmin, and plasminogen, and, therefore, are likely contribute to streptococcal colonization (Esgleas, et al., 2008; Henderson & Martin, 2011; Siemens, Patenge, Otto, Fiedler, & Kreikemeyer, 2011). Surprisingly, experiments suggest that GAPDH also interacts with the cytoskeletal proteins actin and myosin, and with the urokinase plasminogen activator receptor (Jin, Song, Boel, Kochar, & Pancholi, 2005; Pancholi & Fischetti, 1992; Seifert, McArthur, Bleiweis, & Brady, 2003). Alpha-enolase is distributed among different streptococci and is bound in significant amounts to the bacterial surface. Alpha-enolase serves as a major plasminogen-binding protein for streptococci. Similar to fibronectin, plasminogen mediates adherence and subsequent internalization into host cells of *S. pyogenes* (Siemens, Patenge, Otto, Fiedler, & Kreikemeyer, 2011). In addition, after plasminogen is bound, it can be converted to plasmin by *S. pyogenes* streptokinases. Plasmin represents a very potent serine protease that degrades ECM matrix proteins to allow streptococci to come into closer contact with the host cell surface, or to gain access into deeper soft tissue after breaching barriers in the human body. Recently, there have been increasing discussions on the involvement of plasmin in facilitating streptococcal dissemination through epithelial or endothelial barriers (Bergmann, Schoenen, & Hammerschmidt, 2013; Fulde, Steinert, & Bergmann, 2013).

M proteins

M proteins are multifunctional virulence factors on the streptococcal surface, which were the first reported adhesins of *S. pyogenes* (Ellen & Gibbons, 1972). Although they are structurally related, M proteins are a heterogeneous group of adhesins that engage a variety of target cells. These surface proteins bind to a wide range

of different plasma and ECM proteins, including plasminogen, IgA, IgG, factor H, and C4b-binding protein (C4BP) (André, et al., 2006; McArthur & Walker, 2006; Oehmcke, Shannon, Mörgelin, & Herwald, 2010). The B repeats of M proteins bind to fibrinogen, human serum albumin, and IgG; but the capacity to bind these host proteins is highly variable among different M proteins. M proteins are also known to interact with glycosaminoglycanes. These interactions are mediated by their conserved C-terminal domains (Berkower, Ravins, Moses, & Hanski, 1999; Frick, Schmidtchen, & Sjöbring, 2003). The N-terminal sequences of M1 proteins are thought to be responsible for bacterial aggregation, which may also be crucial for colonization, resistance to phagocytosis, and the subsequent invasion of cells (Cue, Lam, & Cleary, 2001; Frick, Mörgelin, & Björck, 2000).

The M6 protein was shown to interact with the membrane-bound cofactor CD46 on keratinocytes. Biochemical data demonstrated that the C-terminal region of this protein and the short consensus domains 3 and 4 of CD46 must interact to establish attachment to keratinocytes (Giannakis, et al., 2002; Okada, Liszewski, Atkinson, & Caparon, 1995). M1 and M24 proteins were shown to be necessary for efficient adhesion to epithelial HEp-2 or and HeLa cells; mutant strains deficient in these M proteins lacked the capacity to adhere to these cell lines (Courtney, Bronze, Dale, & Hasty, 1994; Cue, Dombek, Lam, & Cleary, 1998). On the other hand, M24- and M6-deficient mutants are able to bind to buccal cells, but are unable to adhere to HEp-2 cells, which suggests that another adhesin (and not M protein) was responsible for adhesion to buccal cells. This agreed with another study, which demonstrated that the M6 protein does not contribute to adherence to buccal and tonsillar epithelial cells (Caparon, Stephens, Olsén, & Scott, 1991). M protein also binds to factor H, which may contribute to complementary inhibition by streptococci (Horstmann, Sievertsen, Knobloch, & Fischetti, 1988).

The M3 protein is unique in that it serves as an adhesin to soluble type I and type IV collagen and to native collagen matrices of the host. The highly-specific collagen binding region is located in the N-terminal variable region of this M protein. The M18 protein is the only other M protein with this binding specificity (Dinkla, et al., 2003a). Notably, the collagen binding protein, Cpa, is the only other collagen binding protein expressed by *S. pyogenes* and, to date is limited to M49 isolates (Podbielski, Woischnik, Leonard, & Schmidt, 1999). The role of collagen binding in streptococcal pathogenesis is an important area for future studies.

In summary, the evidence that M proteins mediate adhesion to human cells and tissue is strong, but differences in specificities that reflect the heterogeneity of this protein and expression of other surface adhesins create a complex picture. However, it is clear that streptococci generally do not bind directly to the host cell surfaces, but instead interact with extracellular matrix proteins that form bridges to host cells. It should be noted that some mucin binding cannot be ruled out, which might also contribute to direct binding to host cells.

Fibronectin-binding proteins

Fibronectin is a primary target of the streptococcal adhesion that connects bacterial adhesins to integrin receptors on the surface of eukaryotic cells. Fibronectin is a large glycoprotein that exists both as a soluble protein in plasma and as a fibrillar polymer in the ECM. It is a dimer of two 250 kDa subunits, linked by disulfide bonds at the C-terminal end. Each subunit has three distinct modules, which are termed type I, II and III. The classical fibronectin-binding partner is the $\alpha_5\beta_1$ integrin. Integrin binding is mediated through the RGD sequence within the fibronectin subunits (Pankov & Yamada, 2002).

All streptococci express a complex repertoire of proteins that have different fibronectin-binding capacities. Some strains bind soluble fibronectin with high affinities (in the nanomolar range), whereas others can only bind to immobilized fibronectin. Overall, *S. pyogenes* strains are known to express at least 11 distinct fibronectin-binding adhesins. These include SfbI/F1, protein F2, serum opacity factor (SOF), FbaA, FbaB, FBP54, and several M proteins. SfbI and FBP54 proteins are produced by several *emm* types, while expressions of M1and M24 proteins are restricted to those serotypes (Caparon, Stephens, Olsén, & Scott, 1991; Cue, et al., 2000; Hanski

& Caparon, 1992; Kreikemeyer, Oehmcke, Nakata, Hoffrogge, & Podbielski, 2004a; Natanson, et al., 1995; Neeman, Keller, Barzilai, Korenman, & Sela, 1998).

As expected, the environmental niche influences expression of these fibronectin-binding proteins. For example, at high partial pressures of O_2, the expression of SfbI is increased. On the other hand, M protein is up-regulated in a CO_2-rich environment in some strains of *S. pyogenes*. Therefore, SfbI-protein is presumed to serve as the primary adhesion when colonizing the respiratory tract or skin, but may be less important in deeper tissue infections where higher CO_2 concentrations up-regulate the M protein (Gibson, et al., 1995; Gibson & Caparon, 1996; Kreikemeyer, McIver, & Podbielski, 2003; Kreikemeyer, Klenk, & Podbielski, 2004b).

The SfbI protein and its allelic variant F1 have been extensively studied. Identified in 1992, it became immediately clear that SfbI is an important adhesin due to its wide distribution among 75% of *emm* types and clinical isolates (Hanski & Caparon, 1992; Talay, 2005).

SfbI protein has a modular architecture with a domain rich in aromatic amino acids (ARO) at the N-terminus, a proline rich repeat region (PRR) in the middle of the molecule, and a fibronectin-binding repeat region (FnBR) at the C-terminus (Talay, Valentin-Weigand, Timmis, & Chhatwal, 1994). Sequences of different clinical isolates are variable in the number of repeats in PRR and FnBR regions. Thirty-four distinct alleles of SfbI proteins were described among 54 *S. pyogenes* strains (Towers, et al., 2003). The ARO region also has a high degree of sequence variability and the number of repeat units in PRR (1-11 repeats) and FnBR (1-5 repeats) varies, as well—an apparent consequence of sequence duplication and deletion. The impact of sequence variability on the molecular interactions of SfbI with ECM and on virulence remains is unknown.

Binding to fibronectin is mediated by two distinct domains: the C-terminal fibronectin-binding repeat region and the adjacent non-repetitive domain, which is termed spacer 2 or UR. Both regions bind synergistically to two distinct regions on the fibronectin molecule: the N-terminal fibrin-binding fragment that harbors fibronectin F1 modules 1-5; and the gelatine/collagen binding fragment that harbors F1 modules 6-9 and the two F2 modules. Due to this cooperative binding, the quaternary structure of fibronectin is changed in such a way that the RGD-region in fibronectin is exposed on the outer side of the molecule. Subsequently, the RGD region can bind to $\alpha_5\beta_1$ integrin receptors on the host cell surface (Ozeri, et al., 1996; Sela, et al., 1993; Talay, et al., 2000). These interactions between SfbI and fibronectin were studied in detail through biochemical methods (Marjenberg, et al., 2011; Schwarz-Linek, et al., 2003; Schwarz-Linek, Höök, & Potts, 2004a; Schwarz-Linek, et al., 2004b).

The existing structural model for fibronectin-binding proteins has been comprehensively reviewed (Schwarz-Linek, Höök, & Potts, 2004a). Briefly, SfbI and fibronectin bind to each other in an antiparallel fashion. The C-terminal FnBR in SfbI recognizes the N-terminal domain of fibronectin with high specificity and high affinity (in the nanomolar range) by forming a novel protein-protein interaction mechanism, which is termed the tandem ß-zipper. According to the tandem ß-zipper model, FnBRs in SfbI can bind multiple copies of fibronectin, depending on the number of repeats in the FnBR region. For SfbI from a *S. pyogenes* strain, it was demonstrated that a single SfbI molecule is able to bind up to five fibronectin molecules. For the following adhesion process SfbI-expressing streptococci are covered in a cloud of fibronectin that allows the binding of the $\alpha_5\beta_1$ integrin receptors more easily on the host cell surface. Notably, the observed high affinity is of great importance: high affinity binding is a prerequisite for firm bacterial attachment, because adherent streptococci have to withstand shear forces that occur on the mucosal surfaces, in the blood stream, or during the internalization process itself. Several studies demonstrated that SfbI mediates attachment to epithelial cells of the oral mucosa and the lung, but also to endothelial cells (Molinari, Rohde, Guzmán, & Chhatwal, 2000; Rohde, Müller, Chhatwal, & Talay, 2003). Besides its potential to bind to cellular integrin receptors, SfbI has the ability to recruit collagen to the bacterial surface via pre-bound fibronectin. This allows *S. pyogenes* to form aggregates that adhere to collagen matrices within the body (Dinkla, et al., 2003b). Talay et al. (Talay, et al., 2000) were the first study to define the adhesion protein domain (the FnBR region) within SfbI that specifically interacts with an ECM protein.

Most isolates of *S. pyogenes* that lack the *sfbI* gene express other similar (but distinct) fibronectin-binding proteins. Some examples of these are protein F2 or PFBP (Jaffe, Natanson-Yaron, Caparon, & Hanski, 1996; Kreikemeyer, Oehmcke, Nakata, Hoffrogge, & Podbielski, 2004a; Rocha & Fischetti, 1999). In contrast to SfbI protein, F2 has two binding domains that exclusively interact with fibronectin by targeting the 30-kDa N-terminal fibronectin fragment. Surprisingly, the most common fibronectin-binding protein found in all *S. pyogenes* isolates, FBP54, lacks the LPxTG motif for a membrane anchor. Nevertheless, FBP54 seems to be localized on the streptococcal surface and functions as an adhesin for buccal epithelial, but not HEp2 cells (Chhatwal, 2002; Courtney, Dale, & Hasty, 1996). Some *S. pyogenes* isolates express two other fibronectin-binding proteins, named Fba and FbaB. The *fba* gene was found only in five serotypes of *S. pyogenes*. A mutant that lacked the Fba protein showed reduced adhesion to HEp2 cells, which suggests that Fba is also involved in the adhesion process. The unique fibronectin-binding protein, FbaB, appears to be genetically related to protein F2 and has only been detected in serotype M3 and M18 *S. pyogenes* isolates (Terao, et al., 2001; Terao, Kawabata, Nakata, Nakagawa, & Hamada, 2002). FbaB protein from serotype M3 *S. pyogenes* was shown to be an important invasin for this serotype, and uniquely mediates adherence only to endothelial (HUVEC cells), not to cultured epithelial cells (Amelung, et al., 2011).

Protein H is a fibronectin-binding protein and a member of the M protein family. In contrast to those that bind to type I or type II modules of fibronectin, protein H binds to the type III modules. In addition, protein H was shown to mediate streptococcal aggregation through a so-called AHP sequence that also promoted adhesion to epithelial cells (Frick, Crossin, Edelman, & Björck, 1995; Frick, Mörgelin, & Björck, 2000). M1 protein also binds to fibronectin, which subsequently engages the $\alpha_5\beta_1$ integrin receptors. M1-specific polyclonal antibodies efficiently block adherence to HeLa cells and as anticipated, an M1 protein-deficient mutant was less able to adhere to Hela cells, which confirms its contribution to adhesion for some M1 strains (Cue, Dombek, Lam, & Cleary, 1998; Cue, et al., 2000; Dombek, et al., 1999).

Uptake of streptococci by eukaryotic cells

For decades, streptococci were regarded as extracellular pathogens; however, over the last two decades, considerable experimental evidence from several laboratories has otherwise demonstrated that *S. pyogenes* is a capable intracellular bacterium, and that this intracellular state likely accounts for the commonly observed persistence of streptococcal in the oral cavity following antibiotic therapy. Efficient intracellular invasion was first suggested in 1994, when LaPenta and colleagues used a cell culture infection model to demonstrate that *S. pyogenes* enter non-phagocytic human epithelial cells at frequencies equal to or greater than classical intracellular pathogens, such as *Listeria* or *Salmonella* (Greco, et al., 1995; LaPenta, Rubens, Chi, & Cleary, 1994). Shortly thereafter, immunohistological methods were used to visualize intracellular streptococci in surgically removed tonsils from patients with recurrent infections (Österlund & Engstrand, 1995; Österlund & Engstrand, 1997; Österlund, Popa, Nikkilä, Scheynius, & Engstrand, 1997). These findings were confirmed and extended by a demonstration that showed that excised tonsils from patients with recurrent tonsillitis contain viable streptococci (Podbielski, et al., 2003). Overwhelming evidence demonstrated that other streptococcal species can efficiently invade a variety of epithelial cells, like *S. dysgalactiae subsp equisimilis* and *S. suis* (Benga, Goethe, Rohde, & Valentin-Weigand, 2004; Haidan, et al., 2000; Norton, Rolph, Ward, Bentley, & Leigh, 1999) and that some are efficiently ingested by endothelial cells (Amelung, et al., 2011; Nerlich, et al., 2009; Ochel, Rohde, Chhatwal, & Talay, 2014).

With the advent of field emission scanning electron microscopes (FESEM), pathogen-host interactions can now be imaged at much higher magnifications and resolutions. FESEM has revealed that streptococci use multiple morphologically distinct mechanisms. One invasion mechanism involves the formation of invaginations in the host cell membrane through which bacteria invade (Figure 1, A). Although an overlap exists, streptococcal uptake mechanisms are more varied than those reported for *Listeria* and *Shigella*. Some strains of streptococci exhibit a classical membrane-ruffling pattern (triggering mechanism, Figure 1, B) as described in Salmonella,

while others invade via a well-defined zipper-like mechanism (Figure 1, C), as described in Listeria. Streptococci are not restricted to these two mechanisms; isolates that express the SfbI protein induced a third, previously unknown invasion pathway with morphological features seen only by FESEM. These streptococci induce large invaginations during the internalization process, which sometimes resemble a "hole" in the host cell membrane (Molinari, Rohde, Guzmán, & Chhatwal, 2000; Rohde, Müller, Chhatwal, & Talay, 2003). A variety of invasion mechanisms were also reported for *Streptococcus dysgalactiae subsp equisimilis* (Group C and Group G streptococci) (Haidan, et al., 2000) and non-encapsulated strains of *S. suis* (Benga, Goethe, Rohde, & Valentin-Weigand, 2004) and *Staphylococcus aureus* (Agerer, et al., 2005). Long chains of streptococci invade host cells by yet another mechanism in which engulfment begins at the middle of the streptococcal chain. Host cell microvilli on both sides of the chain start to grow over the adherent chain. The bacteria are physically ingested by a "flap-like" mechanism. Thus, the streptococcal chain is engulfed from the middle when microvilli have fused with each other. In some cases, the remainder of the chain protrudes from both sides of the fused microvilli (see Figure 1, D). Representative scanning images of the different streptococcal invasion mechanisms are shown in Figure 1.

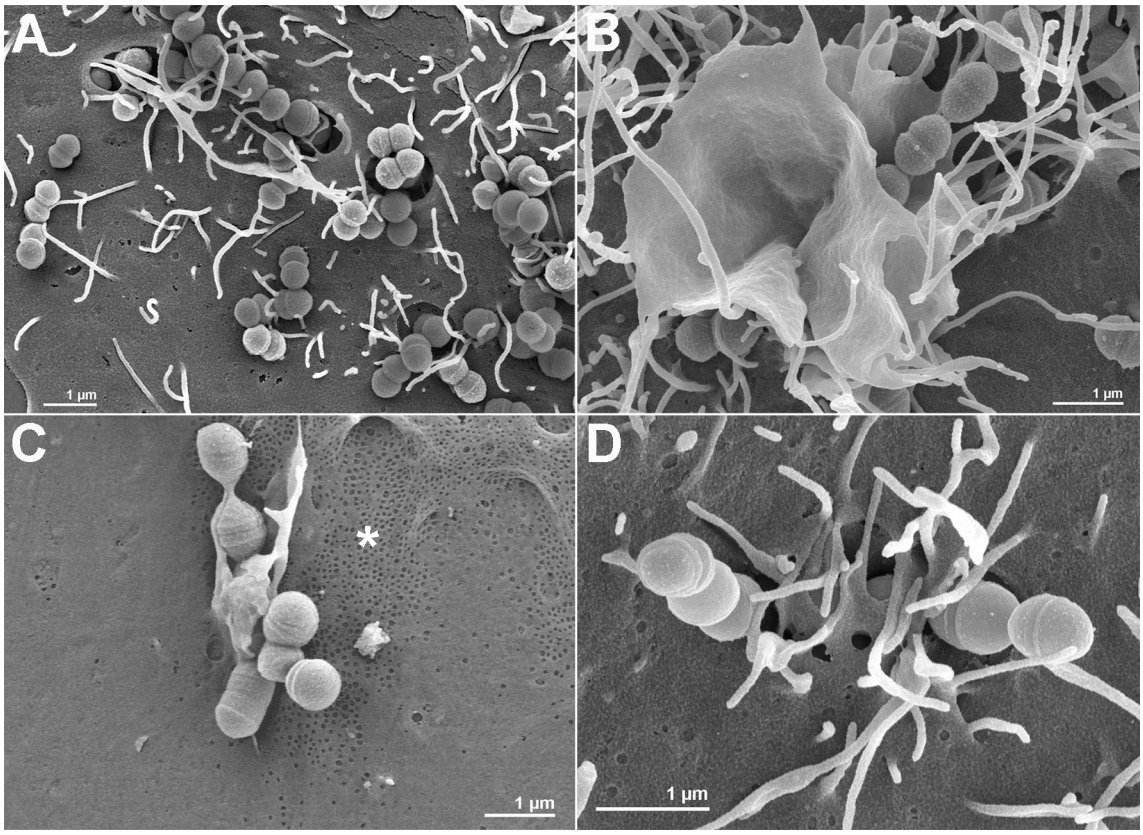

Figure 1. Different invasion mechanisms of *S. pyogenes* into epithelial cells. Field emission scanning electron microscopy (FESEM) depicts different invasion mechanisms of *S. pyogenes* isolates into host epithelial cells. A) Invasion via SfbI-mediated fibronectin binding and integrin clustering leads to the formation of large invaginations in the host epithelial cell (HEp-2) membrane, through which streptococci invade; B) Non-SfbI-expressing isolates induce the formation of large cytoskeletal rearrangements (membrane ruffling); C) Even in the presence of numerous caveolae at the adhesion site (star) non-SfbI expressing isolates trigger signaling events that lead to cytoskeletal rearrangements with subsequent engulfment; D) Chains of non-SfbI-expressing isolates are sometimes taken up by a "flap-like" mechanism, in which the uptake process often starts at the middle of the chain with both ends of the chain still outside the host cell.

Streptococcal invasins are surface exposed and/or diffusible proteins that can promote actin rearrangement of the host cytoskeleton to produce membrane ruffles (Dombek, et al., 1999), or can co-opt host cell endocytic pathways, called caveolae (Rohde, Müller, Chhatwal, & Talay, 2003), which ultimately lead to the internalization of the bacteria. Interactions with specific host cell receptors trigger cellular signalling events that instigate these

physical changes in host cells (Ozeri, et al., 2001; Purushothaman, Wang, & Cleary, 2003; Wang B. , Li, Southern, & Cleary, 2006a; Wang B. , Li, Dedhar, & Cleary, 2007). The most studied *S. pyogenes* invasins are the multifunctional SfbI and M proteins. However, several investigators have linked streptococcal dehydrogenase (Pancholi & Fischetti, 1997), SpeB (extracellular cysteine protease) (Tsai, et al., 1998), superantigen Spe A (Cleary, LaPenta, Vessela, Lam, & Cue, 1998a), and the C5a peptidase (Purushothaman, Wang, & Cleary, 2003) of *S. pyogenes* and/or *S. dysgalactiae subsp equsimilis* with the invasion of host cells. It is unclear whether these proteins contribute directly or indirectly to the invasion process; therefore, this review will focus on the functions of SfbI and the M protein.

SfbI/F1 invasion of epithelial and endothelial cells

The molecular basis for the interaction of SfbI with fibronectin was described by Talay et al. (Talay, et al., 2000). They were the first to separate adherence from the subsequent ingestion process. Co-operative protein interactions were further described on a structural basis to involve a tandem ß-zipper mechanism by Schwarz-Linek et al. (Schwarz-Linek, et al., 2003). The binding of SfbI to fibronectin changes the quaternary structure of fibronectin, which leads to exposure of the RGD-region within the molecule. This permits the RGD sequence to engage $\alpha_5\beta_1$ integrins on host cell surfaces, and the interaction between the RGD-region of fibronectin and integrins can be blocked by antibodies against the ß-subunit of the integrin, or by competitive RGD peptides, which results in significantly reduced invasion frequencies by streptococci (Jadoun, et al., 1998; Molinari, Rohde, Guzmán, & Chhatwal, 2000; Ozeri, Rosenshine, Mosher, Fässler, & Hanski, 1998). Ozeri et al. were the first to suggest that the amount of bound fibronectin on the bacterial surface influences uptake efficiency by host cells (Ozeri, Rosenshine, Mosher, Fässler, & Hanski, 1998). It is reasonable to postulate the existence of a threshold of integrin-bound fibronectin before signalling is initiated. Engagement and clustering of integrin receptors were shown to be a prerequisite for integrin signalling and subsequent invasion of host cells by other bacterial pathogens (Isberg & Leong, 1990; Isberg, 1991; Isberg & Barnes, 2001). The involvement of integrin-clustering during invasion due to multiple binding sites of RGD regions of fibronectin to integrins is strongly supported by the proposed tandem ß-zipper mechanism (Schwarz-Linek, et al., 2003). In this model, a single SfbI molecule is able to bind up to five fibronectin molecules, which results in dense coverage of the streptococcal surface with fibronectin and formation of integrin clusters with a subsequent outside-inside signalling upon contact with host cells. Clustering of integrins underneath attached streptococci was confirmed by staining the ß-subunits of integrins with specific antibodies. The application of high-resolution FESEM to visualize integrin clustering with recombinant SfbI protein coated 15 nm colloidal gold nanoparticles defined the distribution of SfbI on the surface of endothelial HUVEC cells (Rohde, Müller, Chhatwal, & Talay, 2003). Other bacterial pathogens, *Neisseria gonorrhoeae* (van Putten, Duensing, & Cole, 1998), *Staphylococcus aureus* (Fowler, et al., 2000) and *Yersiniae* species (Isberg & Barnes, 2001) specifically bind fibronectin that can engage integrins and lead to intracellular uptake by host cells.

The induction of large invaginations in epithelial and endothelial cells by SfbI-expressing streptococci was a mystery for a long time (see Figure 1, A). However, when FESEM was applied to image the surrounding cellular architecture, aggregates of approximately 80 nm wide depressions in the host cell surface were often observed. Ultrathin sections revealed omega-like structures underneath adherent bacteria, and were concluded to be caveolae from examining their shape (Rezcallah, et al., 2005). Further studies demonstrated that recombinant SfbI protein triggered caveolae aggregation and more completely defined the large invaginations associated with streptococci. Caveolae fuse with each other to form the large invaginations that were observed in FESEM studies. Once inside the host cell, SfbI-expressing streptococci traffic into a new compartment, called the "caveosome." The intriguing aspect of this type of streptococcal invasion is the fact that caveosomes do not fuse with lysosomes. By co-opting the caveolae-mediated cellular pathway, SfbI-expressing streptococci bypass the lysosomal degradation machinery of the host cells (Rohde, Müller, Chhatwal, & Talay, 2003). An identical mechanism for invasion and intracellular trafficking was reported for the simian virus 40 (SV40) (Pelkmans, Püntener, & Helenius, 2002).

S. dysgalactiae supsp equisimilis expresses Group G fibronectin binding protein A (GfbA protein), which also functions as an adhesin and invasin (Kline, Xu, Bisno, & Collins, 1996). GfbA with bound fibronectin interacts with $\alpha_5\beta_1$ integrins, with subsequent formation of membrane ruffles and rearrangements of the host cell cytoskeleton. Large invaginations were only very rarely observed. Immune fluorescence studies that labelled the lysosomal marker enzyme LAMP-1 demonstrated that GfbA-expressing streptococci follow the classical endocytic pathway, with subsequent fusion with lysosomes to form phagolysosomes. This is in contrast to the SfbI-mediated invasion process and was surprising, since both isolates bind similar amounts of fibronectin on their surface (Rohde, et al., 2011).

A definitive method for investigation of the impact of a specific protein on invasion of human cells is to express that protein in Gram-positive non-invasive bacteria, such as *S. gordonii* or *Lactococcus lactis*. Figure 2 shows *Lactococcus lactis* with surface-expressed, SfbI protein-associated caveolae during an invasion event.

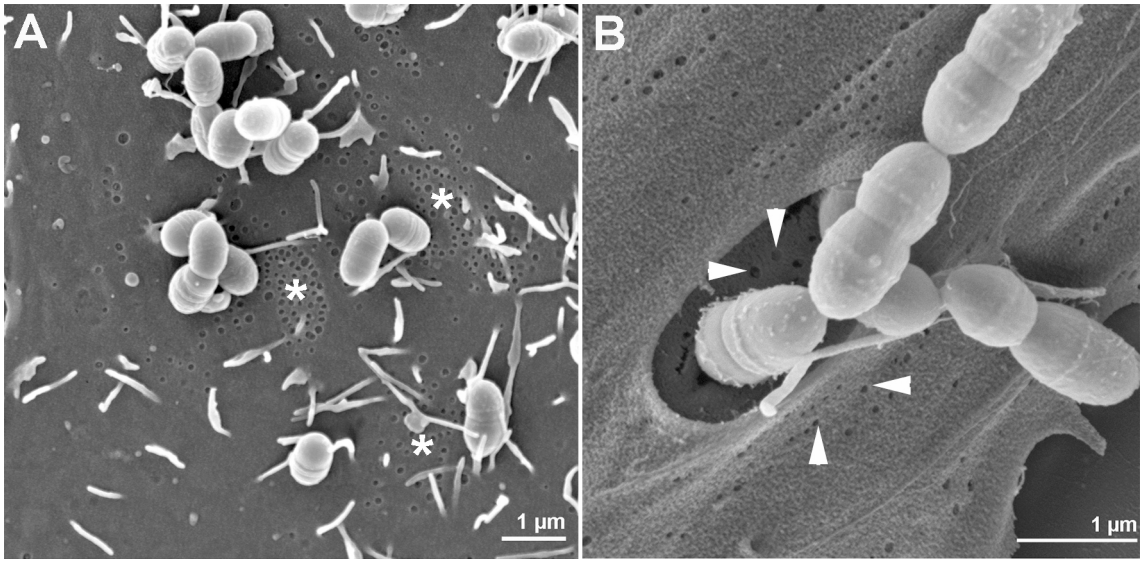

Figure 2. Model for investigation of the role of pathogenicity factors on invasion. A definitive model expresses those factors on the surface of *Lactococcus lactis* or *S. gordonii*. Depicted is the invasion mechanism of surface expressed SfbI in *L. lactis*. As is evident in (A), caveolae (stars) aggregate around adherent SfbI-expressing cells, followed by the formation of large invaginations in the host cell membrane, (B) in which some caveolae can still be detected (shown with arrows) that fuse with the membrane to further enlarge the invagination. The invasion mechanism is identical to that of the SfbI-expressing *S. pyogenes* wild-type isolate, which demonstrates that SfbI alone represents the adhesin and invasin triggering uptake through invaginations into the host cell of the isolate.

The differential impact of Gfb and SfbI on the physical uptake mechanism was investigated in more depth. Sequencing of the GfbA gene revealed that the PRR and FnBR region in the C-terminal part is very similar to the SfbI gene; however, the N-terminal sequence, including the aromatic domain (ARO) of GfbA and SfbI, is significantly different. Therefore, it was postulated that the ARO region was responsible for the morphological differences in the invasion mechanism. This hypothesis was tested by constructing a GfbA mutant protein that lacked the aromatic domain. In addition, the ARO region of the GfbA protein replaced the aromatic domain in SfbI protein. FESEM studies revealed that GfbA protein with a deleted ARO region invades with the formation of large invaginations on the host cell surface, which are structures comparable to those induced SfbI-expressing strains. In addition, intracellular *S. gordonii* that expresses GfbA without the ARO region failed to fuse with lysosomes and instead resided in caveosomes. Moreover, the strain that expressed the SfbI containing the ARO region of GfbA was now able to induce membrane ruffles, and intracellular trafficking resulted in the fusion with lysosomes. Thus, these invasins were altering to trigger an absolutely different invasion mechanism by replacing the ARO region in the protein. In addition, the invasion mechanism clearly influences subsequent intracellular trafficking. FESEM showed that only GfbA without the aromatic domain induced integrin clustering and

signalling, while SfbI with the ARO region of GfbA was unable to induce integrin-clustering and signalling, like wild-type GfbA (Rohde, et al., 2011).

In summary, heterologous surface expression of GfbA in the non-pathogenic *S. gordonii* demonstrated that the ARO region of GfbA alone is responsible for the morphologically distinct invasion mechanism. The invasion mechanism subsequently leads to different intracellular trafficking, as compared to the SfbI-mediated caveolae-dependent invasion mechanism. Thus, these studies have revealed a biological function of the ARO region in a fibronectin-binding protein for the first time (Rohde, et al., 2011).

M protein mediates invasion of human cells

The importance of intracellular invasion to *S. pyogenes* virulence and pathogenicity can be realized from the fact that most (if not all) strains produce one or more invasins. Those strains that lack the potential to produce high-affinity fibronectin-binding proteins, such SfbI, express M proteins that can take over the job and efficiently promote uptake of streptococci by both human endothelial and epithelial cells. To date, M1, M3, M5, M6, M12, M18, and M49 strains have been studied and are shown to invade cultured endothelial and/or epithelial cell lines (Amelung, et al., 2011; Berkower, Ravins, Moses, & Hanski, 1999; Dombek, et al., 1999; Molinari, Rohde, Guzmán, & Chhatwal, 2000; Nerlich, et al., 2009; Rohde, Müller, Chhatwal, & Talay, 2003). The efficiency or frequency of ingestion of different strains can be very different even within an individual serotype, which may reflect minor *emm* gene sequence differences or the acquisition of unidentified invasins encoded by prophages (Cleary, LaPenta, Vessela, Lam, & Cue, 1998a). Only the M18 serotype was found to be less invasive, due to its enormous hyaluronic acid capsule that interferes with initial adherence to cells. The efficiency of ingestion is also influenced by the quality and concentration of fibronectin and glucose in culture medium (Cleary, unpublished data), and by the number of passages of the cell line. Moreover, the genetic stability of the expression of M^+ streptococci following laboratory passage affects the invasion frequency (Cleary, et al., 1998b).

High-frequency invasion of A549 cells and primary cultures of tonsil tissue by the highly virulent M1 clone is dependent on the streptococcal bound fibronectin engagement of $\alpha_5\beta_1$-integrins on those human cells (Cue, Dombek, Lam, & Cleary, 1998; Wang, Li, Southern, & Cleary, 2006a). TGF-ß1 is known to regulate the expression of fibronectin and $\alpha_5\beta_1$-integrins by human cells. Infection of HEp2 cells and intranasal infection of mice with the above M1 strain induced robust TGF-ß1 responses in both. Moreover, the co-incubation of cells with TGF-ß1 increased the frequency of streptococcal ingestion by HEp2 cells. This was attributed to display of more integrins or integrin-binding sites on those cells (Wang, Li, Southern, & Cleary, 2006a).

M1 protein is the primary invasin, as this highly virulent clone lacks other known fibronectin-binding proteins, and in-frame deletion of the *emm1* gene significantly reduced invasion ability. This M1 protein binds both fibronectin and laminin with a much lower affinity than SfbI (Cue, Dombek, Lam, & Cleary, 1998); yet these interactions still promote efficient uptake of these streptococci by epithelial cells. Scanning EM revealed that the invasion of HeLa cells was accompanied by membrane ruffling, which suggests a zipper-like uptake mechanism (Dombek, et al., 1999). Phalloidin labeling showed the rich accumulation of actin around adherent M1 bacteria. Actin polymerisation underneath the port of entry was also observed for an M5 strain (Molinari, Rohde, Guzmán, & Chhatwal, 2000), which suggests a similar uptake mechanism. At later time points, M1 bacteria were found inside membrane-bound vacuoles marked by the lysosome LAMP-1 protein, which strongly suggests that vacuoles that contain this M1 streptococcus fuse with lysosomes and ultimately end up in phagolysosomes (Dombek, et al., 1999). Intracellular trafficking to phagolysosomes accounts for the fact that most internalized M1 streptococci are ultimately killed within seven days. Nevertheless, a few streptococci escape and survive, and thereby provide a reservoir in the human body for recurrent infections. A recent report highlighted that an M1 streptococcus can invade endothelial cells, the cellular barriers that surround blood vessels, and showed that the M1 protein was the primary invasin (Ochel, Rohde, Chhatwal, & Talay, 2014).

Invasive serotype M3 *Streptococcus pyogenes* are among the most frequently isolated streptococci from patients who suffer from invasive streptococcal disease (Sumby, Whitney, Graviss, DeLeo, & Musser, 2006; Terao, Kawabata, Nakata, Nakagawa, & Hamada, 2002). These streptococci also lack genes that encode the high-affinity fibronectin-binding protein, SfbI. Instead, M3 streptococci harbor the *fbaB* gene, which is located within the fibronectin-collagen-T antigen (FCT) locus that contains genes for pilus proteins (Terao, et al., 2001; Terao, Kawabata, Nakata, Nakagawa, & Hamada, 2002). The FbaB fibronectin-binding protein is highly conserved among M3 clinical isolates and was demonstrated to promote efficient invasion of endothelial cells by Nerlich et al. (Nerlich, et al., 2009). A study by Amelung et al. (Amelung, et al., 2011) confirmed the importance of FbaB in the uptake of M3 streptococci by endothelial cells. The uptake of streptococcus was accompanied by F-actin accumulation around adherent streptococci and formation of cellular membrane protrusions adjacent to entering bacteria. In addition, the actin branching protein, Arp2/3, was shown to accumulate near the point of entry. FbaB triggered a phagocytosis-like uptake mechanism, and intracellular streptococci travel the classical endocytic pathway. FbaB was also demonstrated to bind only to endothelial cell lines and not to epithelial cells—a possible FbaB explanation for the highly invasive nature of human M3 streptococci infections.

Dissemination of streptococci from blood vessels into tissue requires them to engage the basal side of the endothelial barrier. Indeed, passage through the endothelial cell barrier of umbilical cords in *ex vivo* experiments by M3/M18 streptococci was demonstrated. A phagocytosis-like uptake delivers streptococci into endocytic vacuoles. Those compartments fuse with lysosomes to form phagolysosomes, but some strains can trigger their own exocytosis by an unknown mechanism that involves the GTPase Rab27 (Talay, Nerlich, Dinkla, Rohde, & Chhatwal, 2008) Talay, personal communication). Figure 3 shows the dissemination of *S. pyogenes* after its intravenous administration to a mouse. Red streptococci have transmigrated through the endothelial cell barrier of the blood vessel, while green streptococci are still attached to the apical side of the endothelial barrier (facing into the blood stream). The blue streptococci may have been just released through that barrier by exocytosis.

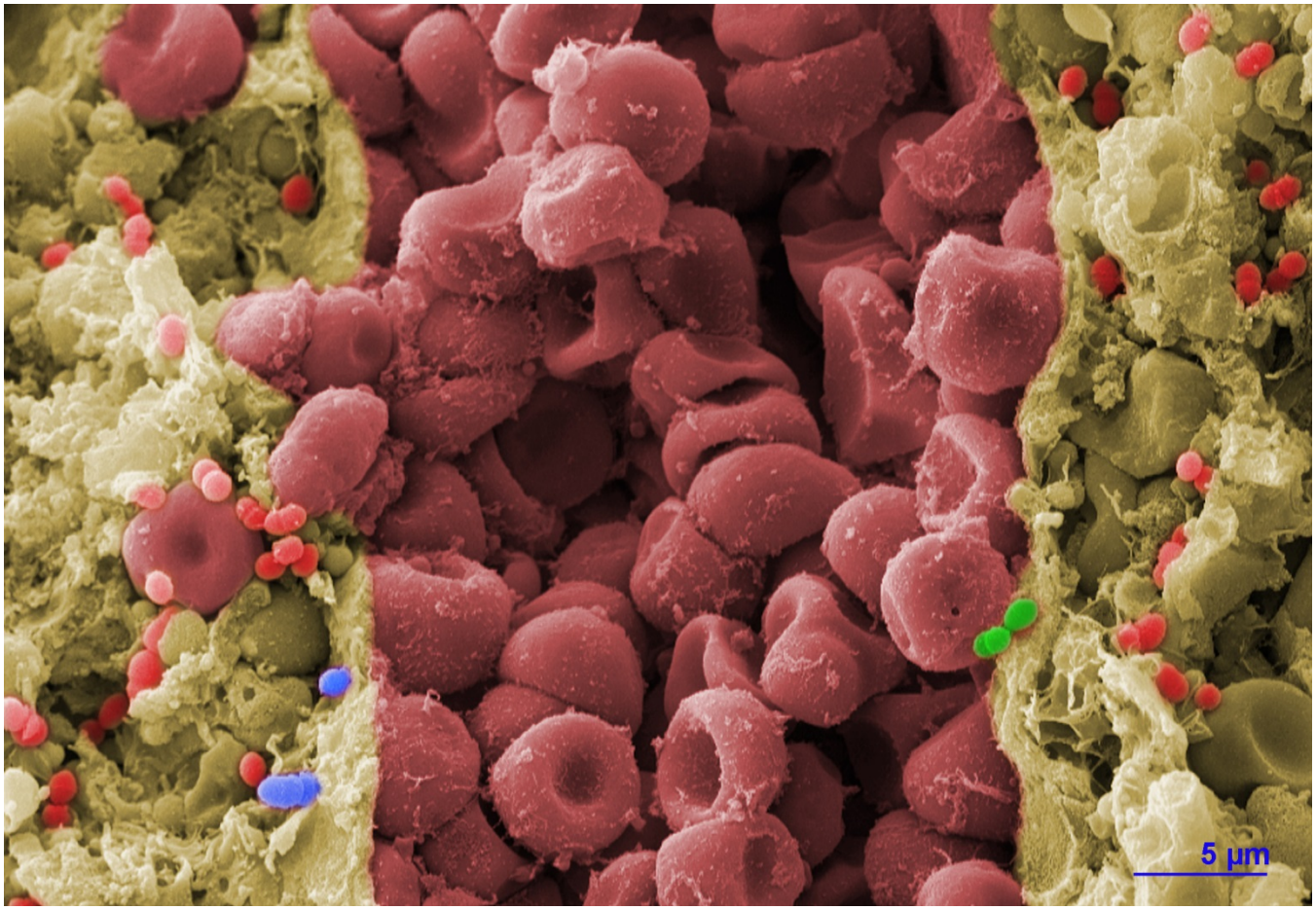

Figure 3. FESEM image of dissemination of *S. pyogenes* in the mouse model. *S. pyogenes* were intravenously administered into a mouse. From the blood stream, streptococci passed through the endothelial barrier layer of the blood vessels by an exocytosis process into deeper tissue (red blood cells are shown in red, streptococci shown in pink are residing in the adjacent tissue, green streptococci are attached to the endothelial barrier layer inside the blood vessel, and blue streptococci are exocytosed after passage through the endothelial layer). Samples are fractured after critical-point drying.

In summary, growing evidence suggests the following scenario for streptococcal invasion into epithelial and endothelial cells. SfbI and M proteins initiate the process by binding fibronectin, which then interacts with integrin receptors. Extensive integrin-clustering triggered caveolae aggregation to form large invaginations that ingest streptococci. Alternately, some streptococci that engage fibronectin with the M protein induce focal adhesion complexes, which are ingested through a zipper-like or membrane ruffling mechanism. The first mechanism ultimately directs streptococci to the safer caveosomal compartment within a cell, while the second mechanism deposits the bacteria into phagolysosomes, where most are destroyed. Figure 4 depicts these two different invasion mechanisms and intracellular trafficking routes.

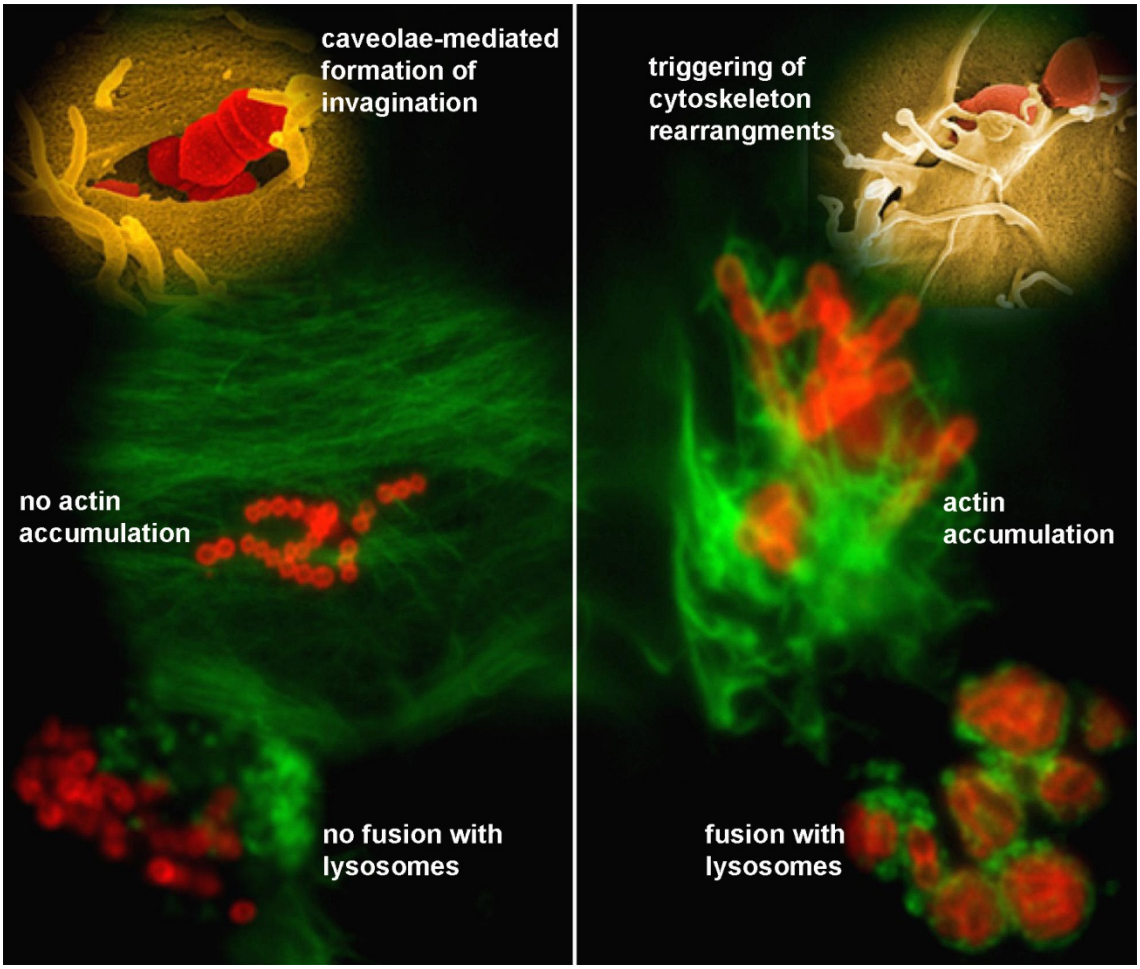

Figure 4. Summary of invasion mechanisms and subsequent intracellular trafficking of *S. pyogenes*. SfbI-expressing isolates co-opt caveolae for invasion into epithelial cells without detectable actin accumulation around invading streptococci. Intracellular trafficking bypasses fusion with lysosomes and intracellular streptococci reside in a safe niche within caveosomes. Non-SfbI-expressing isolates trigger cytoskeletal rearrangements with visible actin accumulation around invading streptococci and traffic inside the host cell through early and late endosomes, and fuse with lysosomes to form phagolysosomes. So far, all *S. pyogenes* invading through cytoskeletal rearrangements (zipper mechanism, membrane ruffling, or "flap-like") form phagolysosomes after fusing with lysosomes.

Streptococcal adhesion complexes, caveolae, and signal transduction

The fact that different strains of *S. pyogenes* express a variety of adhesins and invasins that interact with different host cellular receptors likely accounts for the diversity of tissue tropisms and a wide spectrum of human infections. Uptake by host cells is generated from α5β1 cellular receptors after interaction with fibronectin bound to the streptococci. These interactions trigger a cascade of signals that cause cytoskeleton rearrangements, and can lead to the ingestion of streptococci by non-phagocytic epithelial and endothelial cells. Investigations of signaling pathways generated by these events was motivated by the possibility of discovering targets for drugs that would prevent ingestion of streptococci into antibiotic impermeable cellular compartments, and that could reduce the incidence of antibiotic treatment failures that result in persistent carriage *of S. pyogenes*. Most studies of downstream signaling have focused on SfbI and M1. Although SfbI binds fibronectin with a much higher affinity than the M1 protein, both effectively link streptococci to α5β1 receptors. As described above, SfbI-Fn engagement of the receptor results in integrin clustering that leads to internalization of streptococci into caveosomes. In contrast, M1-Fn engagement of the same receptor results in actin rearrangement to form focal adhesion complexes and internalization of streptococci into phagolysosomes. Figure 5 depicts what is known about the signaling pathways generated by these streptococcal invasins, which lead to these distinct outcomes.

Differential signaling models for SfbI-Fn and M1-Fn mediated *S. pyogenes* invasion of epithelial cells, based on earlier work (Ozeri, et al., 2001) and the more recent work of Wang et al. (Wang, Yurecko, Dedhar, & Cleary, 2006b; Wang, Li, Dedhar, & Cleary, 2007) are proposed in Figure 5. Strain JSR4, an $M6^+$ $SfbI^+$ streptococcus, induced integrin complexes with phosphorylated focal adhesion kinase (FAK) and phosphorylated paxillin, small GTPases Rac, and Cdc42 (Ozeri, et al., 2001; Wang B. , Li, Dedhar, & Cleary, 2007). The activation of PI3K catalyzes phosphorylation of membrane-associated phosphatidylinositol, which binds to downstream targets, including integrin-linked kinase (ILK) (Persad, et al., 2001). Purushothaman et al. and others showed that both SfbI-Fn and M1-Fn mediate invasion of epithelial cells that requires phosphatidylinositol 3-kinase (PI3K) (Purushothaman, Wang, & Cleary, 2003) and ILK (Wang, Yurecko, Dedhar, & Cleary, 2006b). The latter is capable of phosphorylating the β_1 integrin cytoplasmic domain and is known to be a crucial link between integrins and the cytoskeleton. The invasion of epithelial cells by streptococci that expressed either M1 or SfbI proteins was impaired by a specific chemical inhibitor of ILK and by the expression of ILK-specific siRNA in target cells. Control assays using non-integrin dependent invasion of epithelial cells by *Salmonella* confirmed that the chemical inhibition of PI3K or ILK did not have a generally negative impact on the host cell's ability to ingest bacteria, but instead was specific to the Fn mediated uptake of *S. pyogenes*. ILK can indirectly activate Rac and Cdc42, which in turn can regulate actin cytoskeleton rearrangement (Hall, 1998), and can lead to various forms of cell adhesion complexes and stress fibers formation. Therefore, ILK may be a key intermediate protein between integrins and Rac and/or Cdc42—and it's clear that M1-Fn and SfbI-Fn complexes share a common signaling network.

How do these different fibronectin complexes finally direct streptococci into different intracellular compartments? M1-mediated invasion was shown to be significantly less sensitive to genistein than that promoted by SfbI, which suggests a branch in their respective pathways (Wang B. , Li, Southern, & Cleary, 2006a). The common signaling pathway activated by these distinct Fn-binding proteins was reported to diverge at Paxillin (Wang, Li, Dedhar, & Cleary, 2007) (Figure 5). Paxillin phosphorylation was induced by $M6^+$ $SfbI^+$ streptococci (Ozeri, et al., 2001), but the bacterial molecules leading to Paxillin phosphorylation were not defined, nor was the phosphorylated form of Paxillin shown to be required for invasion of epithelial cells in that study. Streptococci and Lactococci that express either surface protein invaded HeLa cells and promoted phosphorylation of Paxillin; however, the inhibition of Paxillin phosphorylation by PP2, a specific tyrosine kinase inhibitor, significantly prevented uptake of $M1^+$ streptococci by HeLa cells, but did not inhibit internalization of streptococci with SfbI on their surface. Still, Paxillin was observed underneath the attached $SfbI^+$ streptococci (Ozeri, et al., 2001) and, therefore, it likely participates in directing signals that lead to caveolae formation. In this case, Paxillin may serve as a scaffold for other components of the signaling complex.

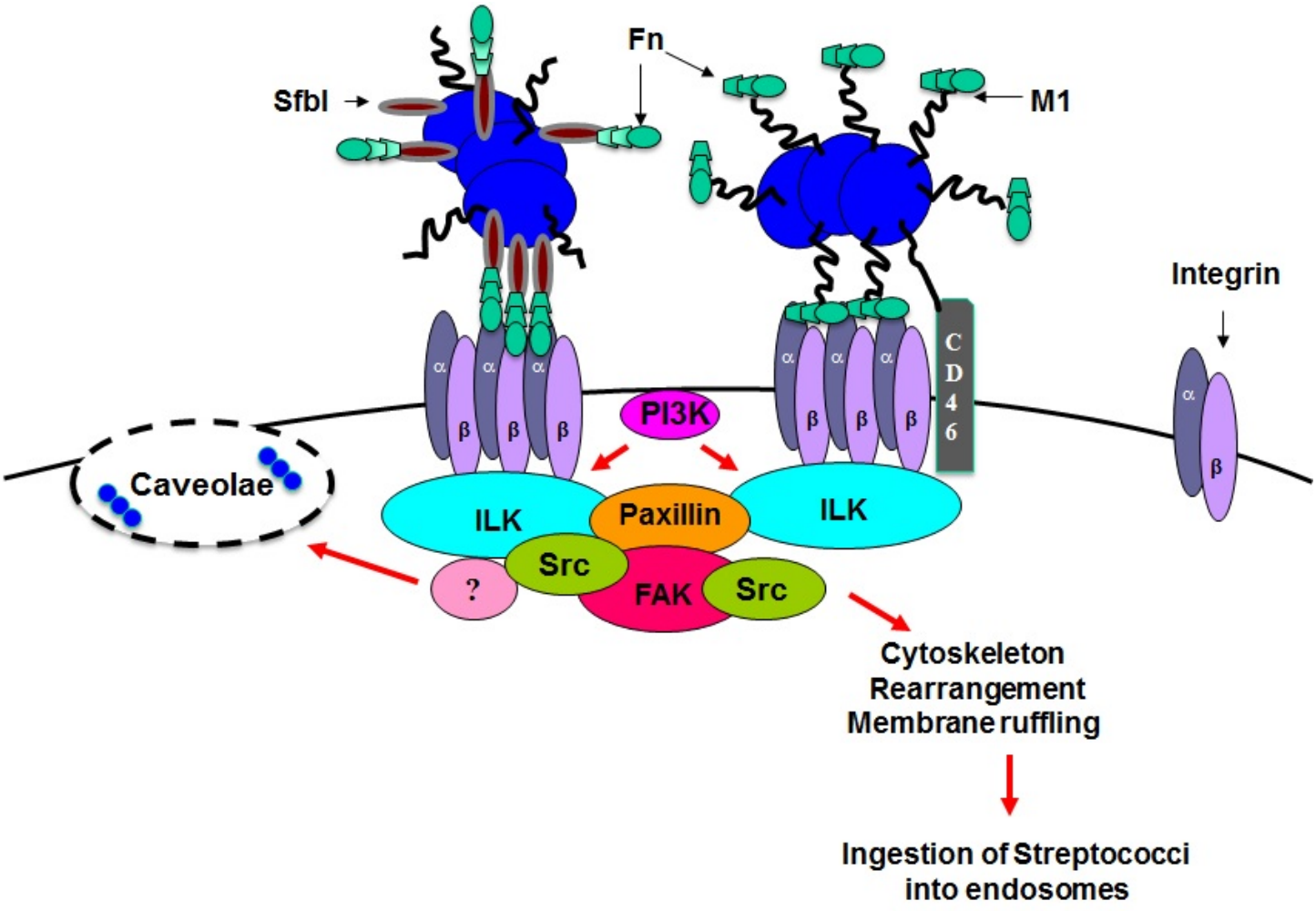

Figure 5. Model for differential ingestion of *S. pyogenes* mediated by Sfb1 and M1 proteins. Interactions of fibronectin bound to Sfb1 and M1 protein with epithelial cells lead to different ingestion pathways. Sfb1-Fn complexes promote uptake of streptococci via caveolae, while M1-Fn complexes foster receptor-mediated endocytosis. The model proposes that these two different streptococcal fibronectin binding proteins direct distinct changes in the quaternary structure of fibronectin, which in turn generate divergent signals when fibronectin engages α5β1 receptors at the surface of epithelial cells. In the end, streptococci are either deposited into relatively safe caveosomes or into bactericidal phagolysosomes. Common components of the signal cascade include PI3K, ILK, and Paxillin; however, data suggest that the signals diverge at the point of Paxillin phosphorylation.

The quaternary structure of fibronectin is partially influenced by the ligands it binds, including bacterial surface proteins. It is reasonable to postulate that the M1 protein and SfbI, which are structurally very different proteins, both induce different fibronectin quaternary conformations that inform the composition of focal adhesion complexes to generate different signals, which ultimately direct streptococci to different intracellular compartments. The impact of the ARO domain of SfbI on invasion supports this idea. As described above, the N-terminal ARO domain of SfbI is critical for ingestion of streptococci into caveosomes, and when removed or replaced by the ARO domain of GfbA, the trafficking route changed (Rohde, et al., 2011). It would be interesting to compare compositions and phosphorylation states of proteins in adhesion complexes generated by these different forms of SfbI.

The M1 protein is able to interact with multiple cellular receptors, either directly or indirectly, including CD46, a cofactor in Factor I-mediated inactivation of complement proteins C3b and C46 (Liszewski, Post, & Atkinson, 1991). CD46 is expressed on the surfaces of most human cells and was shown to enhance invasion of epithelial cells by $M3^+$ and $M1^+$ streptococci (Berkower, Ravins, Moses, & Hanski, 1999; Rezcallah, et al., 2005). Rezcallah et al. demonstrated that over-expression of CD46 with a deletion of the cytoplasmic domain reduced invasion of

epithelial cells. Their results suggested that optimal M1 protein mediated ingestion of *S. pyogenes* requires co-stimulation from both CD46 and α5β1 cellular receptors (Rezcallah, et al., 2005). There are still many important unanswered questions that remain before a clear understanding of the molecular cross-talk between *S. pyogenes* and their host cells can be obtained. The potential to apply this information to the development of drugs, such as kinase inhibitors or peptide mimics that block the bacteria-bound fibronectin engagement with integrins, also remains as an important challenge.

Invasion of professional phagocytes

The classical intracellular pathogens not only efficiently invade epithelial cells, but also survive ingestion by macrophages by blocking intracellular armaments in various ways. Some escape phagosomes and replicate in the cytoplasm of these and other cells. Molinari et al. were the first to identify a strain of *S. pyogenes*, A8, which actually escaped the phagosome and multiplied extensively in the cytoplasm of HEp2 cells (Molinari & Chhatwal, 1998). Resident macrophages and polymorphonuclear neutrophils (PMNs) constitute the first line of cellular defense against invading streptococci. Contrary to the long-held dogma that virulent streptococci are resistant to phagocytosis in blood, some strains of *S. pyogenes* are able to survive the intracellular killing mechanisms of PMNs (Medina, Rohde, & Chhatwal, 2003a). Details of these molecular processes still have to be elucidated, but streptococci clearly have the ability to escape from the phagocytic vacuole into the cytoplasma of PMNs and replicate (Medina, Goldmann, Toppel, & Chhatwal, 2003b). Furthermore, surviving streptococci exhibited increased virulence that was associated with the production of enlarged, hyaluronic acid capsules. Consistent with findings from Medina et al. (Medina, Rohde, & Chhatwal, 2003a), Staali and colleagues reported that M1 protein and the M-like protein H were crucial for the intracellular survival of *S. pyogenes* in PMNs (Staali, Mörgelin, Björck, & Tapper, 2003). Their subsequent work suggested that both M protein and protein H impair the fusion of azurophilic granules with the phagosome, thereby preventing a step that leads to the maturation of bactericidal phagosomes (Staali, Bauer, Mörgelin, Björck, & Tapper, 2006). Moreover, streptococci can accelerate neutrophil apoptosis, which facilitate their escape from host cells (Kobayashi, et al., 2003). The above results support the intriguing hypothesis that PMNs may function as a vehicle for their dissemination through the blood stream; namely, that they serve as a Trojan horse.

Surprisingly, and also in contrast to the assumption that macrophages are responsible for the clearance of streptococci from infected tissue, live streptococci were conclusively found within macrophages in biopsies of patients with soft tissue infections (Thulin, et al., 2006). Small numbers of intracellular streptococci were predominantly found in non-inflamed tissue, while large bacterial loads were observed in inflamed tissue, even after prolonged intravenous treatment with antibiotics. The implication of this study is that intracellular streptococci spread locally to uninfected tissue in low numbers.

Clinical relevance of intracellular streptococci

As described above, the capacity to invade endothelial cells and to survive ingestion by professional phagocytes is consistent with the potential of *S. pyogenes* to cause invasive disease. The epidemiology of *S. pyogenes* infections over the past 20 years confirms that *S. pyogenes* strains are not all equally able to cause serious systemic illness. The high frequency of intracellular invasions of cell lines by multiple strains of the globally disseminated M1 clone (Cleary, et al., 1992; LaPenta, Rubens, Chi, & Cleary, 1994) suggested a positive relationship between that phenotype and the world wide increase in systemic infections in the early 1990s (Musser, et al., 1995). Other M1 subclones isolated from cases of uncomplicated pharyngitis were significantly less able to enter HEp-2 cells (Cleary, LaPenta, Vessela, Lam, & Cue, 1998a). Strains isolated from cases of invasive disease in Spain between 1998 and 2009 were also dominated by the *emm1*/ST28 subclone, which again suggests that the M1 strains represent a hypervirulent lineage of *S. pyogenes* (Montes, et al., 2011). The capacity of M1 and M3 streptococci to be ingested by endothelial cells, which constitute the barriers that line blood vessels (Amelung, et al., 2011; Ochel, Rohde, Chhatwal, & Talay, 2014), also supports the possibility that

intracellular invasion of these cells leads to rapid dissemination into underlying soft tissue after exposure to streptococci. This may explain why cases of invasive infections with these M types have been reported in previously healthy persons without an apparent wound or other port of entry into soft tissue (Stevens, et al., 1989). Perhaps streptococci that hide in the endothelial barrier are also a source of transient bacteremia.

Several more recent surveys that compared strains isolated from sterile tissue to those from non-sterile tissues for the potential to express known invasins or to invade cell lines question the link between high-frequency invasions and disseminated, systemic diseases. Molinari et al. were the first to report that *S. pyogenes* isolated from throats and skin were engulfed by epithelial cells at a much higher frequency than those isolated from blood (Molinari & Chhatwal, 1998; Molinari, Rohde, Guzmán, & Chhatwal, 2000). Analysis of *S. pyogenes* isolates from patients with invasive or uncomplicated infections from Italian and Australian populations found no significant difference in the frequency of genes that encode fibronectin-binding proteins, SfbI, Sfb2, or fba54 (Baldassarri, et al., 2007; Delvecchio, Currie, McArthur, Walker, & Sriprakash, 2002; Musumeci, et al., 2003). The reasons for such systemic dissemination are likely to be complex and to depend on unique expression of an array of multiple virulence genes by highly virulent clones; a variety of host factors must also surely play a role. The capacity to multiply and survive in blood due to mutations in the CovR/S locus that up-regulate the expression of many genes (up to 10% of transcribed genes, including those responsible for hyaluronic acid capsule and a variety of other known determinants of virulence) is one explanation for such hypervirulent clones (Cao, et al., 2014; Sumby, et al., 2005; Sumby, Whitney, Graviss, DeLeo, & Musser, 2006). Although hypervirulent clones of *S. pyogenes* clearly exist, the exact relationship of intracellular invasion to systemic disease is still uncertain.

Several studies suggest that the capacity to hide inside cells is an important characteristic of streptococcal strains that persist in the throats and/or tonsils of asymptomatic carriers or that subsequently resist antibiotic therapy. The intracellular state may significantly increase the capacity of this bacterium to disseminate and persist in human populations.

When strains from carriers and patients with uncomplicated pharyngitis and sepsis were compared, respectively, those from carriers were observed to adhere to and be internalized by HEp-2 cell at higher frequency than strains that were successfully eradicated by antibiotic treatments (Neeman, Keller, Barzilai, Korenman, & Sela, 1998; Sela, Neeman, Keller, & Barzilai, 2000). The former also more often carried the SfbI gene. Contrary to these results a smaller study conducted by Brandt et al. (Brandt, et al., 2001) concluded that the expression of SfbI does not contribute to treatment failure and to the subsequent asymptomatic carriage of streptococci. They reported that only 11 isolates from 4 of 18 patients harbored SfbI. Unfortunately, these authors did not compare strains from treatment failure to those successfully eradicated by antibiotics for their capacity to invade epithelial cells; an essential comparison, since surface proteins other than SfbI can also promote high-frequency invasion (Brandt, et al., 2001). In-vitro serial passage of highly variable M1 cultures through human epithelial cells could enrich more invasive streptococci, presumably concentrating the number of M^+ streptococci in the culture (Cleary, et al., 1998b); therefore, *in vivo* cycling of streptococci between the interior and exterior of the mucosal epithelium may select for variants that are more efficiently internalized.

The earliest, most direct evidence that intracellular streptococci are an important source for the dissemination of streptococci and recurrent tonsillitis is based on microscopic studies of surgically excised tonsils (Österlund & Engstrand, 1995; Österlund & Engstrand, 1997; Österlund, Popa, Nikkilä, Scheynius, & Engstrand, 1997). Most tonsil specimens from children undergoing surgery to stop recurrent tonsillitis harbored viable streptococci within epithelial cells along the tonsillar crypts. *S. pyogenes* was also observed in macrophage-like cells at high frequency in these specimens. Tonsils from control subjects who had their tonsils removed for other reasons did not contain streptococci. More recently, viable intracellular *S. pyogenes* and *Staphylococcus aureus* were found in tonsil specimens of patients plagued by recurrent tonsillitis (Podbielski, et al., 2003; Zautner, et al., 2010). The hyper-invasive M1 subclone was confirmed to efficiently invade primary keratinized tonsillar epithelial cells *in vitro* (Cue, et al., 2000). A tropism for nasal associated lymphoid tissue, tissue known to be functionally

homologous to human tonsils, was discovered using a murine intranasal infection model. M1 streptococci were observed inside M-like cells, sporadically located along the base of the nasal epithelium, within 6 hours after intranasal inoculation. By 24 hours post-inoculation, micro-colonies were observed throughout this lymphoid organ (Park, Francis, Yu, & Cleary, 2003). From 1–10% of streptococci present in single-cell suspensions of nasal-associated lymphoid tissue after intranasal challenge of mice were resistant to penicillin, which confirms their intracellular location.

Surprisingly, *S. pyogenes* has never acquired beta-lactamase genes or penicillin binding protein based resistance to penicillin, even though that antibiotic has been the primary treatment for pharyngitis for decades (Horn, et al., 1998). However, 30 to 40% of children continue to shed streptococci after treatment with penicillin. Brandt et al. reported an 80% relapse by the same initial strain following vigorous penicillin therapy (Brandt, et al., 2001). In vitro, intracellular *S. pyogenes* can resist at least 100 μg/ml of penicillin (unpublished data). Kaplan et al. observed no degradation of intracellular streptococci after exposure to bactericidal levels of penicillin; however, erythromycin or azithromycin that penetrate mammalian cells efficiently can kill intracellular streptococci (Kaplan, Chhatwal, & Rohde, 2006). Those findings support the clinical decision to use these macrolides for a complete elimination of *S. pyogenes* from patients plagued by recurrent tonsillitis. The over-use of penicillin for treatment of pharyngitis may further select for strains that can be efficiently internalized by epithelial cells. High-frequency intracellular invasion of the mucosal epithelium may increase the rate of antibiotic therapy failure, and therefore, increase the size of the human reservoir that can disseminate the organism to others in the population. As this reservoir enlarges, the probability of serious, systemic infection will also increase, and as a result, the strains or serotypes that are less able to acquire an antibiotic-free niche may be less often associated with severe disease. Facinelli and colleagues (Facinelli, Spinaci, Magi, Giovanetti, & Varaldo, 2001) observed an association between genetic resistance to erythromycin (ErmR) and the more efficient uptake of *S. pyogenes* by epithelial cells, which suggests that erythromycin resistance may have been genetically linked to efficient invasion of human cells and thus was co-selected by antibiotic therapy (Facinelli, Spinaci, Magi, Giovanetti, & Varaldo, 2001). SfbI and ErmR genes were commonly present and chromosomally linked in these clinical isolates, which were otherwise genetically diverse.

Considerable evidence leads to the conclusion that intracellular invasion provides a safe haven for pathogens from antibiotics and immune-system defenses, and therefore increases the incidence of asymptomatic carriage of these potentially very dangerous pathogens. Immune carriage of *S. pyogene*s is in some ways analogous to a benign, non-metastatic cancer cell, which is relatively dormant until a mutation converts it to an aggressive phenotype that is able to spread systemically. As reported by Treviño et al. (Treviño, et al., 2009), *S. pyogenes* isolated from the oral mucosa were able to grow in saliva, but grew poorly in blood. On the other hand, these streptococci readily accumulated mutations in the CovR/S master regulator of transcription with striking changes in transcriptome profiles. These changes led to better growth in blood and an inability to compete with wild-type bacteria in saliva (Treviño, et al., 2009). The intracellular persistence and carriage of *S. pyogenes* can also indirectly impact the incidence of systemic, deadly disease. A cluster of toxic shock cases in elderly individuals from southern Minnesota was caused by a serotype M3 clone. That clone was carried in the throats of nearly 40% of school children in nearby communities, which suggests that school children were the reservoir for *S. pyogenes* responsible for that outbreak of toxic shock in older disabled adults (Cockerill, et al., 1997).

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The *Streptococcus pyogenes* Carrier State

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Abstract

The classical features of an individual said to be a carrier of *Streptococcus pyogenes* (Group A streptococcus) is the confirmed presence of the organism in their posterior pharynx, without any of the usual attendant clinical symptoms of acute pharyngitis. This chapter provides an overview and discussion of relevant topics of *S. pyogenes* carriage, including its prevalence, longitudinal studies, transmission from carriers, and identification of carriers in clinical practice. In addition, this chapter provides a distinction between recurrent infections and the carrier state, host and bacterial factors of carriage, and treatment and eradication of the carrier state.

Definition of Carrier

The classical clinical features that can be observed in a child who is believed to have an acute pharyngitis due to *Streptococcus pyogenes* include the abrupt onset of fever with sore throat, and the absence of diarrhea or respiratory symptoms, such as cough and rhinorrhea. In contrast, an asymptomatic child is considered to be a streptococcal pharyngeal carrier if a swab of the posterior pharynx is processed for a bacterial culture or rapid antigen detection test, and if such a test confirms the presence of *Streptococcus pyogenes* (Group A streptococcus). Since the child does not have symptoms of a sore throat and does not have inflammation of the tonsils or pharynx on physical examination, most experts would agree that the child most likely has pharyngeal carriage of the organism. In this circumstance, the pharynx is colonized with *S. pyogenes*, but it does not appear to be causing disease. This would likely be supported by the absence of a serologic antibody rise on serial testing of blood (Kaplan, 1980; Johnson, Kurlan, Leckman, & Kaplan, 2010; Kaplan, Gastanaduy, & Huwe, 1981). As it is not always practical to obtain multiple blood specimens from children, many experts make a presumption of *S. pyogenes* carriage based on the lack of clinical signs and symptoms observed at the time that a rapid test and/or throat culture is positive for *S. pyogenes* (Shulman, et al., 2012).

Prevalence of *S. pyogenes* Carriage

Pharyngitis due to *S. pyogenes* is very common in school-aged children. A meta-analysis based on a systematic review of 29 studies provides a prevalence of information about this condition. When studies of children of all ages with sore throat were analyzed, there was a pooled prevalence of 37% (95% confidence interval (CI) 32-43%) of children who were found to have a positive diagnostic test performed on a pharyngeal swab for *S. pyogenes*. This analysis also demonstrated that the prevalence of *S. pyogenes* carriage among well children with no signs or symptoms of pharyngitis was 12% (95% CI 9–14%) (Shaikh, Leonard, & Martin, 2010). Several other studies support that 15–20% of asymptomatic school aged children are colonized with *S. pyogenes*, and that 25% of asymptomatic household contacts of children with streptococcal pharyngitis have throat cultures that revealed the presence of *S. pyogenes* (Schwartz, Wientzen, Pedreira, Feroli, Mella, & Guandolo, 1981; Shulman, 1994).

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Longitudinal Studies of *S. pyogenes* Carriers

A longitudinal study was conducted in the United States of 100 school-aged children who were followed for up to four years. The mean age was 9.6 years, with a range of 5–15 years. These children had throat swabs cultured for the presence of *S. pyogenes* approximately every two weeks during the school year. An examination of the pharynx was performed at the time that the specimen was acquired, and the child was questioned about upper respiratory symptoms. If the throat culture revealed the presence of *S. pyogenes*, then a parent was contacted and another inquiry was made about the presence of a sore throat in the child. Children with classic symptoms that were consistent with streptococcal pharyngitis were treated with an antibiotic when the result of the throat culture demonstrated *S. pyogenes*. Children without signs or symptoms were not treated with antimicrobial therapy. Children were classified as having asymptomatic pharyngeal carriage if two or more sequential surveillance throat cultures positive for *S. pyogenes* were obtained more than one week apart, in the absence of respiratory symptoms. *S. pyogenes* carriers accounted for 27–32% of the cohort in each year of the study. The mean prevalence of carriers, calculated by month, was 15.9% (standard deviation 4.99%; range 4.2–26%). If children were identified as *S. pyogenes* carriers in the first year that they participated in the study, they were significantly more likely to be carriers in subsequent years, as compared with children who were not identified as *S. pyogenes* carriers in their first year of observation ($p<.0001$). Fifty-three percent of the children who were followed were *S. pyogenes* carriers at some point during their study participation (Martin, Green, Barbadora, & Wald, 2004).

All of the *S. pyogenes* isolates underwent molecular typing by field inversion gel electrophoresis (FIGE). A *S. pyogenes* isolate that represented each distinct FIGE pattern was sent to a reference laboratory for *emm* typing. It was common to find that the children who were *S. pyogenes* carriers switched from colonization with one *emm* type to colonization with another *emm* type. For the majority of the occurrences, there were no clinical signs or symptoms when the switching of *emm* types occurred. However, in 15% of the episodes where type switching occurred, the child reported clinical symptoms consistent with streptococcal pharyngitis at the same time. Occasionally, a child who was carrying one *emm* type had an apparent clinical disease due to a second type, and then resumed carriage with the original *emm* type (Martin, Green, Barbadora, & Wald, 2004). There was no association of *emm* type and carriage with clinical infection. In any given school year, there were 6–11 *emm* types identified in the study population. Each of the *emm* types was observed in children belonging to each clinical classification (Martin, Green, Barbadora, & Wald, 2004).

During the third year of this longitudinal study, there was a clonal outbreak of infections, due to an erythromycin-resistant *S. pyogenes* isolate. The strain was determined to have the M phenotype and was *emm* 6; it was observed in children who were symptomatic with typical symptoms (50%) and atypical symptoms (11%), as well as in asymptomatic children (39%). Typical symptoms were defined as those that occurred in children who had a sore throat as their predominant complaint. Atypical symptoms were defined as children who had rhinorrhea, but no sore throat. This isolate was not observed in the previous two school years (Martin, Green, Barbadora, & Wald, 2002). The same isolate resulted in several different clinical manifestations, including colonization.

Transmission of Streptococcal Infection from Carriers

Immediately after acquisition of a new streptococcal organism, transmission of the bacteria from child to child is thought to occur by large respiratory droplets. However, after several weeks, the child is presumed to no longer be contagious even if they are still colonized. There are several reasons for making this assumption. Patients who are carriers are thought to have a lower density of *S. pyogenes* in their pharynx, compared to those with an acute infection due to *S. pyogenes* (Krause, Rammelkamp, Denny, & Wannamaker, 1962). This may reduce their ability to transmit infection to others. In addition, the lack of respiratory symptoms and therefore respiratory secretions may also impact on their ability to spread the bacteria to others in their environment (Johnson, Kurlan,

Leckman, & Kaplan, 2010; Kaplan, Gastanaduy, & Huwe, 1981). Finally, the bacteria may change over time by becoming less virulent and losing the ability to cause infection (Davies, et al., 1996).

The bacteria that are most likely to colonize individuals are assumed to be less virulent. Davies conducted a population-based surveillance study of invasive disease due to *S. pyogenes* in 1992–1993. During this period, there were 323 cases of invasive disease identified with a variety of presentations. Specimens were obtained for bacterial culture of *S. pyogenes* from adult and child household contacts within two weeks of the identification of invasive disease in 46 patients: 12% had throat cultures which were positive for *S. pyogenes*. By molecular typing methods, all were identical to the strain that was identified in the index patient. Of these household contacts, 33% had pharyngitis at the time the culture was obtained. None had invasive disease or a skin or soft tissue infection. The univariate analysis demonstrated that a younger age and 4 or more hours of contact with the index patient were more likely to be associated with colonization with *S. pyogenes*. It was estimated that the risk of transmitting invasive disease to other household members was low, at 2.9 per 1000 (95% CI, 0.80 to 7.5 per 1000) (Davies, et al., 1996).

Complications from *S. pyogenes* Carriage

Children who are identified as *S. pyogenes* carriers are not thought to be at risk for complications due to *S. pyogenes* (Kaplan, 1980). While the pathogenesis of acute rheumatic fever is not completely understood, there is significant epidemiologic and immunologic evidence to support the theory that the immune response to a *S. pyogenes* infection is a critical factor. There is molecular mimicry between the immune response to the *S. pyogenes* and the heart, synovial, or brain tissue (Kaplan & Bisno, 2006; Stollerman, Lewis, Schultz, & Taranta, 1956; Zabriskie, Hsu, & Seegal, 1970; Krisher & Cunningham, 1985; Wannamaker, et al., 1951). Since *S. pyogenes* carriers do not have evidence of disease due to *S. pyogenes* or confirmation of an immune response to *S. pyogenes*, they are not believed to be at risk for non-suppurative complications (Johnson, Kurlan, Leckman, & Kaplan, 2010).

Pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections (or PANDAS) is a term used to describe the potential relationship between infections with *S. pyogenes* and the abrupt onset of motor tics or obsessive compulsive disorder in children. (See the chapter by Orefici et al in this volume for more details.) Some authors believe that this may be a complication from an acute streptococcal infection and the resultant anti-brain autoantibodies. However, children who are *S. pyogenes* carriers may also be at risk. Murphy conducted a longitudinal school study that followed 693 children, aged 3 to 12 years. While only 4.6% had persistent positive throat cultures for *S. pyogenes*, these children had higher rates of neurologic symptoms, such as choreiform movements and behavioral findings (Murphy, et al., 2007). This data raises the question of a potential relationship of the carrier state to neuropsychiatric symptoms.

Identification of *S. pyogenes* Carriers in Clinical Practice

There are two primary ways that a patient may be recognized as being a streptococcal carrier. A child may have an episode of streptococcal pharyngitis, receive treatment, and have a follow-up throat culture for the presence of *S. pyogenes* at the end of their course of antibiotic therapy. If the follow-up throat culture demonstrates the presence of *S. pyogenes*, the patient is presumed to be a bacteriologic failure. There are several potential explanations for this occurrence. These include: 1) the presence of beta-lactamase–producing oral flora, which prevent the killing of *S. pyogenes*, or the absence of oral flora that are inhibitory to *S. pyogenes*; 2) tolerance of *S. pyogenes* to penicillin; 3) infection in the tonsillar crypts or other compartments where antimicrobial therapy is not effective; or 4) the presence of relatively non-replicating organisms (Smith, Huskins, Kim, & Kaplan, 1987; Roos, Grahn, & Holm, 1986; Gerber, 1994). However, many experts agree that the most likely explanation is that the children who experience bacteriologic failure following antibiotic therapy that is appropriate for streptococcal pharyngitis are *S. pyogenes* carriers (Kaplan, Gastanaduy, & Huwe, 1981; Shulman & Gerber, 2004). The child's initial episode of illness may in fact have been due to *S. pyogenes*; however, the bacteria were not

eradicated by a course of antimicrobial therapy. The pharynx then becomes colonized with *S. pyogenes* without evidence of continuing disease or inflammation. Another possible explanation is that the initial clinical illness may not have been due to *S. pyogenes*, but rather that the symptoms may have been caused by a virus, and that the child was a carrier of this bacterium prior to the recent episode of pharyngitis.

True Recurrent Infections versus *S. pyogenes* Carriage

It is important to attempt to distinguish between children who are having true sequential recurrent episodes of streptococcal pharyngitis from the child who is a *S. pyogenes* carrier, as the management of these two conditions differ. A positive throat culture for *S. pyogenes* or a positive rapid antigen detection test at the time that a child has acute pharyngitis does not prove that the episode is due to *S. pyogenes*. Proof of streptococcal pharyngitis would require obtaining serial blood specimens and observing a rise in antibody titers over time (Kaplan, 1980; Johnson, Kurlan, Leckman, & Kaplan, 2010). This is not practical in the clinical care of children, and is confounded by the fact that prompt therapy may abort or blunt the antibody response. Instead, it is critical to obtain a careful history of the symptoms present with each episode of illness and the child's clinical response to antibiotic therapy.

The child with true recurrent episodes of streptococcal pharyngitis is likely to have classic symptoms with each illness (sore throat, without cough, rhinorrhea, hoarseness) and will experience a prompt resolution of symptoms with the initiation of antibiotic therapy (Nelson, 1984). In contrast, children with a viral illness whose throat cultures are positive for *S. pyogenes* who are actually streptococcal carriers are more likely to have viral symptoms (such as hoarseness, cough, and rhinorrhea) and are less likely to experience a rapid improvement of their symptoms with antibiotic therapy. While these clues are helpful, the best method to distinguish between these two possibilities is to obtain a throat culture after the patient has completed a course of antibiotic therapy, at a time that the patient is without any symptoms. The child with true recurrent infections will test negative for *S. pyogenes*, while the child who is a *S. pyogenes* carrier will have a positive throat culture at a time when they are well (Table 1).

Table 1: Characteristics to Distinguish a Child with True Recurrent Episodes from the Child who is a Streptococcal Carrier

True Recurrent Episodes	Streptococcal Carrier
Illness with classic symptoms: sore throat, fever, tender anterior cervical adenopathy, tonsillar erythema, tonsillar exudate, palatal petechiae	Illness not classic, may include respiratory symptoms such as hoarseness, cough and nasal congestion
Symptoms improve within 24-48 hours after initiation antibiotic therapy	Symptoms do not appear to improve with antibiotic therapy; Illness lasts longer than 5 days
Throat culture or Rapid Antigen Detection Test is negative for *Streptococcus pyogenes* when the child is asymptomatic	Throat culture or Rapid Antigen Detection Test is positive for *Streptococcus pyogenes* when the child is asymptomatic

Eradication of the Carrier State

The child who is a *S. pyogenes* carrier presents challenges in clinical management. In the majority of instances, antimicrobial therapy is not indicated for children with asymptomatic pharyngeal colonization. The guidelines published by the Infectious Disease Society of America suggest that these children do not require antibiotic therapy, since these children are not at risk for developing complications, and that they are not likely to transmit infection to others (Shulman, et al., 2012). The American Academy of Pediatrics Committee on Infectious Diseases suggests several situations when it may be advantageous to eradicate *S. pyogenes* colonization. These include when there is a family history of rheumatic fever or rheumatic heart disease, when the family is extraordinarily anxious or is considering tonsillectomy solely because of the presence of *S. pyogenes* carriage, or when there are community outbreaks of *S. pyogenes* pharyngitis (Kimberlin, Brady, Jackson, & Long, 2015).

Several clinical studies suggest that certain antimicrobial agents are associated with a higher likelihood for successful *S. pyogenes* eradication than others, in the limited circumstances in which it appears desirable to attempt to eradicate carriage (Casey & Pichichero, 2004; Pichichero, et al., 2000; Kaplan, 1980; Kaplan & Johnson, 1988; Chaudhary, et al., 1985). There are two studies whose primary outcome was to determine the efficacy of antibiotics in eliminating *S. pyogenes* carriage. A ten-day course of oral clindamycin was found to be effective, with successful eradication in 85–90% of the children who were *S. pyogenes* carriers (Tanz, et al., 1991). An earlier study demonstrated success with a combination of benzathine penicillin G and oral rifampin (Tanz, Shulman, Barthel, Willert, & Yogev, 1985).

Alternatives that are commonly prescribed include a 10-day course of amoxicillin plus clavulanic acid, or a first-generation oral cephalosporin (Kaplan & Johnson, 1988). If *S. pyogenes* carriage is suspected, the child's subsequent episode of pharyngitis, which is associated with a throat swab positive for *S. pyogenes*, can be treated with one of these options in an attempt to eradicate the *S. pyogenes* (Brook, 2001) (See Table 2). There are no randomized controlled clinical trials to compare treatment regimens for children who are thought to have true recurrent episodes of streptococcal pharyngitis, or for those who are believed to have asymptomatic pharyngeal carriage.

Table 2: Antibiotic Dosages Commonly Used For Eradication of the Streptococcal Carrier State in Children

Agent	Route	Dosage	Duration
Clindamycin	oral	20-30 mg/kg/d in 3 doses (max.= 300 mg/dose)	10 days
Penicillin and Rifampin	oral	Pen V 50 mg/kg/d in 4 doses x10 days (max.=2000 mg/d) Rifampin: 20 mg/kg/d in 1 dose for each of the last 4 days of treatment (max.=600 mg/d)	10 days
Amoxicillin clavulanic acid	oral	40 mg /kg/d in 3 doses based on the amoxicillin (max.= 2000 mg amoxicillin/d)	10 days
Benzathine penicillin G *Plus* Rifampin	IM	600,000 units for < 27 kg 1,200,000 units for ≥ 27 kg	1 dose
	oral	20 mg/kg/d in 2 doses (max.=600 mg/d)	4 days

mg = milligrams, kg = kilograms, d = days, max = maximum, IM= intramuscular

Host Factors for *S. pyogenes* Carriage

It is not clear if host factors, the specific characteristics of the bacterial isolates, or both play a role in determining whether a patient becomes a *S. pyogenes* carrier. It is believed that type-specific immunity does not prevent episodes of pharyngitis or asymptomatic carriage, particularly in view of the large number of different M types (Guirguis, Fraser, Facklam, El Kholy, & Wannamaker, 1982). From early studies, it is known that a patient who is not treated with an antibiotic for *S. pyogenes*, eradication of the *S. pyogenes,* presumably due to host response, occurs over several weeks in 50% of the patients (Brink, Rammelkamp, Denny, & Wannamaker, 1951; Catanzaro, et al., 1954; Krause, Rammelkamp, Denny, & Wannamaker, 1962). In her longitudinal study, Martin found that children who were *S. pyogenes* carriers were likely to remain carriers over time, while others never became carriers (Martin, Green, Barbadora, & Wald, 2004). In addition, children who also had siblings in the study were more likely to be a *S. pyogenes* carrier if their sibling was also colonized with *S. pyogenes* (unpublished data). Repeated environmental contact may also play a potential role. These observations suggest that there may be specific host and exposure factors that increase the likelihood that a child will become asymptomatically colonized with *S. pyogenes*.

Bacterial Factors

The M protein, which is encoded by the *emm* gene, is used for classifying types of *S. pyogenes* isolates. A longitudinal study did not demonstrate an association of *S. pyogenes emm* type with the likelihood of becoming a *S. pyogenes* carrier. Each *emm* type was observed in each clinical classification: typical infection, atypical infection, or carrier state (Martin, Green, Barbadora, & Wald, 2004). Mengeloglu studied clinical isolates from patients with infections due to *S. pyogenes* (n=79) and from carriers (n=60) and found no differences when the groups were compared by *emm* typing (Mengeloglu, et al., 2013). Some studies suggest that certain *emm* types are more likely to produce a biofilm than others (Ogawa, et al., 2011). *S. pyogenes* isolates may be able to evade host factors and antibiotics when contained within a biofilm, which may enable these isolates to be associated with asymptomatic colonization.

Some isolates of *S. pyogenes* have an external capsule and are described as mucoid. Capsule production can be upregulated, as seen in invasive infections, or can be downregulated. While mucoid strains of *S. pyogenes* have been associated with outbreaks of acute rheumatic fever, it is believed that isolates with less capsule production may be more likely to be isolated from children who are probable streptococcal carriers (Veasy, et al., 2004; Veasy, et al., 1987).

S. pyogenes is considered to be an extracellular pathogen. It can cause pharyngitis by adhering to epithelial cells, and several studies have demonstrated internalization into epithelial cells (Molinari & Chhatwal, 1998). There are several studies that demonstrate that children who are asymptomatic carriers of *S. pyogenes* are more likely to have a *S. pyogenes* isolate that contains the *prtF* genes (Neeman, Keller, Barzilai, Korenman, & Sela, 1998). PrtF1 and PrtF2 are considered to be major streptococcal virulence factors that may allow strains of *S. pyogenes* to enter respiratory epithelial cells. They are thought to play a role in the adherence and internalization of the bacteria, and may be critical in invasive *S. pyogenes* infections (Cunningham, 2000). Neeman examined the frequency of *prtF1* containing *S. pyogenes* isolates in children with pharyngitis. In 54 children with bacterial eradication after a course of antibiotics, 16/54 (30%) of the isolates were *prtF1* positive, compared to 9/10 (90%) of the isolates recovered from children who were colonized with the same strain before and after treatment and were presumed to be *S. pyogenes* carriers (Neeman, Keller, Barzilai, Korenman, & Sela, 1998). Other studies support these findings (Molinari, Talay, Valentin-Wiegand, Rohde, & Chhatwal, 1997; Molinari & Chhatwal, 1998; Hotomi, et al., 2009). Musumeci compared *S. pyogenes* isolates obtained from patients with asymptomatic carriage (n=30) and with pharyngitis (n=32) and found no differences in *prtF1*-positive strains (70% vs. 69%). However, the proportion of isolates with the *prtF2* gene was higher in those isolates from asymptomatic carriers (80% vs. 53% $p < 0.05$) (Musumeci, et al., 2003). Investigators further examined *prtF1* positive *S. pyog*enes isolates using a HEp-2 cell model, and found that adherence and internalization were higher in the strains from *S. pyogenes* carriers, as compared to those who had experienced successful bacterial eradication (Sela, Neeman, Keller, & Barzilai, 2000), while other studies also support that *prtF2* positive isolates are associated with a greater efficiency for internalization (Gorton, Norton, Layton, Smith, & Ketheesan, 2005). This supports the hypothesis that internalization of *S. pyogenes* may contribute to bacteriologic failure and the persistent carriage of *S. pyogenes*.

There may also be an association between *S. pyogenes* isolates that are erythromycin resistant and their ability to invade respiratory epithelial cells. Facinelli found that erythromycin-resistant isolates were more likely to be *prtF1* positive (89%) than erythromycin-susceptible isolates (21%). The presence of *prtF1* was also associated with higher cell invasion efficiency (Facinelli, Spinaci, Magi, Giovanetti, & Varaldo, 2001). Cocuzza examined *S. pyogenes* isolates obtained from children with pharyngitis (n=837) both before and after antibiotic treatment. Overall, 33% were positive for *prtF1*. There was a higher prevalence of *prtF1* in isolates that were erythromycin-resistant (45%), with the highest proportion observed in the iMLS phenotype with inducible resistance (84%) (Cocuzza, Lanzafame, Sisto, Broccolo, & Mattina, 2004).

In asymptomatic carriage of *S. pyogenes*, complex and varied expression of virulence traits appear to be critical for allowing the bacteria to evade the host immune response. It appears that *prtF1* permits cell adherence and invasion. The presence of virulence factors and macrolide resistance genes may be associated with certain *emm* types, rather than with the source of the bacterial isolate (Creti, et al., 2005; Blandino, Puglisi, Speciale, & Musumeci, 2011; Baldassarri, et al., 2007; Ogawa, et al., 2011). This may explain the variety of clinical manifestations that may be observed within a population that has active infections with the same *emm* type of *S. pyogenes*.

Conclusion

S. pyogenes infections are common among school-aged children. The majority of positive throat cultures observed in a longitudinal study of school-aged children were obtained from children who were carriers of *S. pyogenes*. Carriers switched *emm* types, but tended to become carriers repeatedly during the study with different *emm* types. Practitioners should consider treating children known to be *S. pyogenes* carriers when they develop a new illness with symptoms that are consistent with streptococcal pharyngitis. This may represent a new infection leading to disease, and these children then may be at risk to transmit the infection, as well as for complications, such as rheumatic heart disease. Further investigations regarding host and bacterial factors will be needed to fully understand the asymptomatic carriage of *S. pyogenes*.

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Epidemiology of *Streptococcus pyogenes*

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Introduction

While the incidence of many diseases has declined in developed countries, regions of the world with low income and poor infrastructure continue to suffer a high burden of *Streptococcus pyogenes* (group A streptococci) diseases with millions of deaths yearly (Carapetis, Steer, Mulholland, & Weber, 2005). The majority of these deaths follow the development of rheumatic heart disease (RHD), which remains a concern in both developed and developing countries. In more affluent countries, the prevalence of RHD is much lower; the majority of *S. pyogenes*-associated deaths are attributed to the clinical manifestations associated with invasive disease.

Our general understanding of the epidemiology of group A streptococci and their related diseases remains relatively poor in comparison to other infectious diseases. Many countries with established infectious disease surveillance programs undertake relatively little surveillance of diseases caused by *S. pyogenes* and other pyogenic streptococci. However, this has improved over the years with many countries establishing the presence of invasive group A streptococcal infections as a statutory notifiable disease. To fully understand the epidemiology of these diseases in terms of how they disseminate, the host and strain characteristics of importance to onward transmission, disease severity, and both inter- and intraspecies competition for ecological niches, researchers would need to undertake comprehensive investigations that follow a large cohort of individuals for a substantial period of time. Understanding these factors would also allow for the development of effective prevention strategies. The size and severity of the burden of *S. pyogenes* disease highlights the importance of epidemiologic surveillance to detect changes in disease distribution in various populations.

Since the early 1980s, there have been some remarkable changes in the worldwide epidemiology of group A streptococcal infections, particularly in the reporting of invasive group A streptococcal infections. Outbreaks of infection of both suppurative and non-suppurative *S. pyogenes* sequelae were frequently reported in the 1980s and 1990s (Efstratiou, 2000). The increase in the incidence of invasive *S. pyogenes* infections has frequently been associated with specific clones, which raises the possibility that the rise of particularly virulent clones was responsible for this re-emergence—in particular, the MT1 clone which is dominant among invasive *S. pyogenes* isolates in most developed countries (Luca-Harari, et al., 2009; O'Loughlin, et al., 2007). The incidence of invasive *S. pyogenes* infection varies by time and geographic region, which presumably reflects a population's susceptibility to particular strains, but also the natural variation in the predominant types (O'Brien, et al., 2002). Variation in the type distribution may also lead to fluctuations in the severity of infections and in overall mortality rates.

S. pyogenes infections may be observed in persons of any age, although the prevalence of infection is higher in children, presumably because of the combination of multiple exposures (in schools or nurseries, for example) and host immunity. The prevalence of pharyngeal infection is highest in children older than three years and has been described as a 'hazard' in school-aged children (Martin, Green, Barbadora, & Wald, 2004). Disease in neonates is uncommon, which may reflect a protective, transplacentally-acquired immunity.

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For this chapter, we will focus upon the epidemiology of *S. pyogenes* infection, with emphasis on the novel molecular genomics approaches that are being applied to global epidemiology, as well as the prevention, control, and management of these devastating diseases.

Surveillance and statutory notifications

Surveillance of infectious diseases forms the bedrock of control and prevention, facilitating the identification of changes warranting investigation and implementation of control measures. Longer-term monitoring provides opportunities to assess the changes in disease burden and impact of control measures. Historical review of statutory notifications and death registrations from the UK illustrates the dramatic change in the epidemiology of *S. pyogenes* disease over the past century (Figure 1). Incidence and mortality remained high in the pre-antimicrobial (penicillin) era, although both started to fall prior to penicillin's widespread availability after the Second World War, which suggests that other host, pathogen, or environmental factors played a key role in diminishing the impact of these diseases. Modern-day surveillance programs tend to focus on invasive *S. pyogenes* disease, with legislation in place in many countries that requires statutory notification, in recognition of the importance of rapid public health action following the diagnosis of a single case (see Control and prevention). Laboratory-based surveillance systems are commonly adopted as a means to monitor invasive *S. pyogenes* infections. Surveillance case definitions vary by country but most identify cases with *S. pyogenes*-positive blood cultures, with or without inclusion of additional cases diagnosed through other sterile sites.

Surveillance systems and methods for common superficial *S. pyogenes* manifestations are more variable and sparse between countries. Primary care-based surveillance networks provide valuable means of quantifying and monitoring the burden of upper respiratory tract and skin or soft tissue infections. Although these clinical case definitions may lack specificity, they may provide sensitive measures for diseases that are not normally subject to microbiological investigation.

Epidemiology of invasive disease

Disease incidence

With the establishment of surveillance systems for invasive *S. pyogenes* infections in many developed countries, data are being accumulated to allow researchers to assess longitudinal patterns in disease incidence. While concerns about the escalation in invasive disease have been widespread since the 1980s, evidence from surveillance systems to substantiate these claims is more elusive. (Steer, Lamagni, Curtis, & Carapetis, 2012a). Recent surveillance data from Utah do point to worrying trends of sustained increase in disease incidence, with rates rising to reach a surprising 9.8 per 100,000 population in 2010 (Stockmann, et al., 2012). Short-lived periods of intensification of disease incidence have been reported in many countries (Steer, Lamagni, Curtis, & Carapetis, 2012a). These may represent natural cycles driven by an accumulation of susceptible individuals as result of waning immunity and an influx of unexposed birth cohorts. Historical time series document such epidemic cycles for scarlet fever and other formerly severe *S. pyogenes* manifestations (Figure 1). The introduction of novel strains within a population has long been demonstrated as a cause of upsurge in *S. pyogenes* disease incidence, and the advancement of whole genome sequencing is beginning to provide evidence for this phenomenon (Al-Shahib, et al., 2014; Beres, et al., 2004; Tyrrell, et al., 2010; Turner, et al., 2015). Periodic upsurges may also be the result of intensified transmission within specific risk groups: for example, increases in the incidence of *S. pyogenes* disease may follow the introduction of unfamiliar strains within drug-injecting communities (Lamagni, et al., 2008c; Sierra, et al., 2006), or may occur during an influenza epidemic (Zakikhany, et al., 2011).

The dynamic nature of *S. pyogenes* infection notwithstanding, contemporary data suggest an invasive *S. pyogenes* infections incidence of around 2 to 4 per 100,000 population in developed countries (Steer, Lamagni, Curtis, &

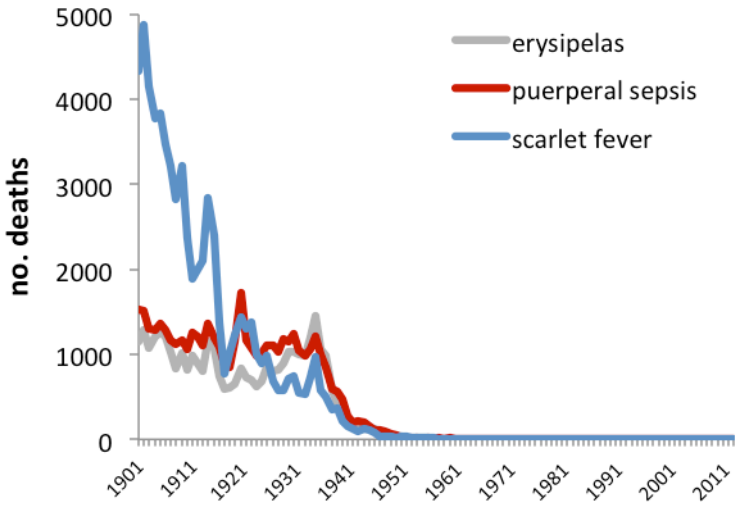

Figure 1: Certified deaths attributed to erysipelas, puerperal sepsis and scarlet fever, England and Wales, 1901-2012

Carapetis, 2012a). Considerably higher rates are observed in developing countries and within indigenous populations in developed countries, such as the USA and Australia, which range from 12 to 83/100,000 (see the below section on Demographic risk factors).

Seasonal patterns of disease

A distinct seasonal pattern of invasive *S. pyogenes* disease incidence can be noted in many temperate climates within Europe and North America (Lamagni, et al., 2008a; Lamagni, et al., 2009b). While cases occur throughout the year in these countries, disease incidence is typically lowest in the autumn and then steadily rises towards its peak incidence in December through to April (Figure 2). The drivers for this seasonal pattern remain unexplained to date, and may reflect an interplay between climatic factors, behavioral patterns, and the incidence of predisposing viral infections (Lamagni, et al., 2008a; Lamagni, et al., 2009b; Zakikhany, et al., 2011).

Demographic risk factors

Numerous epidemiological studies have identified high rates of invasive *S. pyogenes* infection in men rather than women, a pattern that can be observed for many other invasive bacterial infections and one that is not fully understood. Age-specific incidence rates show a typical J-shaped distribution, with highest rates in the elderly, followed by infants. Assessment of rates of disease according to patient ethnicity show generally higher rates of disease in individuals of non-white European descent. These observations have been made in a diverse range of populations, including indigenous populations of Australia, New Zealand, the Pacific Islands, and circumpolar regions of the northern hemisphere. The reasons behind these excesses in risk are poorly understood and could reflect differential access to healthcare or general living conditions—but could also encompass some genetic predisposing factors. Future studies will assist in identifying potential strategies to mitigate this risk.

Acute and chronic risk factors

While numerous observational studies have described the frequencies of potential risk or predisposing factors in patients with invasive *S. pyogenes* disease, rigorous assessment through analytical means has been rather more limited. Nonetheless, some commonalities are found across studies (Table 1). The relative importance of these factors may change over time as the prevalence of the acute or chronic predisposing factors changes in frequency, such as influenza activity (Zakikhany, et al., 2011). As a result, continuous monitoring or periodic reassessment is essential as a means to recognize secular trends.

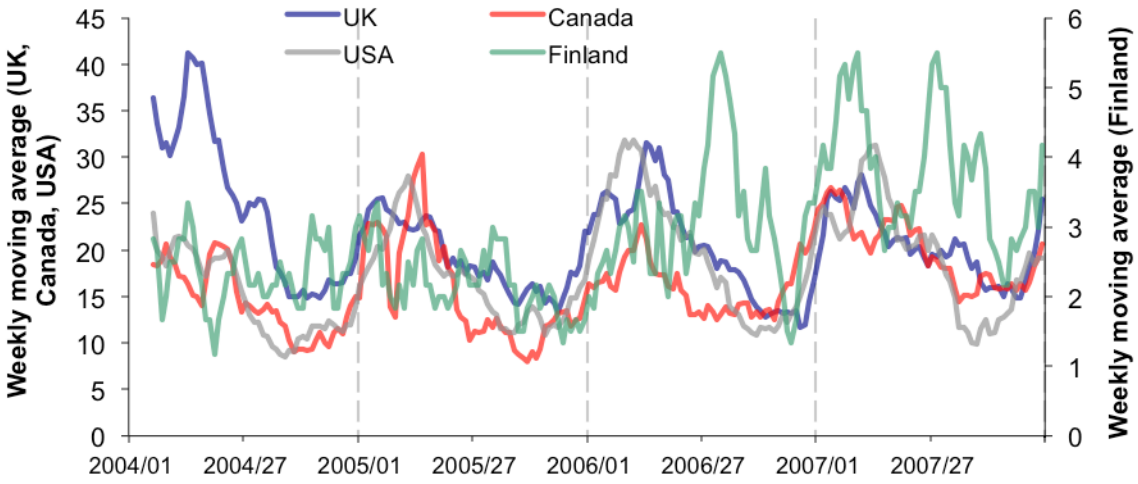

Figure 2: Seasonal patterns of invasive *S. pyogenes* infection by country (six-week moving averages) (Lamagni, et al., 2009b)

A key epidemiological feature of invasive *S. pyogenes* disease is its occurrence in individuals with no identified risk factors or predisposing conditions, which occurs in around 20-30% of all invasive *S. pyogenes* cases (Lamagni, et al., 2008a; O'Loughlin, et al., 2007). This proportion is higher in children with invasive disease, with estimates of 50–80% of pediatric cases having no identified risk factors (Lamagni, et al., 2008b; O'Loughlin, et al., 2007).

Of cases with identified acute or chronic risk factors, skin lesions, including traumatic, surgical, or chronic, are the most common risk factors identified, as they provide a portal of entry for the *Streptococcus* bacterium. These are typically reported in 17-25% of all cases (Lamagni, et al., 2008a). Blunt trauma predisposing to necrotizing fasciitis has been reported in many cases (Lamb, Sriskandan, & Tan, 2015). Acute viral respiratory infections, and influenza in particular (Morens, Taubenberger, & Fauci, 2008), are recognized risk factors for invasive *S. pyogenes* infection, with secondary infections generally occurring within one week of influenza diagnosis (Zakikhany, et al., 2011). The relative importance of influenza as a risk factor for invasive *S. pyogenes* infection will therefore be highly dependent on the levels of circulating influenza and the populations affected.

Among children, recent varicella infection is a common risk factor that can be identified in 14%–16% of pediatric invasive *S. pyogenes* cases in the absence of universal varicella vaccination programs (Lamagni, et al., 2008b; Laupland, Davies, Low, Schwartz, Green, & McGeer, 2000; Patel, Binns, & Shulman, 2004). The invasive presentations among cases with chicken pox are varied, although they most commonly manifest as severe soft tissue infections, including necrotizing fasciitis. The interval between chicken pox onset and *S. pyogenes* infection is typically around 4–5 days after the onset of chicken pox, but can occur up to 12 days later (Laupland, et al., 2000). Estimates during the two weeks after varicella onset suggest between a 40- and 60-fold elevation in the risk of invasive *S. pyogenes* infection during this period. While this excess risk may relate directly to inoculation of pox lesions with *S. pyogenes*, the varied range of focal and non-focal presentations suggests that additional factors, such as immunosuppression, play a role (Laupland, et al., 2000).

A number of co-morbidities have been associated with an excess risk of invasive *S. pyogenes* infection, of which heart disease, diabetes, and malignancy have more robust supporting evidence (Table 1). Controversy remains over the potential role of non-steroidal anti-inflammatory drugs in elevating the risk of invasive *S. pyogenes* disease. While several studies have reported an association with necrotizing fasciitis and streptococcal toxic shock syndrome, these may not reflect a causal relationship; other confounding factors may have an influence, such as delays in seeking and receiving appropriate treatment or self-medication of severe pain associated with

necrotizing fasciitis. Further studies are needed to explore this potentially important factor (Factor, et al., 2005; Lamagni, et al., 2008b; Zerr, Alexander, Duchin, Koutsky, & Rubens, 1999).

Table 1: Documented risk and predisposing factors associated with invasive *S. pyogenes* infection

Demographic factors		
	Age (infants & elderly)	**Ethnicity** ~
	Male sex	**African American**
		Bedouin population
		Canadian Arctic aboriginal
		East African
		Jewish
		Native American
		Native Alaskan
		Indigenous Australian
		Pacific Islanders
Underlying conditions		
	Alcoholism	**Injecting drug use**
	Benign tumour	Liver disease
	Chronic respiratory conditions	**Malignancy**
	Chronic & traumatic skin lesions	Metabolic disorders
	Congenital abnormalities	**Non steroidal anti-inflammatory drug use**
	Diabetes	Neurological & psychological disorder
	Endocrine disorders	Obesity
	Epilepsy	**Pregnancy & childbirth**
	Gastrointestinal disorders	Prematurity
	Glaucoma	Renal diseases
	Heart disease	Rheumatoid arthritis & polymyalgia rheumatica
	Hypertension	**Smoking**
	Immunosuppression (non-HIV related)	**Steroid use**
		Trisomy
		Vascular disease
Antecedent /concurrent infection		
	Epstein-Barr virus	Pneumonia
	Herpes zoster	Rotavirus
	HIV	Scabies
	Influenza	**Varicella (children)**
Living conditions & socioeconomic factors	**Co-habitation with a child High number of household members**	**Low number of rooms in household** Living in unhygienic conditions
	Hypothermia	**Malnutrition (children)**

Table 1 continued from previous page.

Demographic factors		
Ecological factors		
Winter onset		

* bold font indicates excess risk demonstrated through analytical epidemiological studies comparing incidence to normative population data or control group
~ compared to populations of white European decent

Pregnancy and the puerperium

Many of the factors identified to confer an excess risk of invasive *S. pyogenes* infection also serve to elevate the risk of susceptibility to a number of infectious diseases. Two are more specific to *S. pyogenes* infection: varicella (as described above) and pregnancy and childbirth. The latter has long been recognized as a risk factor for severe sepsis, but has perhaps been under-considered in modern times. While the incidence of puerperal sepsis and associated mortality has dramatically fallen over the course of the last century (Figure 1), pregnancy and the puerperium remain periods of considerable risk.

Maternal invasive *S. pyogenes* infections are particularly associated with late pregnancy (beyond 30 weeks gestation) and four weeks post-delivery (Lamagni, et al., 2011; Yamada, et al., 2010). Although the number and relative proportion of invasive *S. pyogenes* infections associated with late pregnancy or recent childbirth is low, typically 2–4% of all invasive *S. pyogenes* infections, this represents a substantial elevation in risk during a relatively short period, and which is estimated at 20–100 fold higher than in age-sex matched controls (Deutscher, et al., 2011; Chuang, Van Beneden, Beall, & Schuchat, 2002; Lamagni, et al., 2008b; Lamagni, et al., 2011). Of note is the range of focal and non-focal clinical manifestations of *S. pyogenes* disease in these women, including pneumonia, septic arthritis, necrotizing fasciitis, and genital tract sepsis (Chuang, Van Beneden, Beall, & Schuchat, 2002; Lamagni, et al., 2011; Sriskandan, 2011). The source of these infections is poorly understood, but seminal work undertaken at Queen Charlotte's hospital in London during the 1930s suggested that the woman's genital tract is an uncommon source, with either her throat or that of a close contact (family member or healthcare staff) a more common source for the infection (Colebrook, 1935). This correlates with vaginal carriage studies that indicate very low *S. pyogenes* carriage rates (<1%) (Hassan, et al., 2011; Mead & Winn, 2000). The reasons for the excess risk during the puerperium are not well understood, but may relate to immunological changes during pregnancy, coupled with specific characteristics of the organism (Mason & Aronoff, 2012). (See the section on Trends in *emm* type prevalence and disease associations).

While other organisms play an important etiological role in maternal sepsis (Acosta, et al., 2014; Maternal, Newborn and Infant Clinical Outcome Review Programme, 2014), outcomes can be especially severe for *S. pyogenes* maternal sepsis, with case fatality rates of around 2% are reported for the USA and UK (Deutscher, et al., 2011; Lamagni, et al., 2009a). In developing countries, between 8 and 12% of all maternal deaths can be attributed to sepsis, and while the full role of *S. pyogenes* in this considerable global burden is not well understood, it is likely to be significant (Khan, Wojdyla, Say, Gülmezoglu, & Van Look, 2006). Of note is the onward risk of invasive disease to neonates born to mothers who developed *S. pyogenes* infection (see Control and Prevention) and the poor outcomes for many of these infants (Hamilton, Stevens, & Bryant, 2013; Lamagni, Oliver, & Stuart, 2015; Mahieu, Holm, Goossens, & Van Acker, 1995; Miyairi, Berlingeri, Protic, & Belko, 2004).

Epidemiology of superficial disease

The main focus of epidemiologic research on *S. pyogenes* infections has been and still is invasive *S. pyogenes* disease, RHD, and toxin-mediated diseases. Our understanding of the epidemiology of less severe (but still extremely common) superficial infections is limited, despite the substantial burden presented by these diseases,

especially streptococcal pharyngitis and tonsillitis. This can and does predispose sufferers to other more serious infections, such as scarlet fever and invasive *S. pyogenes* infections. Such superficial diseases still represent a significant burden on healthcare providers, and are also a constant reservoir for deep-seated infections.

Epidemiology of pharyngitis

According to the Royal College of Physicians, "Pharyngitis is one of the most common reasons for patients to consult with their general practitioner. Acute tonsillitis and pharyngitis account for over 800 consultations per 10,000 patients annually, in addition to the economic impact of days missed from school or work" (European Medical Alliance, 2015).

Pharyngitis is diagnosed in approximately 11 million people in the United States each year. Although most cases are viral, *S. pyogenes* is the cause in 15–30% of the pharyngitis cases in children and 5–20% in adults. Cases usually occur in late winter and early spring (Choby, 2009). In Australia, the incidence of acute sore throat among school-aged children with culture-positive *S. pyogenes* has been estimated at 13 per 100 person-years with one in four of all children with acute sore throat having serologically confirmed *S. pyogenes* pharyngitis (Danchin, et al., 2007). In addition, 43% of families with an index case of *S. pyogenes* pharyngitis have a secondary case. Again, late winter and early spring are peak *S. pyogenes* seasons (Danchin, et al., 2007). It is also estimated that 15% of school-age children in developed countries will develop a symptomatic case of *S. pyogenes* pharyngitis each year, whereas the incidence of *S. pyogenes* pharyngitis in less developed countries may be five to ten times that number (Carapetis, Steer, Mulholland, & Weber, 2005).

Epidemiology of scarlet fever

Disease incidence

While the incidence of scarlet fever has dramatically fallen over the last century, there has been a resurgence in interest in studying scarlet fever after recent reports of increased incidence and large-scale outbreaks. While scarlet fever is no longer generally the life-threatening condition that it once was (Figure 1), scarlet fever outbreaks can spread rapidly and cause considerable public alarm. Notification data provide a means of monitoring disease in some countries, along with primary care surveillance networks. Rates of infection in the UK have been around 4 per 100,000 population prior to recent events, which is similar to rates in Hong Kong (Guy, et al., 2014; Luk, et al., 2012).

An upsurge in scarlet fever was reported in Vietnam in 2009 with a 40% increase in disease incidence (ProMED-Mail, 2009). Two years later, this was followed by reports of remarkable increases in disease incidence in Hong Kong and mainland China between 2011 and 2012. (ProMED-Mail, 2009; ProMED-Mail, 2012; ProMED-Mail, 2011). Scarlet fever rates in Hong Kong reached 24/100,000 population in 2011, a nine-fold increase over recent years (Luk, et al., 2012). During 2014, the UK became the latest country to report on remarkable increases in scarlet fever, as it reached its highest rate of incidence since the 1960s. Elevations in disease incidence were reported across the entire UK, with rates reaching 49 per 100,000 in some parts of the country (Guy, et al., 2014). Investigations in each country have failed to fully explain why these phenomena have occurred. As with all re-emerging infections, campaigns to re-educate clinicians on signs and symptoms to look out for are important, as well as optimal management of the disease. Continued vigilance will remain essential to monitor disease incidence in these countries and to assess changes in countries that have yet to be affected.

Risk factors

Scarlet fever incidence follows a similar seasonal pattern to invasive disease, with the highest incidence of cases and outbreaks occurring in the spring (Briko, et al., 2003; Guy, et al., 2014). Children under 10 years of age are

primarily affected, although children and adults of all ages are susceptible to infection. Residential institutions for children are settings with particularly heightened transmission (Briko, et al., 2003).

Epidemiology of rheumatic fever, rheumatic heart disease, and other sequelae

Acute rheumatic fever is an inflammatory response to *S. pyogenes* infection that typically occurs two to three weeks after a throat infection. Worldwide, approximately 500,000 new cases occur annually, and at least 15 million people have chronic rheumatic heart disease (Carapetis, Steer, Mulholland, & Weber, 2005; Webb, Grant, & Harnden, 2015). Reviews of population based data have estimated that approximately 336,000 cases of acute rheumatic fever (ARF) occur yearly in children aged 5–14 years, Additionally, ARF was at one time the leading cause of death in children in some parts of the world (Bland, 1987). More than 471,000 cases of ARF occur in all age groups (Carapetis, Steer, Mulholland, & Weber, 2005). Populations that are affected by ARF and RHD are most frequently found in developing countries in impoverished settings that do not have adequate medical or health infrastructure. Data collection is not often possible in those countries and as a result, the incidence of RF and RHD is likely to be underestimated.

The highest incidence of RF and RHD reported globally is among Pacific populations, the New Zealand Maori (Jaine, Baker, & Venugopal, 2008). The majority of cases occur in low socioeconomic communities in the northern and central North Island and pockets around Wellington, the capital city (BPAC NZ, 2011) Maori children are about 20 times more likely to be hospitalized for rheumatic fever, and Pacific children are about 40 times more likely to be hospitalized for rheumatic fever than non-Maori/non-Pacific children—a result which has been attributed to *S. pyogenes* pharyngitis that goes untreated among Maori and Pacific people (Ministry of Health – Manatū Hauora, 2015). Rheumatic fever can be prevented by prompt diagnosis of an *S. pyogenes* throat infection and treatment with antibiotics. The New Zealand Ministry of Health's Rheumatic Fever Prevention Programme (RFPP) was established in 2011 to improve access to timely treatment for *S. pyogenes* throat infections among its at-risk communities; to increase awareness of rheumatic fever; and to reduce household crowding and therefore reduce the household transmission of *S. pyogenes* throat bacteria (Ministry of Health – Manatū Hauora, 2015).

A systematic review of ten population-studies was documented from 1967 to 1996 and described the mean global incidence of ARF at 19 per 100,000 (Tibazarwa, Volmink, & Mayosi, 2008). The highest reported annual incidence rate was 51 per 100,000, which came from a study conducted in India. The lowest incidence rates were found in America and Western Europe, while higher rates were found in Eastern Europe, Asia, Australasia, and the Middle East. Information from the Africa Region was unavailable although it is known that African nations have a high incidence of RHD (Carapetis, Steer, Mulholland, & Weber, 2005).

ARF occurs even within populations at high socioeconomic levels within industrialized countries (Veasy, Tani, & Hill, 1994). In the United States, the incidence of ARF is generally lower than that in developing countries, which reports an incidence that ranges from 2–14 per 100,000 (Bland, 1987; Carapetis, Steer, Mulholland, & Weber, 2005). The higher estimates are probably due to regional outbreaks documented in certain US regions, including Tennessee, Ohio, and Pennsylvania. These outbreaks have caused considerable concern: in particular, an outbreak in Salt Lake City, Utah in the 1980s occurred where the incidence of ARF among children aged 3–17 years approached 12 per 100,000 (Veasy, Tani, & Hill, 1994).

The overall decline in ARF outbreaks is believed to be due to improvements in aspects of primary prevention, including access to healthcare and use of antibiotics. It has also been hypothesized that evolving differences in the streptococcal M protein type plays a major role in the number of diminishing cases (Shulman, Stollerman, Beall, Dale, & Tanz, 2006). However, it still remains unclear why the trend has moved away from rheumatogenic

strains of *S. pyogenes* to those that do not commonly cause ARF; the answers may well be resolved with the advent and application of genomics.

Carriage and transmission of group A streptococci

While group A streptococci are known colonizers of the oropharynx, genital mucosa, rectum, and skin (especially at the site of lesions), rates of carriage in present-day populations are poorly understood. Studies examining carriage rates in healthy adults suggest low levels of carriage, typically less than 5% and 1% for throat and vaginal/rectal carriage respectively (Steer, Lamagni, Curtis, & Carapetis, 2012a). Estimates of pharyngeal *S. pyogenes* carriage in healthy children vary considerably from 2% up to17% (Marshall, et al., 2015; Gunnarsson, Holm, & Söderström, 1997; Martin, Green, Barbadora, & Wald, 2004).

Group A streptococci are transmitted through a number of modalities. Direct person-to-person transmission occurs through the inhalation of respiratory droplets or through skin contact. Transmission through environmental reservoirs has been strongly implicated in experimental or outbreak investigations, either through direct contact with contaminated objects and surfaces or through dust particles. Seminal investigations conducted in the late 1940s and 1950s in the context of high incidences of respiratory tract infection and rheumatic fever at the Warren Air Force base (Wyoming, USA) yielded a considerable body of knowledge on the transmission of *S. pyogenes*, and in particular, the influence of physical proximity on transmission rates. While transmission through consumption of food inoculated by food handlers colonized with *S. pyogenes* has become less common, it still occasionally occurs and does result in outbreaks (Kemble, et al., 2013).

Control and prevention

In the absence of licensed vaccines, modern day public health strategies for *S. pyogenes* disease focus on measures to minimize transmission and to provide protection for individuals who are at risk of invasive disease. Concern over the increasing incidence of invasive disease has led to the assessment of opportunities for controlling the spread of infection. As most cases of invasive disease occur sporadically rather than in identified clusters, opportunities for prevention through outbreak control are somewhat limited.

Secondary household risk

As largely community-acquired infections, with around 1 in 10 linked to healthcare interventions, the initial focus of public health guideline developments has been to assess clustering patterns of invasive *S. pyogenes* disease within households to evaluate the overall risk of secondary transmission. Given the relatively low frequency of primary cases, such assessments require a large study population. To date, these have been undertaken by Canada, USA, Australia, and the UK. Very small numbers of household pairs have been identified through these systematic assessments, with many of these "secondary" cases being co-primary cases, by virtue of simultaneous presentation with the index case. For these pairs of cases, there's no opportunity for intervention to reduce the risk of invasive *S. pyogenes* disease or mitigate its impact (Lamagni, Oliver, & Stuart, 2015). Intervals between index and secondary cases have varied, but generally fall within a month of the onset of the index cases. While the numbers of such clusters are small, this represents a substantial risk of disease in household contacts that is variously estimated from 800 to over 5000 cases per 100,000 person-years (Table 2) (Lamagni, Oliver, & Stuart, 2015). This clearly represents a significant elevation in risk over background incidence, although further studies are needed to add precision to these estimates. Different strategies for managing this elevated risk have been adopted by different countries and include antibiotic prophylaxis to all close contacts or in individuals at substantially greater risk (Steer, Lamagni, Curtis, & Carapetis, 2012a). Given the rapid onset of invasive *S. pyogenes* infection, advising close contacts on the need to seek medical attention if they develop the signs and symptoms of invasive *S. pyogenes* infection is an essential component of an overall risk management approach.

Table 2: International population-wide assessments of risk of invasive *S. pyogenes* infection in household contacts of index cases (Lamagni, Oliver, & Stuart, 2015)

Location	Year(s)	Background incidence	Cases in contacts	Attack rate in contacts		Rate Ratio
		rate/100,000 person-yrs	no.	rate/100,000 person-yrs	(95% CI)	
Ontario, Canada	1992-93	2.4	4	3581	(976 - 9168)	1492
USA	1997-99	3.5	1	804	(20 - 4480)	229
UK	2003	3.5	5	2579	(837 - 6018)	731
Victoria, Australia	2002-04	2.7	3	5468	(1128 - 15979)	2011
All countries	-	-	13	2681	(1428 - 4585)	-

Outbreak management

While the focus of public health guidelines has been on the management of outbreaks of invasive disease, many of the same principles apply to the control of less severe outbreaks, such as scarlet fever. Prevention measures can include:

- environmental decontamination and improved hygiene
- communication of elevated risk
- exclusion from workplace or school
- antibiotic prophylaxis and treatment

Epidemiological investigation of outbreaks is essential as a means to identify or exclude transmission routes, and therefore, to target prevention measures. Bacteriological screening of potentially exposed individuals can provide evidence of ongoing transmission within a given setting or target population, or a potential point source of transmission to others. Negative results should be cautiously interpreted, given that screening will miss colonized individuals by virtue of poor sampling technique, failure in processing and testing of specimens, or, at a more fundamental level, colonization at non-swabbed body sites. While rectal/vaginal carriage of *S. pyogenes* in healthcare staff has been implicated in several outbreaks, investigators may be reluctant to suggest swabbing such intimate sites in the absence of compelling epidemiological evidence. As a result, epidemiologic data identifying common links between cases and potential sources remains a key part of any investigation.

The role of the environment in facilitating the spread of *S. pyogenes* is potentially under-recognized, despite well-documented accounts that suggest a key environmental role in facilitating disease transmission. *S. pyogenes* are shed in the immediate environment of infected, untreated individuals in large numbers with viable bacteria cultivated from clothing and bedding belonging to the infected person, as well as in accumulated dust. Similarly, food can become inoculated and may facilitate the spread of infection to numerous recipients of foods prepared by infected kitchen staff (Kemble, et al., 2013). Therefore, identifying and decontaminating the environment is a fundamental component of outbreak management strategy.

Antibiotic treatment is recognized as an effective means to reduce transmission of the organism particularly for respiratory and cutaneous infections (Steer, Lamagni, Curtis, & Carapetis, 2012a). Studies assessing the effectiveness of antibiotics at decolonizing infected individuals have found that the majority of individuals no longer have viable *S. pyogenes* in their throat 24 hours after starting therapy. Therefore, this forms the minimum exclusion period for colonized or infected individuals to prevent onward transmission, albeit with the recognition that a certain number of individuals may become recolonized through contact with non-treated carriers.

Outbreak settings

Few countries have systematic surveillance systems that provide comprehensive data on outbreaks of all types of *S. pyogenes* infection, which precludes our ability to make a meaningful assessment of the burden and characteristics of these events. Outbreaks of scarlet fever and pharyngitis continue to occur and are primarily reported in schools and preschool settings. Secondary prevention measures in these situations include reinforcement of hand hygiene and ensuring exclusion from school/nursery for a minimum of 24 hours after the initiation of antibiotics. Co-circulation of varicella represents a significant threat, and as such, varicella vaccination in groups that are likely to be susceptible (such as pre-schoolers) may also be considered in populations without universal varicella immunization programs.

Outbreaks of invasive *S. pyogenes* infection are uncommon, but do regularly occur primarily in hospital settings and facilities that offer long-term institutional care (Cummins, Millership, Lamagni, & Foster, 2012; Daneman, et al., 2007; Jordan, Richards, Burton, Thigpen, & Van Beneden, 2007). The institutionalized nature of the care provided in these environments translates to the potential for many residents and patients to develop infections through contact with colonized staff. When coupled with the inherent vulnerability of these populations, rapid investigation of *S. pyogenes* infections acquired in these settings is essential as a means to identify the source and instigate effective control measures. Colonized staff, whether symptomatic or asymptomatic, as well as potential environmental reservoirs that are epidemiologically linked to cases, should both be identified as part of outbreak investigations. Screening of staff, residents, and the environment can assist in understanding patterns of transmission and identify targets for prevention measures, including stepped-up decontamination and antibiotic prophylaxis to clear carriage in staff and residents. Invasive disease outbreaks affecting children are rare, but have been noted, particularly in cases where *S. pyogenes* and chicken pox have co-circulated in nursery or school settings.

Microbiologic surveillance

An important part of epidemiologic surveillance for *S. pyogenes* disease has been the characterisation of bacterial isolates. The pivotal work of Rebecca Lancefield led to the development of the classic serologic typing scheme based on the M protein, with more than 120 M proteins validated to date (Facklam, et al., 1999). Advances in the molecular field saw the emergence of the *emm* typing scheme based on the *emm* gene that encodes M protein. More than 234 *emm* types are recognized, with >1200 distinct allelic forms of the *emm* type-specific regions of *emm* genes, which are known as the *emm* subtypes (Beall, et al., 2000). Virtually all epidemiologic studies define *S. pyogenes* isolates according to their *emm* type, and therefore, the *emm* type provides the primary basis for understanding the epidemiology, biology, and genetic structure of the species. *Emm* typing has served the streptococcal scientific community for several decades and is still relevant to tracking outbreaks and surveillance. However, the differences in *emm* type diversity and disease associations have created challenges when extracting and analyzing data from developed countries to reach conclusions about developing countries. Comparative *emm* typing studies to determine prevalence in different regions and countries is made more complex by the different methods used. Some studies represent single time point surveillance, while others analyze isolates collected over time (Shulman, et al., 2009). Isolates from outbreaks and epidemics are also likely to differ from non-related sporadic isolates within the same location (McMillan, Sanderson-Smith, Smeesters, & Sriprakash, 2013). In both its serologic and nucleotide-based based "formats," *emm* typing has been used extensively to examine both geographic strains distribution and disease association. Large scale studies have been undertaken that have used *emm* sequence typing, particularly in the USA, Canada, and Europe (Lamagni, Efstratiou, Vuopio-Varkila, Jasir, & Schalén, 2005; O'Loughlin, et al., 2007; Luca-Harari, et al., 2009; Imöhl, Reinert, Ocklenburg, & van der Linden, 2010; Friães, Lopes, Melo-Cristino, & Ramirez, 2013; Tamayo, Montes, García-Arenzana, & Pérez-Trallero, 2014).

There are also two other serologic typing schemes that have an important role in the understanding of the molecular epidemiology and genetic organization of the species, based on the serum opacity factor (SOF) and T protein. The original SOF typing scheme was serologic, where SOF type specific antiserum neutralized the enzymatic activity. The *sof* sequence types have been defined by Beall and colleagues (Beall, et al., 2000). Despite the close physical distance between *sof* and *emm* on the chromosome, several *emm* types are found in association with >1 *sof* type, and vice versa, which is indicative of horizontal genetic transfer of *emm* or *sof* to new genetic backgrounds (Efstratiou, 2000; Beall, et al., 2000). The T protein serologic typing scheme is based upon the trypsin-resistant T antigens that have been described as surface pili, which mediate adherence and promote biofilm formation (Manetti, et al., 2007).

A new cluster typing system based on *S. pyogenes emm* types was recently developed that uses the portion of the *emm* genes that encode the entire surface-exposed region of M proteins, for >1000 *emm* genes that correspond to 175 *emm* types. The 175 *emm* types can be grouped into two clades, two sub-clades, and 48 *emm* clusters, 16 of which encompass 82% of the *emm* types. The *emm* clusters represent functionally distinct groups of M proteins, as shown by the characterization of the host binding protein binding of 24 representative *emm* types. For the first time, the classification enabled a model where functionality attributes could potentially be ascribed to proteins from the same *emm* cluster. This novel complementary tool to *emm* typing should add meaningful information and could be widely used for *S. pyogenes* molecular epidemiology. This classification system will be hosted on the website from the streptococcal reference laboratory at the Centers for Disease Control and Prevention (CDC), Atlanta, Georgia (National Center for Immunization and Respiratory Diseases, 2012). The new typing system does not replace *emm* typing, but adds meaningful information to the current, broadly used typing scheme. A recent study that used the "cluster typing system" to assess the disease burden in New Caledonian, Australia, and Fiji identified a common point between the *emm* types present in these countries, whereas very few similarities could be found among the *emm* types, as only a limited number of *emm* clusters were responsible for most of the disease burden. Therefore, this study confirmed the high burden and supported the added value of the *emm*-cluster typing system to analyze the epidemiology and to contribute to global vaccine development efforts by informing vaccine formulation (Baroux, et al., 2014).

Trends in *emm* type prevalence and disease associations

Given the diversity of the structure and function of M protein, it is not surprising that *S. pyogenes* of certain M/*emm* types (and in particular, M1 and M3), are strongly associated with invasive infections. This is true for countries within Europe and the USA, in contrast to other regions of the world where types differ quite significantly.

There have been many large scale *emm*-typing surveillance studies undertaken over the last decade in almost all global regions, and it has become clear that the epidemiology of *S. pyogenes* differs between developing and developed regions of the world. Systematic reviews have documented the global epidemiology of these diseases (Smeesters, Mardulyn, Vergison, Leplae, & Van Melderen, 2008; Steer, et al., 2009a), and have highlighted that in developing countries, the *emm* type not only differs, but is much more diverse in comparison to the *emm* type found in developed countries. This has been observed in India, Fiji, Ethiopia, and Brazil (Abdissa, et al., 2006; Dey, et al., 2005; Smeesters, et al., 2006; Smeesters, Mardulyn, Vergison, Leplae, & Van Melderen, 2008; Smeesters, Dramaix, & Van Melderen, 2010; Steer, et al., 2009a). These studies have also showed that single-type dominance did not exist. The systematic review of the global *emm* type distribution undertaken by Steer and colleagues (Steer, et al., 2009a) revealed distinct differences in the *emm* type distribution and diversity of types between global regions and particularly in the molecular epidemiology in Africa and the Pacific (Figure 3). This could be due to the different clinical presentations prevalent within these regions: for example, there are differing presentations of skin infections, impetigo, RF, and RHD, with large numbers of circulating strains. The review further emphasized the important need for further molecular epidemiologic data from regions where the disease burden is greatest.

Certain *emm* types regularly feature among the prevalent types that cause invasive disease; in particular, types 1, 3, 12, 28, and 89 are seen most frequently (Steer, et al., 2012b; O'Loughlin, et al., 2007). The most common *emm* type recovered in association with *S. pyogenes* invasive disease in developed countries globally is *emm*1. This *emm* type ranks very high in pharyngitis (National Center for Immunization and Respiratory Diseases, 2012; Shulman, et al., 2009; Steer, et al., 2009a). Studies on *emm*1 isolates that spread globally in the 1980s and early 1990s had two prophages that were absent from strains isolated prior to those periods (Cleary, LaPenta, Vessela, Lam, & Cue, 1998). Over the last few years, the evolution of *emm*1 isolates has been intensively studied through genome sequencing and other methods (Aziz & Kotb, 2008; Nasser, et al., 2014).

The prevalence of certain types in invasive or non-invasive infections is a reflection of the circulating types of *S. pyogenes* in the general population at a given time. The diversity of types in developing countries such as Ethiopia, India, Fiji, and Brazil is larger, and the type distributions differ significantly to those reported from Europe or the USA (Abdissa, et al., 2006; Sagar, Kumar, Ganguly, & Chakraborti, 2008; Steer, et al., 2008). Significant differences between genders regarding their infection with particular types have also been documented. For example, in a Pan-European study of type distributions, *emm*28 and *emm*87 were prevalent in females (Luca-Harari, et al., 2009). The role of *emm*28 isolates in puerperal fever is well recognized (Areschoug, Carlsson, Stålhammar-Carlemalm, & Lindahl, 2004; Mihaila-Amrouche, Bouvet, & Loubinoux, 2004). In contrast, *emm*83, *emm*81, and *emm*43 were associated with intravenous drug use and were preferentially found among males (Luca-Harari, et al., 2009).

Molecular epidemiology

In a relatively short period, studies of the molecular epidemiology of *S. pyogenes* have progressed from the study of single genes to population-based genomic comparisons, which significantly highlight the dynamic nature of the organism (McMillan, Sanderson-Smith, Smeesters, & Sriprakash, 2013). Epidemiologic investigations have repeatedly found non-random *S. pyogenes* serotype and disease type associations (Musser & Shelburne, 2009). Thus, molecular pathogenomic approaches have been applied to *S. pyogenes* for over a decade now and studies have revealed new information on molecular epidemiology and pathogenesis, particularly with regards to clone emergence and strain genotype/disease phenotype relationships (Musser & Shelburne, 2009).

It is well known that strains of the same serotype/*emm* type and MLST type can differ extensively in their pathogenomic/virulence gene content. Recent studies on the molecular anatomy of strain genotype with patient phenotype associations at the nucleotide level in M/*emm*3 have clearly identified unique and novel differences by using whole-genome sequence (WGS) approaches (Beres, et al., 2006; Al-Shahib, et al., 2014).

A large-scale analysis of 3,615 genome sequences, combined with virulence studies, was recently published by Nasser and colleagues (Nasser, et al., 2014). The study eloquently delineated the nature and timing of molecular events that contributed to an ongoing "global epidemic" caused by *S. pyogenes* M-type 1. The analysis of population-based sporadic strains from seven countries identified strong patterns of temporal population structure. The study concluded that "the molecular evolutionary events transpiring in just one bacterial cell ultimately produced many millions of human infections worldwide." Results from other well-documented studies (Athey, et al., 2014) that used WGS approaches in epidemiologic situations also highlight the important fact that high quality, well-curated databases are crucial to fully take advantage of the data generated by WGSs.

Conclusions

Studies that expand *emm* typing surveillance to population genomics are providing new insights into not only the epidemiology, but also the pathogenesis and biology of the organism. Recent advances in molecular microbial characterization by whole-genome analysis are opening up tremendous new opportunities for a better understanding of the pathogenicity, evolution, and spread of *S. pyogenes* and the epidemiology of the diseases they cause. WGS holds the promise of improving the resolution and predictive value of typing, as applied to

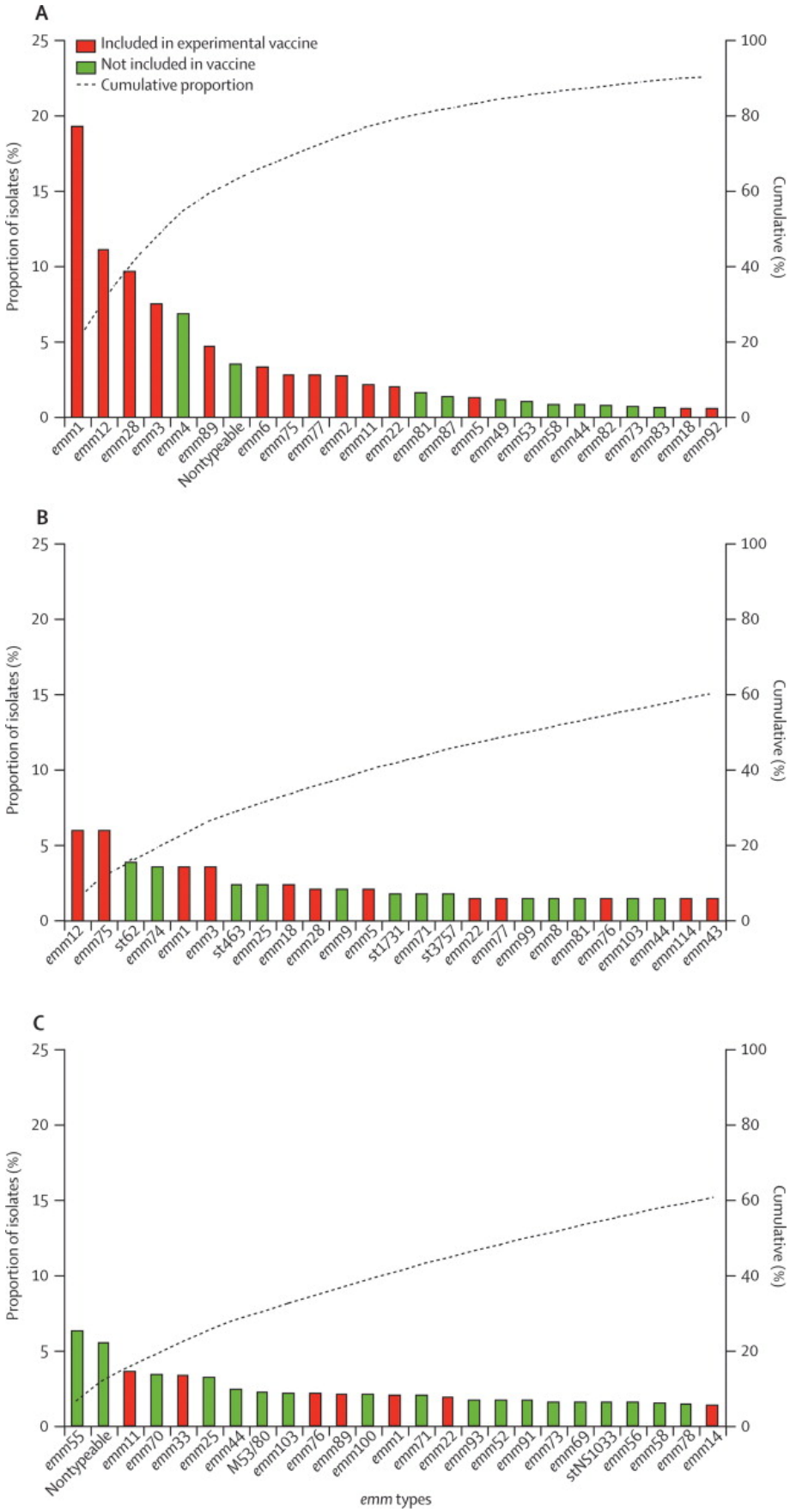

Figure 3: The 25 most common *emm* types as proportions of all isolates in high-income countries (A), Africa (B), and the Pacific region (C) In Africa, *emm* 112 and in the Pacific region *emm* 74 were equal to 25th, but are not included (Steer, Law, Matatolu, Beall, & Carapetis, 2009b)

disease surveillance and outbreak investigations. However, there is a need to provide backward compatibility with currently used typing schemes that are well validated, to facilitate comparison and enhanced understanding of epidemiologic trends, in addition to gathering further epidemiologic data from regions where the burden of *S. pyogenes* disease is greatest.

The resurgence of *S. pyogenes* as a cause of serious human infections in the USA, Europe, and elsewhere has been thoroughly documented over the last few decades and has heightened public awareness about this organism, but only in recent years. An overall resurgence of disease, coupled with the lack of a licensed *S. pyogenes* vaccine and ongoing concern about the acquisition of penicillin resistance, remains a major concern and highlights the importance of strengthening global surveillance of this pathogen.

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Animal Models of *Streptococcus pyogenes* Infection

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Introduction

An established principle of modern research into bacterial pathogenesis is that no *in vitro*, *in situ*, or *in silico* model can accurately account for the myriad host defense mechanisms and host tissue complexity that a pathogen will encounter in a live animal. This fact was appreciated over 100 years ago by Robert Koch and the early pioneers of pathogenesis research, who recognized that an animal model of infection that mimicked a human disease was a crucial component in establishing a microbial etiology for any given disease (Kaufmann & Schaible, 2005). The postulates described by Koch continue to the present day to be the "gold standard" by which etiology is established (Kaufmann & Schaible, 2005). In the spirit of Koch, the early pioneers of the molecular era of pathogenesis research (most notably Stanley Falkow) have expanded on the principles elaborated by Koch to use animal models to develop our modern understanding of the molecular basis of microbial pathogenesis, in order to establish a functional link between any given gene product of a pathogen and its capacity to cause disease (Falkow, 1988; Falkow, 2004) (Table 1). Thus, the availability of animal models that can faithfully reproduce a human disease continues to be a foundational component of modern microbial pathogenesis research.

For research into the pathogenesis of diseases caused by *Streptococcus pyogenes*, the groundbreaking work of the labs of Joseph Ferretti, Patrick Cleary and June Scott (Ji, McLandsborough, Kondagunta, & Cleary, 1996; Perez-Casal, Price, Maguin, & Scott, 1993; Simon & Ferretti, 1991) established a methodology for the manipulation of the *S. pyogenes* genome, and opened the door for the analysis of pathogenesis that followed the principles elaborated by Falkow. This work spurred the development of new *in vivo* models that could be used to investigate the role of specific virulence factors in *S. pyogenes* pathogenesis. However, for *S. pyogenes*, the development of *in vivo* models has proven to be challenging for a number of reasons: First, *S. pyogenes* is a strictly human pathogen and is exquisitely adapted to its human host to the extent that many of its important virulence factors (for example, its several secreted superantigens and its plasminogen activator streptokinase (Kasper, et al., 2014; Sun, et al., 2004; Reglinski & Sriskandan, 2014)) only have activity against humans cells and proteins. The second issue reflects *S. pyogenes'* remarkable versatility as a pathogen, as it is capable of causing diseases that result from very different pathogenic mechanisms. Most of these fall into one of three broad classes (Reglinski & Sriskandan, 2014; Cunningham, 2000; Ralph & Carapetis, 2013; Wong & Stevens, 2013; Cunningham, 2012): first, local, lesional diseases in soft tissue characterized by inflammation, which can result in considerable damage to tissue in more severe manifestations; second, both local and systemic diseases that arise from damage caused by secreted streptococcal toxins; and third, immune dysfunction that results from an inappropriate immune response to streptococcal antigens. The third challenge to model development arises from the range of different tissue compartments that *S. pyogenes* can damage, which ranges from skin and soft tissue to internal organs like the heart and kidneys and to any number of different sites in the skin and other soft tissues. A final major challenge to model development is the population of *S. pyogenes* itself, which has proven to have extensive strain diversity despite its restriction to a human habitat (Bessen, 2009). This means that there is

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no single strain of *S. pyogenes* that can be considered representative of the population as a whole and also, that relatively few strains have been shown to be virulent in any given animal model.

Despite these challenges, the prior 15 years has seen the development of an impressive number of *in vivo* models in a diversity of animal species, ranging from invertebrates to primates, that have proven useful in the dissection of *S. pyogenes* gene/pathogenesis relationships (Figure 1). In considering these models, it is important to note that there is no single comprehensive model of *S. pyogenes* infection. In fact, there is no single model that can accurately reproduce the authentic pathogenesis of any specific *S. pyogenes* disease. Instead, various models have been developed to model different aspects of various pathogenic mechanisms, and as a result, a thorough understanding of any particular model's strengths and weaknesses is an important consideration for experimental design, for interpretation of results as they apply to understanding pathogenesis in that model system, and for extrapolation to the mechanism by which any *S. pyogenes* gene may contribute to human disease. In the following sections, we will review the salient features of the animal models that have proven particularly useful in modern analyses of *S. pyogenes* pathogenesis, including their utility, strengths, and limitations, as well as some examples of the types of strains and mutants whose pathogenic mechanism a given model has been shown to resolve. For purposes of organization, the various models will be grouped together with the host animal upon which they are based.

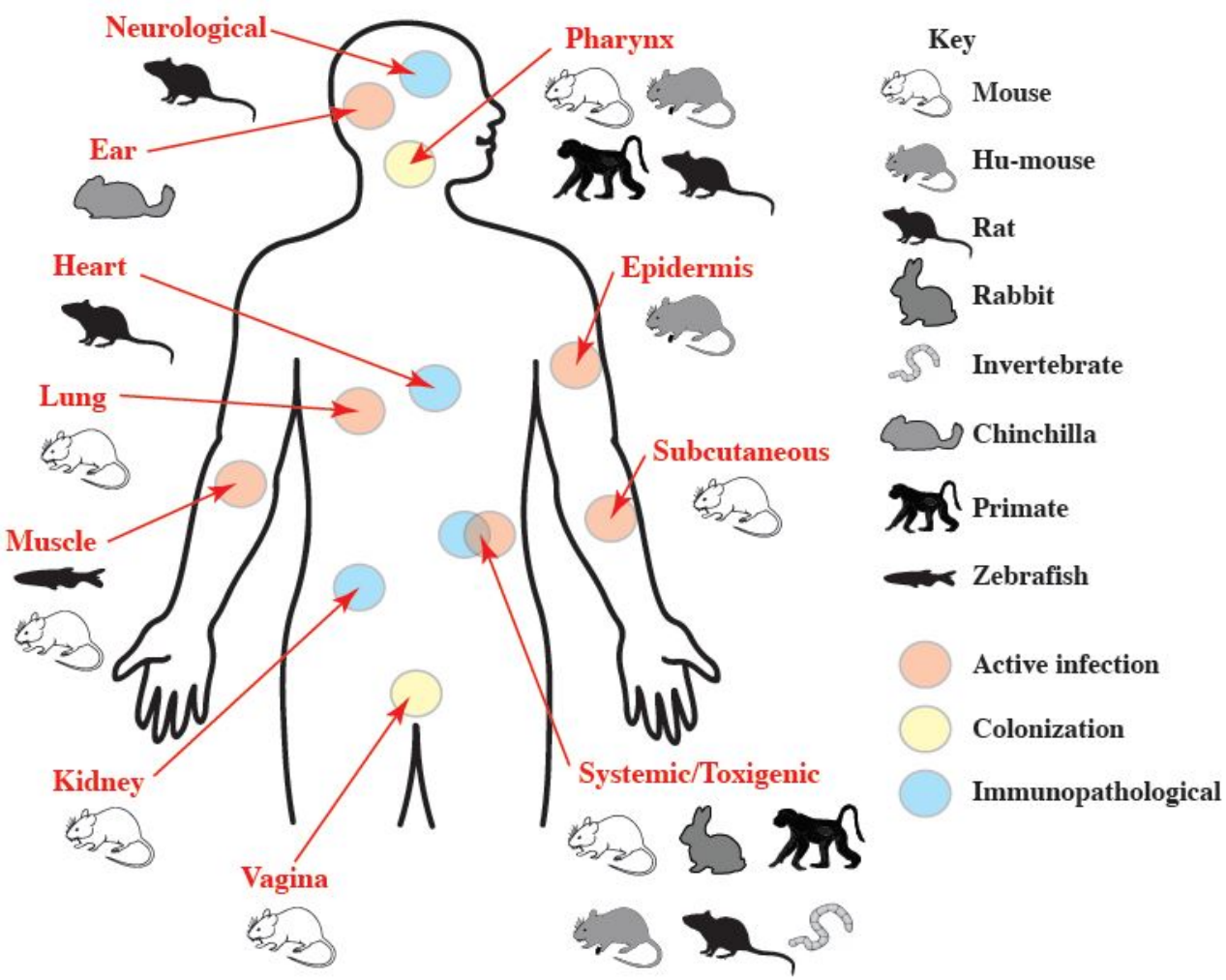

Figure 1. Animal models for analysis of *S. pyogenes* pathogenesis. This figure shows the animal models that have been developed for various human *S. pyogenes* diseases. The models are presented based on the animal species used and the human tissue that is infected, damaged, or colonized in the corresponding human disease. The icons that represent each animal species are defined in the key and the colors show the type of infection that is modeled. The strengths and limitations of each model for analysis of their corresponding human disease are discussed in the text.

Table 1. Molecular Koch's postulates

The phenotype or property under investigation should be associated with pathogenic members of a genus or pathogenic strains of a species.
The gene(s) associated with the supposed virulence trait should be isolated by molecular methods. Specific inactivation or deletion of the gene(s) should lead to a measurable loss in pathogenicity or virulence.
Restoration of pathogenicity should accompany the reintroduction of the wild-type gene(s).

As proposed by Stanley Falkow (Falkow, 1988). Adapted from (Falkow, 2004).

Murine Models

Subcutaneous ulcer/air sac model

One of the most widely used animal models for the analysis of *S. pyogenes* virulence factors is the murine subcutaneous ulcer model and its air sac derivative. This model is particularly attractive because it reproduces

many of the core features that are held in common by a broad range of *S. pyogenes* diseases. These include that the infection results in a highly inflammatory localized lesion in a soft tissue, that there is a robust level of bacterial proliferation, and that the lesion elicits the extensive recruitment of inflammatory cells. Introduced in its modern form by the group of Neil Barg (Bunce, Wheeler, Reed, Musser, & Barg, 1992), it originally involved injecting a mixture of bacteria and cytodex beads into the subcutaneous tissue of a mouse flank. However it was subsequently found that the beads were not necessary to produce an infection (Schrager, Rheinwald, & Wessels, 1996). Typically, 10^6–10^8 CFU of the *S. pyogenes* strain of interest is injected into the tissue, which results in a well-defined area of induration that is apparent by 8–12 hours post-infection. The lesion that forms is characterized by the recruitment of a large number of inflammatory cells, of which the majority are neutrophils that form a loose abscess-like structure that surrounds the area of bacterial growth (Figure 2). Macrophages and the cytokines IL-12, Interferon-γ and TNF-α appear to be important for the formation of this structure, which limits dissemination of bacteria from the site of the lesion (Mishalian, et al., 2011; Raeder, Barker-Merrill, Lester, Boyle, & Metzger, 2000). By 18–24, hours the region of induration ulcerates and develops an eschar, followed by the gradual expansion of the margins of the ulcer to reach a maximum at about Day 3 (Figure 2). At approximately Day 8, the lesion begins to resolve, and by Day 14, it is typically healed. Many different *S. pyogenes* strains have been shown to be virulent in this model, and these fall into two general classes: those that remain localized to the lesion for the duration of the infection (such as those found in (Brenot, King, Janowiak, Griffith, & Caparon, 2004)), and those that have a propensity to disseminate into the vasculature to cause systemic disease after a period of several days (such as those found in (Limbago, Penumali, Weinrick, & Scott, 2000)). Interestingly, mutations have been described, most notably in the CsrR/S (CovR/S) two-component transcription regulator, which can convert a primarily resident strain into a highly invasive strain (Engleberg, Heath, Miller, Rivera, & DiRita, 2001). Many strains of mice are sensitive to this infection, including strains like the BALB/c and C57BL6 strains, as well as their various transgenic and knock-out variants that are commonly used in immunological analyses. However, a commonly used mouse strain is the outbred SKH1 mouse that has a defect in the *Hr* gene located on chromosome 14 that renders it hairless (Benavides, Oberyszyn, VanBuskirk, Reeve, & Kusewitt, 2009), which eliminates the requirement for depilation for injection and observation of the lesions. This model also offers a large number of different infection parameters to assess virulence. A commonly used approach is to quantitate the surface area of the resulting ulceration and how it changes over time. Typically, a conclusion regarding virulence between the wild-type *S. pyogenes* strain and its mutant derivatives is made by a comparison of the lesion area at the time point when the wild-type strain's lesion reaches a maximum area (usually by Day 3). Since the resulting ulcer will have an irregular border, its area is most accurately measured by the use of digital photography and image processing software (Brenot, King, Janowiak, Griffith, & Caparon, 2004). Other parameters that can be assessed include change in weight over time, numbers of colony forming units (CFU) that are recoverable from the lesion, the number of mice that do or do not develop a ulcer following infection, the time to maximal lesion area, and the time to healing of the ulcer. For *S. pyogenes* strains that disseminate from the lesion, additional parameters can be measured, which typically include CFU recovered from the spleen and inguinal lymph nodes and lethality. This model has also proven its utility for the monitoring of the behavior of the *S. pyogenes* transcriptome during growth in a soft-tissue environment. The fact that the bacteria remain confined to a necrotic lesion that is not conducive for maintaining the integrity of host RNAs has allowed the efficient recovery of *S. pyogenes* RNA directly from the infected tissue for analyses of gene expression (Brenot, Weston, & Caparon, 2007; Loughman & Caparon, 2006; Kietzman & Caparon, 2011).

The subcutaneous air sac model is a derivative of the subcutaneous ulcer model. The same subcutaneous tissue compartment is infected in both models. A distinction for the air sac model is that prior to infection, an air pouch is created under the skin by the injection of air into which the bacteria are subsequently injected (Raeder & Boyle, 1993). This method's principal advantage is that it facilitates the recovery of host inflammatory cells, which migrate into the sac lumen and can be recovered by lavage for analyses *in vitro* by FACS and other methods that require a suspension of single cells.

Like any of the animal models for analysis of *S. pyogenes* pathogenesis, the subcutaneous ulcer model has a number of important strengths and limitations that must be taken into account when designing experiments to provide insight into the role of a specific *S. pyogenes* gene product in virulence. As discussed, the model reproduces features of a highly inflammatory infection caused by *S. pyogenes* growing extracellularly in the types of cutaneous tissue that *S. pyogenes* infects in humans. On the other hand, the histopathology of the infection does not closely reproduce that observed for human cutaneous diseases, including impetigo, pyoderma, erysipelas, cellulitis, or necrotizing fasciitis. These observations can be associated with differences in the anatomy of the murine cutaneous tissues as compared to humans, as the mouse has a much higher density of flank skin hair follicles and a much thinner epidermal layer. Also, there is a considerable variation among the virulence of *S. pyogenes* strains in this model, and the virulence of any particular strains in humans is not predictive of its virulence in this model. Certain virulence factors may play a more important role in the mouse rather than the human, since mice lack the human-specific targets of factors, like streptokinase and the superantigens. In association, mutations of some *S. pyogenes* virulence factors do not always result in the same degree of attenuation in different strains. A classic example is the case of the SpeB cysteine protease, whose loss of function in different strains has been associated with significant attenuation or no attenuation at all (Ashbaugh, Warren, Carey, & Wessels, 1998; Lukomski, et al., 1999). This has suggested that different *S. pyogenes* strains may have unique virulence profiles that are comprised of overlapping, redundant and/or combinatorial sets of virulence genes (Saouda, Wu, Conran, & Boyle, 2001; Sierig, Cywes, Wessels, & Ashbaugh, 2003). In examining the immune system, the subcutaneous model has proven valuable for analysis of the role of innate immunity in the host's response to *S. pyogenes* and how specific virulence factors modulate innate immunity. It is less clear that this model will be useful for analyses of the adaptive arm of the immune response, since a protective immune response apparently does not develop after the resolution phase of the infection (Bisno & Gaviria, 1997). However, the limitations of this model are counter-balanced by a number of important strengths. These include its simplicity, its relatively high-throughput and low cost, and the ability to apply the power of mouse genetics to test the role of various host factors-virulence factor relationships (for one example, see (Cusumano, Watson, & Caparon, 2014)). In addition, because the bacteria grow in a defined lesion, it reproduces the dynamic host environment that is remodeled both by host immunity and by microbial metabolism, which consumes host-derived substrates and accumulates metabolic end-products, like organic acids (Loughman & Caparon, 2006). Alterations to the transcriptome that accompanies adaptation to this dynamic environment are closely linked to changes in virulence factor expression (Kreikemeyer, McIver, & Podbielski, 2003). These many advantages have made the subcutaneous ulcer model one of the most versatile and useful models for the analysis of *S. pyogenes* pathogenesis.

Impetigo model

The histological differences between the human and murine epidermal tissues noted above have made the mouse unsuitable for modeling impetigo and other *S. pyogenes* infections of superficial cutaneous tissues. Bessen and colleagues have developed an innovative solution to this problem (Scaramuzzino, McNiff, & Bessen, 2000). Their approach was to create a humanized mouse (hu-mouse) by engrafting human epidermal tissue from neonatal foreskin onto the flanks of the SCID mouse. The absence of an adaptive immune response in this mouse line prevents the rejection of the tissue grafts. Once the grafts are established, they are superficially damaged by cross-wise cuts with a scalpel blade, bacteria are topically applied, and are then occluded with a bandage. Both damage and occlusion were required for establishing the infection, similar to what has been observed in experimental infections conducted in humans (Leyden, Stewart, & Kligman, 1980). This results in an impetigo-like lesion that is characterized by erosion of the stratum corneum, with infiltration of murine polymorphonuclear leukocytes (PMNs) that eventually contribute to the formation of pus, which is surrounded by clumps of proliferating streptococci. Virulence is measured by a semi-quantitative visual assessment of histopathology or by the determination of colony forming units to monitor bacterial growth. The degree of damage generally correlates with the magnitude of streptococcal growth (Scaramuzzino, McNiff, & Bessen,

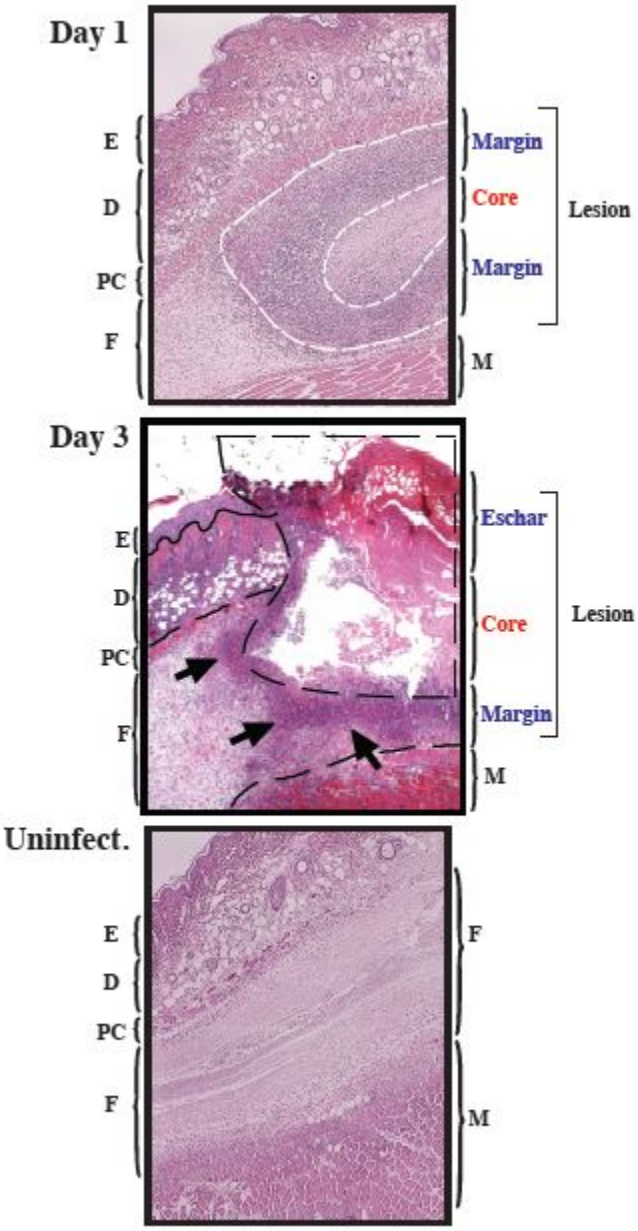

Figure 2. Murine subcutaneous ulcer model. This figure shows the histopathology of infection by *S. pyogenes* HSC5 following the subcutaneous inoculation of 1 x 10^6 CFUs into the flank skin of SKH1 mice. Shown are hematoxylin & eosin (H&E)-stained sections of infected tissues in comparison to uninfected (uninfect.) tissue. By Day 1 post inoculation, a well-defined subcutaneous lesion forms that consists of a necrotic Core that contains replicating streptococci, along with debris from destroyed host cells, surrounded by a Margin that consists of a dense concentration of inflammatory cells, predominantly PMNs, recruited from the vasculature. By Day 3, the lesion has reached its maximal size and the upper margin has eroded through the superficial tissue layers to form an open ulcer with formation of an eschar. Replicating streptococci continue to be confined to the lesion Core by the inflammatory cells in the Margin (shown by arrows). For many strains, including HSC5, the lesion will heal over the course of the next 10 days and the streptococci will be cleared from the tissue (Brenot, King, Janowiak, Griffith, & Caparon, 2004). For other strains and certain mutants (most notably in the CsrR/S [CovR/S] two-component transcription regulator) the infection may progress to systemic disease. Abbreviations: E, epidermis; D, dermis; PC, panniculus carnosus; F, fascia; M, muscle. Magnification, 40X.

2000). Other characteristics that reproduce elements of the human disease include the finding that *S. pyogenes* strains of lineages that are most likely to be associated with impetigo tended to cause more severe tissue damage than those lineages that are more likely to cause pharyngitis (Scaramuzzino, McNiff, & Bessen, 2000), and that

virulence requires known *S. pyogenes* virulence factors, which include the virulence gene transcription regulator Mga (Luo, Lizano, Banik, Zhang, & Bessen, 2008).

Despite these strengths, the hu-mouse model does have some important limitations. It is technically complex and requires both a source of human tissue and surgical skills for reproducible production of the hu-mice themselves. The effort required to generate the hu-mice limits the throughput of the assay, as there is a limited quantity of the animals themselves. Also, although human tissue is infected, the model still lacks the human-specific targets of several important virulence factors, including streptokinase and superantigens, which are the serum protein plasmin and HLA on bone marrow-derived T cells, respectively. The use of the immunocompromised SCID mouse means that the infection proceeds in the absence of adaptive immunity. However, because the model can faithfully reproduce many features of human impetigo, the hu-mouse model is one of the most useful and accurate models available for analysis of *S. pyogenes* infection of the superficial skin.

Models of systemic disease

The common element of the various models of systemic disease is that the major mechanism of damage to the host arises from the presence of streptococci actively multiplying in the bloodstream. With the exception of puerperal sepsis (childbed fever), which is now a rare disease, sepsis is a relatively rare manifestation of infection by *S. pyogenes*, and often occurs after a localized infection in soft tissue (Reglinski & Sriskandan, 2014; Wong & Stevens, 2013; Rantala, Vuopio-Varkila, Vuento, Huhtala, & Syrjänen, 2009). The murine models of systemic infection do have utility for modeling human disease, since growth in human blood *in vitro* is the classic assay for assessment of *S. pyogenes* virulence, for testing its ability to evade phagocytosis, and for testing the protective efficacy of antibodies (Lancefield, 1962). In the mouse, the different types of systemic disease models are distinguished from each other by their route of inoculation, which may occur intravenously (IV), intraperitoneally (IP), intranasally (IN), or intratracheally (IT). The IN route has also been adapted for analysis of oropharyngeal colonization (see below). The IV route introduces the bacteria directly into the bloodstream, while the IN and IT routes require that the streptococci invade across a tissue barrier to reach the vasculature. Each of these routes involves a direct injection or instillation of the bacterial inoculum and is technically simple to perform. It has been proposed that the IN and IT routes may yield more useful information, as they bypass the least number of the steps of infection since they require the bacteria to invade across the lung epithelium in order to reach the bloodstream (Roberts, Scott, Husmann, & Zurawski, 2006). However, to improve upon the upper airway model, Husmann et al. infected mice via IT inoculation to bypass the initial site of upper airway colonization and found that infected mice more quickly developed signs of illness within a few days following infection, as compared to IN infection (Husmann, Dillehay, Jennings, & Scott, 1996). Furthermore, examination of these IT-infected mice found a higher frequency of acute, suppurative bronchopneumonia with two-thirds of these mice developing bacteremia (Husmann, Dillehay, Jennings, & Scott, 1996). Therefore, IT inoculation may be a more efficient mechanism to investigate the role of streptococcal virulence factors in inducing significant airway and systemic disease (Roberts, Scott, Husmann, & Zurawski, 2006; Husmann, Dillehay, Jennings, & Scott, 1996; Husmann, Yung, Hollingshead, & Scott, 1997). Virulence of strains is typically determined by monitoring lethality, by using a single concentration of bacteria to infect a group of mice. Differences in virulence can be tested for significance by the Kaplan-Meier survival probability estimate model (Brenot, King, Janowiak, Griffith, & Caparon, 2004). A typical dose for a strain that is virulent in these assays is approx. 10^5-10^6 CFU and numerous strains have been found to be virulent, although it has long been recognized that there is no correlation between the virulence of strains in humans and in systemic models (Lancefield, 1962). This likely indicates that the mouse and human have a differential sensitivity to certain *S. pyogenes* virulence factors. For those strains that are less virulent, useful information can often be acquired by examination of the kinetics of clearance from the vasculature by enumeration of CFUs recovered from highly perfused organs like the spleen, liver, and lungs. The choice of mouse strain and gender are important considerations, as mice with different genetic backgrounds can have significant differences in sensitivity to systemic infection, with male mice being generally more susceptible (Medina, Goldmann, Rohde, Lengeling, & Chhatwal, 2001). The genes responsible for

these differences are apparently located outside of the MHC locus (Goldmann, et al., 2005), and an absence of adaptive immunity does not alter the course of infection (Medina, Goldmann, Rohde, Lengeling, & Chhatwal, 2001). In addition to existing strains, transgenic technologies have been employed to expand the types of strains that can be useful for the analysis of pathogenesis in systemic models. The most interesting application of this approach has been to overcome the limitation noted above that results from certain virulence factors of *S. pyogenes* having evolved to recognize exclusively human targets. In the case of streptokinase, a transgenic hu-mouse has been developed that expresses human plasminogen (Sun, et al., 2004). Analysis of these mice conclusively establishes streptokinase as virulence factor for systemic infection, as these mice have a markedly increased susceptibility to lethal infection, which is dependent on the expression of human streptokinase (Sun, et al., 2004).

Implanted chamber models

In the models discussed so far, pathology results from streptococci damaging the tissue that are directly associated with or by dissemination through the vasculature. However, many *S. pyogenes* diseases are caused by toxins or other bacterial products that are released by streptococci growing in a localized lesion that then traffic to tissues and organs at a distant site to cause damage (Reglinski & Sriskandan, 2014; Cunningham, 2000; Ralph & Carapetis, 2013; Wong & Stevens, 2013). In some cases, these diseases can be modeled in mice by using an implanted chamber model. The approach is to surgically-implant a "cage" made of steel or Teflon into the subcutaneous tissues on the mouse flank. The incision is allowed to heal over the course of several weeks to allow a fibrous capsule to form around the implant. The infection is initiated by injection of the bacteria through the skin and into the inside of the cage, which allows the influx of nutrients and the release of streptococcal products, but the retention of streptococcal cells. Implanted chambers have been used to produce a glomerulonephritis-like disease that is characterized by deposition of streptokinase and host complement components in the glomerular basement membrane (Nordstrand, Norgren, Ferretti, & Holm, 1998) and to monitor toxin production in an *in vivo* environment (Kazmi, et al., 2001). A similar approach using rabbits has been employed for modeling streptococcal toxic shock syndrome (Schlievert, 2007).

Footpad model

Most animal models of infection quantitatively assess events that are associated with the very late or terminal stages of disease, including bacterial proliferation, damage to tissue and lethality. However, many virulence factors act during the very early stages of infection to help establish a foothold for the bacteria in the infected tissue. Since there are relatively few bacteria present at this stage, and often no gross alterations to tissue, it can be a challenge to observe the streptococcal cells or to measure any parameter of early infection to assess the contribution of specific virulence factors. Recent advances in imaging have begun to open a window into this critically important aspect of *S. pyogenes* pathogenesis. One approach is to use the power of two-photon microscopy, a technology with the resolution to image streptococcal bacteria in live un-fixed tissue at a depth of several millimeters, to examine bacteria over the first hour following their introduction into tissue. In one application, fluorescent dyes were used to label bacterial cells and blood vessels in combination with LysM-eGFP mice, whose macrophages and neutrophils express eGFP. Two-photon microscopy was used to examine the behavior of neutrophils immediately following the injection of wild-type and mutant bacteria into the mouse footpad (Lin, Loughman, Zinselmeyer, Miller, & Caparon, 2009). In addition to positional information both inside and outside of vessels, imaging in real-time allows for the quantitation of neutrophil velocity and meandering for assessment of chemotactic behavior. The application of this method revealed a previously unrecognized role for streptolysin S in altering neutrophil extravasation (Lin, Loughman, Zinselmeyer, Miller, & Caparon, 2009). While this is a promising technique, its general adoption has been slowed by its requirement for expensive and highly specialized equipment.

Oropharyngeal colonization models

A major reservoir for *S. pyogenes* in humans is the posterior oropharynx, especially the mucosal-associated lymphoid tissue known as Waldeyer's ring, which includes the tonsils. This tissue is the site of one of the most common manifestations of *S. pyogenes* infection (pharyngitis), and as a result, there has been considerable interest in developing animal models to attempt to replicate and study this disease process (Cunningham, 2000). However, rodents lack a Waldeyer's ring homologue in the pharynx, and experimental colonization of the rodent oropharynx has been both challenging and limited primarily to only a few select *S. pyogenes* strains. Early experiments by Rebecca Lancefield's group (Hook, Wagner, & Lancefield, 1960) took advantage of a mouse-pathogenic strain of *S. pyogenes*, the M type 50 strain B514/33 that was originally isolated from an epizootic infection of a mouse colony. More recent experiments using this lineage have used strain B514-Sm, a spontaneous streptomycin-resistant derivative of the original strain, whose high level of antibiotic resistance allows direct selection for the determination of recovered CFUs. Strain B514-Sm seems exceptionally capable of colonizing the murine oropharynx, and approximately 60% of inoculated mice remain colonized for over a month. Mice are infected by IN inoculation with 10^7 CFU in a 10 µl normal saline drop that is placed into one nostril and inhaled, which distributes the inoculum throughout the upper airway. Colonization is monitored by throat swabs that are resuspended and diluted in saline and plated onto selective media (THY agar with 1000 µg/ml streptomycin) (Roberts, Scott, Husmann, & Zurawski, 2006). As discussed above, with IN inoculation some mice will develop bronchopneumonia and die; however, the dose-response ratio is non-linear, given the random distribution of the inoculum in the airway. More recently, the group of Alam et al. refined delivery by the IN route to expand the number of strains that can be analyzed in the model, and found that the volume of the inoculum is a critical variable. They found that IN inoculation of a serotype M18 strain in a volume of 2.5 µl to each nare of a mouse resulted in consistent nasopharyngeal deposition without lung involvement or significant nasal clearance (Alam, Turner, Smith, Wiles, & Sriskandan, 2013). In addition, they used bioluminescence imaging of a strain that was engineered to express luciferase as a non-invasive method for monitoring the progress of infection (Alam, Turner, Smith, Wiles, & Sriskandan, 2013). The strengths of these models include the ability to colonize the murine upper airway and oropharynx, which is a site that is highly relevant to human streptococcal disease. This advantage has been used to show that mutation of the CovR/S virulence regulator in a serotype M18 strain attenuates infection of the oropharynx, in contrast to the hypervirulence of mutants in subcutaneous models (Alam, Turner, Smith, Wiles, & Sriskandan, 2013). Some disadvantages of these models include a limited number of *S. pyogenes* strains capable of colonizing the mouse nasopharynx, that strain B514 has an atypical pattern of virulence gene expression (Yung & Hollingshead, 1996), and the significant differences between human and murine nasopharyngeal lymphoid tissue architecture. However, despite these differences, the murine oropharyngeal model has proven to be useful for analysis of both passive and active mucosal immunization using various streptococcal antigens. For example, passive administration of lipoteichoic acid (LTA) or anti-M protein secretory IgA can significantly reduce colonization in the model (Bessen & Fischetti, 1988b; Dale, Baird, Courtney, Hasty, & Bronze, 1994). Active immunization of the nasal mucosa with several surface proteins, including the hemoprotein receptor Shr (Huang, Fisher, Nasrawi, & Eichenbaum, 2011), the fibronectin-binding protein SfbI (Guzmán, Talay, Molinari, Medina, & Chhatwal, 1999), and various M protein-based vaccines, including peptides conjugated to cholera toxin b subunit or diphtheria toxoid (Bessen & Fischetti, 1988a; Olive, et al., 2006; Olive, Clair, Yarwood, & Good, 2002), have proven to be efficacious in reducing mucosal colonization after subsequent IN challenges of vaccinated mice.

Nasopharyngeal-associated lymphatic tissue colonization model

Despite a lack of Waldeyer's ring tissue in the pharynx, mice and other rodents have a pair of lymphoid lobes located along the lateral nasopharyngeal wall, termed nasal-associated lymphoid tissue (NALT), which function analogously to human tonsils (Asanuma, et al., 1997; Koornstra, de Jong, Vlek, Marres, & van Breda Vriesman, 1991). To investigate whether NALT may be a more appropriate model of *S. pyogenes* airway colonization, Pat

Cleary's group infected 8- to 11-week-old BALB/c mice IN with 5 x 10^8 CFU of *S. pyogenes* clinical isolate strain 591 (serotype M49), strain 90-226 (serotype M1), or derivatives (Park, Francis, Yu, & Cleary, 2003; Park, et al., 2004). Their group determined NALT to be a significant site of *S. pyogenes* colonization following intranasal inoculation, and by using strain 591, was able to recover viable CFUs, both intra- and extra-cellularly, for about 8 days post-inoculation (Park, Francis, Yu, & Cleary, 2003). They also introduced bioluminescent imaging as a non-invasive method for the assessment of colonization, using a *S. pyogenes* strain that was modified to express luciferase (Park, Francis, Yu, & Cleary, 2003). An obvious advantage of this model is the ability to directly examine adaptive immune responses following *S. pyogenes* colonization of the NALT tissue, which has proven useful for studies that have investigated potential vaccine candidates. The Cleary group has shown that following intranasal inoculation with *S. pyogenes*, NALT develops a rapid and prolonged activation of $CD4^+$ T cells of the IL-17-producing Th17 subclass, and that antigens of *S. pyogenes*, including sortase A, are capable of inducing an antibody-independent, neutrophil-based immune response that promotes the clearance of NALT colonization (Park, et al., 2004; Wang, et al., 2010; Dileepan, et al., 2011; Fan, et al., 2014). Some disadvantages of this model include a short duration of colonization, a limited number of *S. pyogenes* strains with demonstrated activity in the model, a higher degree of technical difficulty in isolating NALT tissue for experimental analysis, and a requirement for specialized equipment when using bioluminescent imaging.

Vaginal colonization model

Given the limitations of the oropharyngeal and NALT models for carriage, with their limited number of *S. pyogenes* strains capable of causing infection and relatively short duration of infection, a model capable of sustaining a longer period of colonization is required. As a result, the estradiol-primed mouse vaginal colonization model was developed in C57BL/6 and BALB/c mice and was based on previously developed vaginal colonization models for group B streptococcus, *Neisseria gonorrhoeae*, and *Candida albicans* (Watson, Nielsen, Hultgren, & Caparon, 2013; Sheen, et al., 2011; Jerse, 1999; Fidel, Lynch, & Sobel, 1993). While not as prevalent in human female genitourinary disease as some of the other previously mentioned pathogens, *S. pyogenes* is nonetheless a significant cause of bacterial vulvovaginitis and puerperal sepsis (or "childbed fever"), and asymptomatic vaginal carriage has been linked to nosocomial transmission from healthcare workers to patients, causing wound infections (Stamm, Feeley, & Facklam, 1978; Berkelman, et al., 1982; Stricker, Navratil, & Sennhauser, 2003; Anteby, Yagel, Hanoch, Shapiro, & Moses, 1999). In each of the murine vaginal models, pathogen colonization is enhanced and carriage is extended by one or more administrations of up to 0.5 mg ethinyl estradiol dissolved in sterile sesame oil given 24 to 48 hours prior to vaginal inoculation of ~1 x 10^6 CFU *S. pyogenes* in 20 µl of PBS. Estradiol supplementation syncs all of the mice into the estrous phase of the estrous cycle, a period associated with epithelial cell proliferation and minimal inflammatory cell infiltrate; this phase seems to be when mice are the most receptive to colonization with bacterial or fungal pathogens in the vaginal tract, which is likely due to a combination of a hospitable epithelial cell bed and estradiol-induced anti-inflammatory properties. The estrous phase can be monitored by microscopic examination of vaginal lavages with sterile PBS stained with a modified Wright-Giemsa stain and differential counting of epithelial cells and leukocytes (Watson, Nielsen, Hultgren, & Caparon, 2013; Marcondes, Bianchi, & Tanno, 2002). Vaginal lavages can be serially diluted and plated onto selective media to which the *S. pyogenes* strain is resistant (such as Todd Hewitt yeast extract (THY) agar, supplemented with 1000 µg/ml streptomycin); this allows for the determination of streptococcal colony counts over time (Figure 3). With *S. pyogenes*, many strains from a diversity of backgrounds will continue to colonize the murine vagina over a period of several weeks to months, with strains exhibiting variability in the overall rate of clearance from the vaginal mucosa (Watson, Nielsen, Hultgren, & Caparon, 2013) (Figure 3). The significant duration of mucosal carriage this model permits allows for the investigation of the role of streptococcal virulence factors and regulatory mechanisms important for chronic mucosal persistence. The model previously demonstrated attenuated vaginal mucosal carriage of strains that lacked the carbon catabolite repressor, CcpA, and the arginine deiminase (Arc) operon, which suggests these two important streptococcal factors are involved in promoting mucosal survival (Cusumano, Watson, &

Caparon, 2014; Watson, Nielsen, Hultgren, & Caparon, 2013). A major strength of the vaginal model is that carriage is asymptomatic; mice do not exhibit signs of distress or pain and do not lose weight or fur ruffles, among others (Watson, Nielsen, Hultgren, & Caparon, 2013). Furthermore, the ability to sample vaginal lavages for CFU counts, cytokine levels by ELISA, and other inflammatory cell markers implies that fewer mice are required to complete the experiment, given that mice can be sampled serially over time without requiring sacrifice. Disadvantages of this model include the need for a *S. pyogenes* strain carrying a selectable marker (namely, a spontaneous mutation in *rpsL* that produces streptomycin resistance) to eliminate normal vaginal flora, and the requirement for mice to have received prior estrogenization, which may interfere with certain immune responses. Overall, the murine vaginal carriage model should permit investigation of factors that are critical to influencing host-pathogen interactions and persistence at the mucosal surface, a key immunologic barrier to disease.

Rat Models

Oropharyngeal colonization

A variant of the murine models described above has been developed using rats to expand the number of streptococcal strains accessible to investigation. In this model, 8- to 12-week-old Fischer CDF344 rats were treated with streptomycin (1000 μg/mL) in their drinking water to disrupt their normal flora and then inoculated either IN or orally for pharyngeal colonization (Hollingshead, Simecka, & Michalek, 1993). IN infection utilizes 20 μl inocula, while oral inoculations are 50 μl, with both containing ~10^8 CFU. The Fischer rat was found to support colonization with additional strains of *S. pyogenes*, as compared to the mouse oropharynx model. For example, Hollingshead et al. colonized Fischer rats with the M6 serotype strain JRS4, isogenic M-negative mutant derivatives of JRS4, and an additional unrelated M6 serotype strain S43-29R; in these experiments, strains that expressed M protein exhibited significantly longer oropharyngeal carriage than isogenic M-negative strains (Hollingshead, Simecka, & Michalek, 1993). The duration of colonization in the rat model was several weeks for some individual rats, although the numbers of CFUs recovered from throat swabs (which ranged from a few to ~200 CFUs per rat) remained relatively small over the course of the experiment. Mice colonized with the M6 serotype strain developed serum and salivary antibody responses directed against the M protein, which indicates that the model may have utility for the analysis of the development of serotype-specific immunity. A limitation to the model is that due to streptomycin treatment of the rats, it requires that the infecting *S. pyogenes* strain be resistant to streptomycin, although spontaneous streptomycin-resistant derivatives of many *S. pyogenes* strains can readily be obtained with a single-hit frequency in *rpsL*, the gene that encodes the 30S ribosomal protein S12 (Watson, Nielsen, Hultgren, & Caparon, 2013).

Autoimmune carditis

In addition to the many diseases it causes by active multiplication and direct damage to tissues or toxigenic diseases, *S. pyogenes* can also cause a plethora of diseases with an autoimmune character (Cunningham, 2000; Ralph & Carapetis, 2013). One of the most prominent of these is acute rheumatic fever (Cunningham, 2012). The heart is particularly sensitive and damage to it is manifested as carditis and valvulitis. The latter is the cause of most morbidity and mortality that results from mitral valve stenosis and aortic regurgitation. Most evidence indicates that this pathology results from autoimmunity that is triggered by a response against *S. pyogenes* antigens that cross-react with heart tissue. For modeling these diseases in the laboratory, the Lewis rat has been the species of choice. Since the disease is a sequela to an active infection and is driven by adaptive immunity, rats are not infected, but are immunized with *S. pyogenes* antigens that are implicated as cross-reactive with human tissues. Immunization with streptococcal M protein, a surface protein that is the serotype-specific antigen of *S. pyogenes*, results in the development of a myocarditis and a valvulitis. The pathology that develops resembles human rheumatic disease, including the formation of Anitschkow cell-containing granulomas (called Aschoff bodies) and infiltration of CD4+ and CD8+ T cells into the valvular lesions (Gorton, Govan, Olive, & Ketheesan,

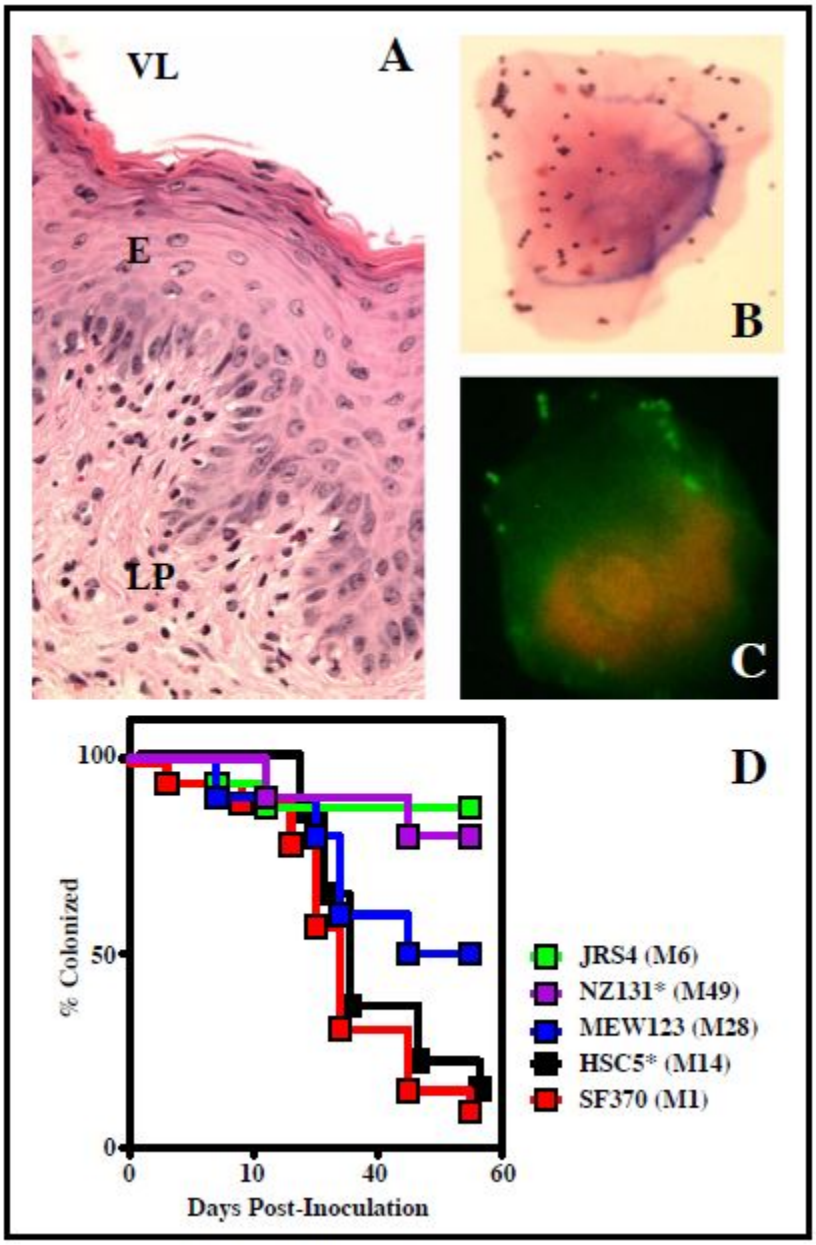

Figure 3. Vaginal model of asymptomatic mucosal carriage. A. Presented is an H&E-stained cross-section of vaginal wall from an estradiol-treated mouse colonized with *S. pyogenes* HSC12 for 8 days (magnification, 40X). Anatomy landmarks include vaginal lumen (VL), epithelium (E), and lamina propria (LP). Note the absence of significant inflammation, which is consistent with the asymptomatic nature of the carriage. **B**. Gram-stained murine vaginal epithelial cell from a vaginal wash. Numerous *S. pyogenes* bacteria are apparent as Gram-positive cocci in pairs and chains attached to the cell surface (magnification, 100X). **C**. Immunofluorescent micrograph of a murine vaginal epithelial cell stained with a FITC-conjugated anti-*S. pyogenes* antibody (ViroStat, Inc., Portland, ME) and counterstained with Evan's Blue (ViroStat) (magnification, 100X). **D.** Multiple *S. pyogenes* strains from diverse M protein serotypes and clinical origin are compatible with the murine vaginal colonization model. Shown are the percentage of mice that are colonized with each strain at the indicated time points, following inoculation of 1 x 10^6 CFU on day 0, as recovered by vaginal washes. The *S. pyogenes* strains are streptomycin-resistant or are a streptomycin-resistant derivative of the listed strain (indicated by "*") to enable direct selection on Todd Hewitt yeast (THY) extract agar supplemented with streptomycin (1000 μg/ml) (Asanuma, et al., 1997).

2006; Kirvan, Galvin, Hilt, Kosanke, & Cunningham, 2014; Quinn, Kosanke, Fischetti, Factor, & Cunningham, 2001). Cross-reactive epitopes have been mapped by using peptides that correspond to defined regions of the M

protein, both by direct immunization and by the adoptive transfer of peptide-reactive T cells to naïve rats to induce valvulitis (Kirvan, Galvin, Hilt, Kosanke, & Cunningham, 2014).

PANDAS

Acute rheumatic fever is only one of several autoimmune-type diseases that are caused by *S. pyogenes* (Cunningham, 2000). Of these, Sydenham's chorea, obsessive-compulsive disorder, Tourette's syndrome, and pediatric autoimmune neuropsychiatric disorders associated with streptococcus (known as PANDAS) all have a prominent neurological or neuropsychiatric component (Esposito, Bianchini, Baggi, Fattizzo, & Rigante, 2014; Macrì, Onori, Roessner, & Laviola, 2013). As with rheumatic fever, it is hypothesized that autoantibodies that react with neuronal tissue in the brain are produced against cross-reactive *S. pyogenes* antigens during an active infection. These antibodies persist, long after the initial streptococcal infection is cleared, to produce chronic symptomatology. The Lewis rat has also been used to model the pathological consequences of streptococcal-induced neuronal autoantibodies (Lotan, et al., 2014). In this model, rats that are immunized with a whole cell lysate of *S. pyogenes* proceed to develop quantifiable alterations to several behavioral and motor functions, as compared to sham-immunized animals. Serum from these animals reacts with brain tissue *in vivo* and to dopamine and serotonin receptors *in vitro*. Purified IgG from these cross-reacting sera could at least partially reproduce symptomatology, following its direct perfusion into the striatum region of the brain (Lotan, et al., 2014). Further analysis using this model may help to identify those neuronal structures that are susceptible to streptococcal-induced autoimmunity.

Chinchilla Models

Model of Otitis Media

Interestingly, while *S. pyogenes* causes less than 10% of the cases of culture-positive acute otitis media (ear infection), it was the most common agent of this disease prior to the 1950s and remains the fourth most common cause of bacterial culture-positive otitis media today (Segal, et al., 2005). Modern analyses have strongly implicated biofilm formation in the pathogenesis of ear infections caused by numerous bacterial species (Post, 2001), and the most powerful analysis of biofilm formation in otitis media has come from the use of the chinchilla ear infection model (Post, 2001; Hong, Pang, West-Barnette, & Swords, 2007). This model has been adapted for analysis of the contribution of biofilm to the pathogenesis of *S. pyogenes* otitis media (Roberts, Connolly, Doern, Holder, & Reid, 2010). In this model, approximately 10^5 CFU of the strain of interest are inoculated via transbullar injection into the ear of a chinchilla. Infection is monitored by otoscopy over the course of 7 days. By 2 days, the majority of ears show evidence of tympanic membrane and inner ear inflammation with an associated serous middle ear effusion. Biofilm formation is apparent as macroscopic structures that contain bacterial microcolonies in localized three-dimensional communities (Roberts, Connolly, Doern, Holder, & Reid, 2010). Numerous parameters can be quantitated, including histopathology, the enumeration of CFUs recovered from the macroscopic structures and from the serous effusion, and by monitoring survival of the animals over the 7-day course of infection. Biofilm ultrastructure can be further assessed by electron and fluorescent microscopic analyses of the recovered macroscopic material (Roberts, Connolly, Doern, Holder, & Reid, 2010). This model has been used to show that the transcription regulator Svr enhances biofilm formation *in vivo* (Roberts, Connolly, Doern, Holder, & Reid, 2010).

Primate Models

Non-human primate model of pharyngitis

Despite the relatively low cost and accessibility of rodent models of *S. pyogenes* pathogenesis, there has long been a desire to develop non-human primate models of disease to more closely mimic the human condition of

pharyngitis. Rodent models have failed to establish significant oropharyngeal colonization with *S. pyogenes* of any sufficient duration and do not develop evidence of symptomatic infection. Non-human primates offer advantages of a more similar development and components of immune responses to humans, as compared to rodents, as well as easier access to the site of infection, larger blood volumes to conduct biochemical analyses, and the ability to use multiple different strains of *S. pyogenes.* Initial reports of primate models for group A streptococci began in the early twentieth century and have evolved over the past 100 years to several different non-human primate species, including the baboon, rhesus monkey, chimpanzee, and the cynomolgus macaque (Taranta, Spagnuolo, Davidson, Goldstein, & Uhr, 1969; Vanace, 1960; Watson, Rothbard, Swift, & de Mello, 1946; Krushak, Zimmerman, & Murphy, 1970; Sumby, Tart, & Musser, 2008). *S. pyogenes* effectively colonizes the oropharynx of these primates, and there is an associated characteristic humoral immune response with type-specific M-protein antibodies detectable in serum (Taranta, Spagnuolo, Davidson, Goldstein, & Uhr, 1969; Vanace, 1960; Watson, Rothbard, Swift, & de Mello, 1946; Krushak, Zimmerman, & Murphy, 1970; Ashbaugh, et al., 2000). For example, in the baboon model, inoculation with ~3 x 10^5 CFU of a serotype M3 *S. pyogenes* strain establishes pharyngeal colonization for at least 6 weeks; in contrast, acapsular derivatives or M-protein–deficient mutants of the parent strain were significantly attenuated for carriage (Ashbaugh, et al., 2000). A hyaluronic acid capsule was further shown to be important for oropharyngeal colonization, as expression of the hyaluronic acid synthase (*has*) operon was significantly upregulated following inoculation into the baboon pharynx (Gryllos, et al., 2001), which likely served to inhibit phagocytosis by approaching leukocytes at sites of inflammation. Transcriptome analysis indicates similar expression profiles of a subset of *S. pyogenes* genes in the macaque pharynx, as compared with the human pharynx (Virtaneva, et al., 2003). Advantages of the primate model include the extended persistence of *S. pyogenes* in the primate pharynx, as compared to rodents, and the subsequent development of type-specific humoral immune responses—both features that are believed to be necessary for the development of rheumatic fever. Limitations of this model include the prohibitive expense and limited availability of primate colonies and the fact that baboon and rhesus monkeys do not develop symptomatic pharyngitis following inoculation. On the other hand, chimpanzees and macaques do develop significant pharyngeal erythema, fever, tonsillar enlargement, and palatal petechiae following infection, which is reminiscent of human *S. pyogenes* pharyngitis (Virtaneva, et al., 2003; Friou, 1950). Given the limited ability of *S. pyogenes* to colonize the rodent oropharynx, the non-human primate has become, and currently remains, the gold-standard model for upper airway and oropharyngeal *S. pyogenes* colonization.

Non-human primate model of sepsis

Hypotension and multi-organ failure are the signature features of streptococcal toxic-shock syndrome. This is a devastating disease with mortality rates that can be as high as 80%, despite the availability of aggressive antibiotic and supportive therapies (Stevens, et al., 1996). The disease has a very rapid time-course and patients often succumb even before an *S. pyogenes* etiology can be ascertained. Thus, a priority for understanding the pathogenesis of this disease is to elucidate the critical early checkpoints that can be exploited to develop therapeutic strategies for blocking the subsequent onset of multi-organ failure. As discussed above, many of the toxins that are critical for the pathogenesis of this disease, including the superantigen toxins, only recognize species-specific targets. Thus, animal models sensitive to these toxins are required for a critical analysis of the pathogenesis of this disease. This requirement has made the non-human primate model of sepsis (Creasey, et al., 1993) particularly attractive for this analysis (Stevens, et al., 1996). In this model, baboons (*Papio cynocephalus cynocephalus*) under light anesthesia are intravenously infused with a high dose of *S. pyogenes* (approx. 10^{10} CFU of log-phase, washed *S. pyogenes* cells) over a 2 hour period (Stevens, et al., 1996). Analyses of physiology and blood chemistry are then conducted over a 10-hour period, which results in a mortality rate that approaches 100% (Stevens, et al., 1996). Parameters of infection assessed include body temperature, heart rate, mean systemic arterial blood pressure and serum chemistry, and cytokine profiles (Stevens, et al., 1996). In this model, blocking the cytokine TNF-α using a therapeutic monoclonal antibody significantly improved both mean arterial blood pressure and survival (Stevens, et al., 1996).

Invertebrate Models

Silkworm and wax worm models

The principal advantages of infection models using easily bred invertebrates include that they are simple to perform, are inexpensive, do not require extensive animal husbandry, and offer a relatively high-throughput. One invertebrate model that has been used to analyze the virulence of human pathogens is the silkworm (*Bombyx mori*). Fatal infections occurred with inoculation of pathogenic pathogens (*S. aureus, P. aeruginosa, V. cholerae*), but not by non-pathogenic strains (Hamamoto, et al., 2004; Kaito, Akimitsu, Watanabe, & Sekimizu, 2002). Inoculation into the silkworm is through injection into the haemolymph (comparable to the bloodstream) of approximately 9 x 10^8 CFU of *S. pyogenes*, followed by monitoring survival for 5 days at 27°C (Kaito, et al., 2005). A similar model uses the wax worm (*Galleria mellonela*), whose principal advantage is that unlike for other invertebrates, infections can be conducted at 37°C (Loh, Adenwalla, Wiles, & Proft, 2013; Olsen, et al., 2010). Both of these models have proven sensitive to known *S. pyogenes* virulence factors and have been used to identify novel factors (Kaito, et al., 2005; Loh, Adenwalla, Wiles, & Proft, 2013; Olsen, et al., 2010).

C. elegans model

The well-known *Caenorhabditis elegans* invertebrate animal model has also been used to analyze *S. pyogenes* virulence (Bolm, Jansen, Schnabel, & Chhatwal, 2004; Jansen, Bolm, Balling, Chhatwal, & Schnabel, 2002). While most *C. elegans* virulence assays involve growing the worms on a plate inoculated with the pathogen, a liquid assay was developed to increase reproducibility. Two hundred sterile worms were added to ~2 x 10^7 CFU of *S. pyogenes* in a 24-well plate and monitored every 24 hours. More than 80% of the *C. elegans* organisms were killed by *S. pyogenes* in under 24 hours. However, colonization of the intestine does not occur with *S. pyogenes*, as when the worms are infected with *Enterococcus faecalis* strains. Interestingly, killing is not toxin-mediated, as strains with mutations in the genes encoding SLO and SpeB did not inhibit killing. Further analysis determined that killing was mediated by the production of hydrogen peroxide by *S. pyogenes*, which could be abrogated by the addition of catalase to the cultures (Jansen, Bolm, Balling, Chhatwal, & Schnabel, 2002).

Zebrafish Model

Myonecrosis model

The zebrafish (*Danio rerio*) infectious disease model has been particularly successful for analysis of *S. pyogenes* acute, deep tissue, myonecrotic infections. The symptoms observed mimic those reported for *S. pyogenes* deep tissue infections in humans, including lack of inflammatory cell infiltrates, large aggregates of bacteria dissecting along tissue planes, and extensive areas of necrotic tissue damage. While *S. pyogenes* does disseminate to the spleen, its systemic spread to other organs is not observed (Neely, Pfeifer, & Caparon, 2002). Experimental evidence suggests that fatality results from toxic shock, and most likely occurs through the systemic dissemination of streptococcal toxins (Benavides, Oberyszyn, VanBuskirk, Reeve, & Kusewitt, 2009; Rosch, Vega, Beyer, Lin, & Caparon, 2008). Inoculation is usually by IM injection into the dorsal muscle, although IP injections have also been analyzed (Phelps, Runft, & Neely, 2009). The IP LD_{50} for a serotype M14 strain of *S. pyogenes* in adult zebrafish was 2.5 x 10^2 CFU, while the IM route resulted in an LD_{50} of 3 x 10^4 CFU (Neely, Pfeifer, & Caparon, 2002). Over the course of 24 hours, an IM injection results in the formation of a hypo-pigmented lesion at the site of injection; this is indicative of extensive muscle necrosis that is apparent when the tissues are examined by histology (Figure 4). The lesions continue to enlarge until the death of the animal at 36 to 96 hours post injection. When analyzing pathogenesis with strains that carry mutations in known or putative virulence genes, a dose of 10 to 100 times the LD_{50} is used to determine survival, in comparison to fish injected with the wild-type strain using the Kaplan-Meier survival probability estimate model to test for significant differences (Neely, Pfeifer, & Caparon, 2002). Colonization can also be quantified over time by euthanization and

dissection of the muscle tissue to determine replication and/or clearance of the pathogen at the site of injection. Dissemination to the spleen can be determined by aseptic dissection of the organ, followed by serial dilution plating (Phelps, Runft, & Neely, 2009).

Multiple strains of *S. pyogenes* have been successfully used in the zebrafish model, including M59 (Bates, Toukoki, Neely, & Eichenbaum, 2005; Fisher, et al., 2008; Montañez, Neely, & Eichenbaum, 2005), M14 (Brenot, King, Janowiak, Griffith, & Caparon, 2004; Benavides, Oberyszyn, VanBuskirk, Reeve, & Kusewitt, 2009; Rosch, Vega, Beyer, Lin, & Caparon, 2008; Neely, Lyon, Runft, & Caparon, 2003), and M1 strains (Neely M. , 2015). Analyses of these strains have been used to examine multiple characteristics of *S. pyogenes* pathogenesis, including nutrient acquisition (Bates, Toukoki, Neely, & Eichenbaum, 2005; Fisher, et al., 2008; Montañez, Neely, & Eichenbaum, 2005), virulence gene regulation (Neely, Lyon, Runft, & Caparon, 2003), protein secretion (Rosch, Vega, Beyer, Lin, & Caparon, 2008), tissue-specific gene expression (Cho & Caparon, 2005) and toxin-mediated inhibition of host responses (Benavides, Oberyszyn, VanBuskirk, Reeve, & Kusewitt, 2009; Lin, Loughman, Zinselmeyer, Miller, & Caparon, 2009; Rosch, Vega, Beyer, Lin, & Caparon, 2008; Phelps & Neely, 2007) (Figure 4). However, like any animal model, inoculation with some strains does not cause disease and a notable example is the commonly used M6 strain JRS4 (Neely, unpublished research).

One of the major advantages of this model is that the zebrafish is one of the smallest animals that has both an adaptive and an innate immune system. In fact, analysis of its completely sequenced genome (Genome Research Limited, 2015) reveals a high degree of synteny with the human genome (Postlethwait, Amores, Force, & Yan, 1999) and similarity to the mammalian immune system (for further review, see (Hsu, et al., 2004)). This includes homologs to all of the mammalian Toll-like receptors (Jault, Pichon, & Chluba, 2004; Meijer, et al., 2004) and the complement system (Hsu, et al., 2004). The small size and fecundity of the zebrafish and the relatively low cost and simplicity of natural breeding allows for the production and housing of thousands of animals in a small space. This facilitates large-scale screens, including pathogen-centric signature tagged transposon mutagenesis screens (Kizy & Neely, 2009), and host-centric genetic or small molecule-based screens using embryos or larvae arrayed in 96-well plates or via automated microinjection (Wang, Liu, Gelinas, Ciruna, & Sun, 2007). There is an extensive methodology available for analysis of different life stages of zebrafish, and several of these have been successfully employed for analysis of the effect of *S. pyogenes* toxins on immune cell infiltration (Lin, Loughman, Zinselmeyer, Miller, & Caparon, 2009). Zebrafish genes can be selectively and transiently knocked-down using morpholino technology (Nasevicius & Ekker, 2000) and extensive collections of modified and mutant zebrafish lines are available. These include lines that are transparent (Ren, McCarthy, Zhang, Adolph, & Li, 2002; White, et al., 2008) and lines where cells of the myeloid lineage express GFP (for one example, see (Gray, et al., 2011)). The Zebrafish International Resource Center (ZIRC) (The Zebrafish International Resource Center, 2006) maintains a searchable database for finding zebrafish strains with specific mutations. Recent developments in genetic technology has made creating transgenic zebrafish with specific genetic knock-outs highly successful, including the use of Zinc-finger nucleases, TALENs, and the CRISPR/Cas9 systems (for further review, see (Schulte-Merker & Stainier, 2014)).

As with other models, the zebrafish model is not without its weaknesses. These include that the development of reagents, like monoclonal antibodies for cells and markers of interest, lags behind those available for murine models, and it has been difficult to establish zebrafish-specific cell cultures *in vitro*. While zebrafish do have an immune system, some important differences remain. For example, one interesting difference is a lack of discernable lymph nodes, although evidence of a zebrafish lymphatic system was recently reported (Küchler, et al., 2006; Yaniv, et al., 2006). Lastly, the optimal temperature for the incubation of zebrafish is 27–29^{o}C, which is problematic for those *S. pyogenes* virulence genes that may be temperature-regulated.

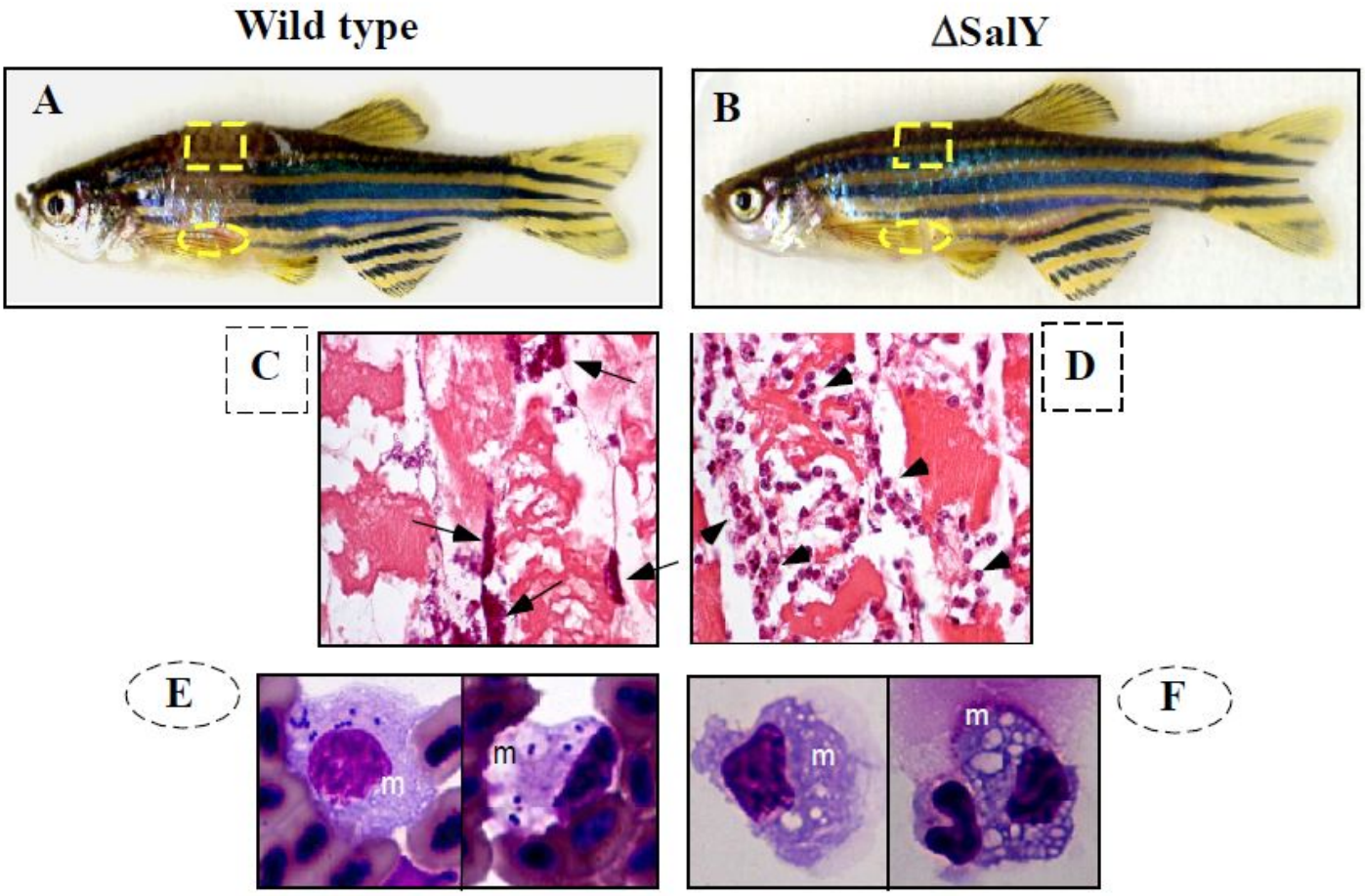

Figure 4. Zebrafish myonecrosis model. Zebrafish-infected IM with 10^5 CFU of wild type *S. pyogenes* strain HSC5 and a HSC5 *salY* mutant (ΔSalY), as indicated. At 24 hours post-infection, fish are euthanized and further processed as described (98). SalY is a component of a lantibiotic ABC transporter that is required for the survival of *S. pyogenes* in zebrafish macrophages (Rosch, Vega, Beyer, Lin, & Caparon, 2008). Comparison of intact zebrafish reveals that a large hypo-pigmented lesion in the dorsal muscle has formed around the site of injection of the wild-type strain (outlined by the dashed-box) (**A**), but that no lesion is apparent in the ΔSalY mutant (**B**). Examination of H&E-stained paraffin embedded sections of skeletal muscles reveals that the hypo-pigmented lesion caused by the wild-type strain is accompanied by large aggregates of streptococcal bacteria in the muscle (black arrows) and no inflammatory cell inflammation (**C**). In contrast, muscle infected by the ΔSalY mutant contains few cocci and a large infiltration of inflammatory cells (black arrowheads) (**D**). Macrophages (m) recovered from a spleen homogenate from wild type-infected zebrafish contain intracellular cocci (**E**) that are not observed in splenic macrophages of the ΔSalY mutant, which instead contain empty vacuoles (**F**). The location of the spleen is shown by the dashed oval.

Conclusion

In his commentary on the fifteenth anniversary of the publication of his "molecular Koch's postulates," Stanley Falkow made the observation that his experimental approach for the identification of pathogenicity genes remains relevant even in an age where the complete genomes of pathogens are readily available, noting that "Genomes do not necessarily reveal their secrets about pathogenic traits by simple visual inspection or by sophisticated bioinformatics analysis" (Falkow, 2004). Thus, the various animal models described in this chapter will continue to play an important role in the analysis of *S. pyogenes* gene/pathogenesis relationships for the foreseeable future.

When applying the Falkow strategy (Table 1), there are some important considerations to be made when reaching conclusions about the role of any *S. pyogenes* gene in an *in vivo* model. First, it is always important to

determine the growth rate of a mutant as compared to its wild type parent using an *in vitro* medium. This is not because genes that promote growth are less interesting or unimportant, but because the pathogenesis in virtually all of the models described in this chapter extends from the ability of *S. pyogenes* to rapidly proliferate in tissue. Thus, a decrease in growth rate will almost always result in attenuation. The choice of an *in vitro* medium is an important consideration, given the complexities of the tissue environment. As a result, strategies have been developed for the comparison of *in vivo* and *in vitro* growth conditions (Loughman & Caparon, 2006; Cho & Caparon, 2005). Secondly, as described by Falkow in his postulates, it is important to establish that re-introduction of the wild-type gene is accompanied by a restoration of virulence (Table 1). In cases where plasmid-based complementation is used for *S. pyogenes*, it is always important to determine that the complementing plasmid is stably maintained in an *in vivo* environment in the absence of antibiotic selection. If the plasmid is not stable, an alternative is to restore the wild-type gene at its resident locus on the chromosome (for an example, see (Watson, Nielsen, Hultgren, & Caparon, 2013)).

Finally, it is important to keep in mind that the various models described here each have important limitations, and have all been designed to model specific aspects of the various and varied *S. pyogenes* diseases. Thus, care should be taken when concluding that a particular gene may not be a virulence factor, when a more likely explanation is that the particular animal model used cannot resolve its contribution. This often occurs because the step of virulence process it is required for is bypassed in the model. Modern genomics and genetic technology, combined with the many creative *in vivo* models described here, have opened a golden era of research into the pathogenesis of *S. pyogenes*.

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Global Disease Burden of Group A Streptococcus

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Introduction

Despite being in existence for hundreds of years, *Streptococcus pyogenes* (group A streptococci) remains a significant cause of global morbidity and mortality, with a particular impact in resource-limited settings. The vast majority of cases of acute rheumatic fever (ARF), rheumatic heart disease (RHD), acute post-streptococcal glomerulonephritis (APSGN), and invasive *S. pyogenes* cases occur in low-resource settings (Carapetis, 2005) (Figure 1).

Accurate estimates of the global disease burden are important in planning for disease control, allocating resources, advocating for interventions, and prioritizing new strategies and research. This is particularly relevant in light of the potential to invest in the development of a vaccine to control *S. pyogenes* disease (Moreland, et al., 2014) (for more information, see the chapter on vaccine development in this book). However, overall disease burden estimates are difficult, mainly because of the scarcity of comprehensive disease registries, the reliance on passive surveillance systems, and the underreporting of both acute and chronic cases. This is true in all regions, and particularly in countries where *S. pyogenes* diseases occur most frequently (Marijon, Mirabel, Celermajer, & Jouven, 2012).

Clinical manifestations of *S. pyogenes* are among the most diverse of any human pathogen (Ralph & Carapetis, 2013). *S. pyogenes* has a varied presentation that ranges from seemingly benign pharyngitis and skin infections to more severe glomerulonephritis and sepsis, as well as devastating rheumatic heart disease (RHD), toxic shock syndrome, and necrotizing fasciitis.

In 2005, a review of the global burden of group A streptococcal diseases was published, using conservative methodologies that were deliberately designed to under-estimate the burden (Carapetis, 2005; Carapetis, Steer, Mulholland, & Weber, 2005b). At that time, it was estimated that a minimum of 18.1 million people were suffering from invasive *S. pyogenes* diseases, with an additional 1.78 million incident cases occurring each year. These estimates did not include over 111 million cases of streptococcal pyoderma and 616 million cases of *S. pyogenes* pharyngitis each year (Carapetis, Steer, Mulholland, & Weber, 2005b). A subsequent review published in 2008 provided updated data on the RHD burden from Asia, which estimated between 1.96 and 2.21 million cases of RHD in Asian children 5 to 14 years of age (Carapetis, 2008). These studies also highlighted the dramatic gaps in reliable recent disease burden data from many regions, particularly Asia, Eastern Europe, and Latin America.

In this chapter, we will build on the 2005 review by including the considerable amount of studies that have been performed since that review. Improved capacity for case detection that includes the use of echocardiography, as well as an increase in notification, have been associated with increases in reported rates of *S. pyogenes*–related diseases in low-resource settings.

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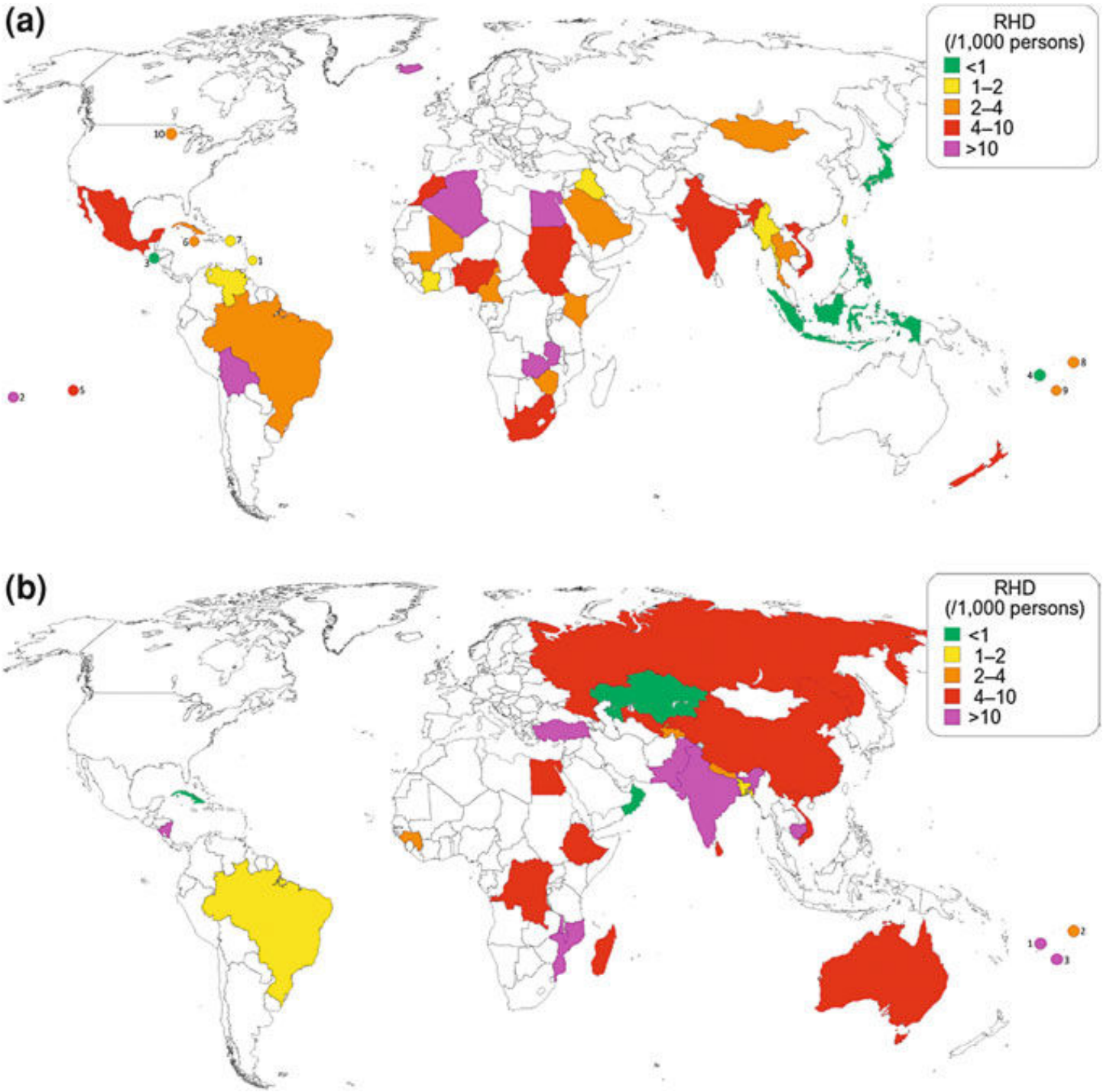

Figure 1. (a) Map of RHD prevalence, 1970–1990; (b) Map of rheumatic heart disease prevalence, 1999–2011. Reproduced from (Seckeler & Hoke, 2011).

Methods

We conducted a systematic review with the goal of including population-based data published between 2005 and October 2014. Information on post-streptococcal glomerulonephritis (Table 3) was expanded to include older studies, because of the lack of recent studies in this area. We searched the PubMed database for the following terms. The number of publications returned is in parentheses.

1. Rheumatic fever or rheumatic heart disease AND incidence or prevalence (1120)

2. Rheumatic fever or rheumatic heart disease AND mortality or cause of death or burden of disease (685)
3. Glomerulonephritis AND (streptococcus or streptococcal or post-streptococcal or post-streptococcal or streptococcus) AND incidence or prevalence (89)
4. Invasive or bacteremia or bacteremia or toxic shock syndrome or necrotizing fasciitis or necrotizing fasciitis AND incidence or prevalence AND group A *Streptococcus* or group A streptococcal or *Streptococcus pyogenes* (345)
5. Stroke AND Rheumatic fever or rheumatic heart disease (147)
6. Endocarditis AND Rheumatic fever or rheumatic heart disease (298)
7. Impetigo or pyoderma or scabies AND incidence or prevalence (603)
8. Bacteremia or bacteraemia and developing country (101)
9. Pharyngitis AND incidence or prevalence (>9,000)

Because of the large number of publications on pharyngitis, and because other summarized sources of information were available for this endpoint, we chose not to review those abstracts for this chapter. For all other *S. pyogenes* diseases, we reviewed abstracts, and those that seemed to offer population-based incidence or prevalence data were selected for inclusion in the tables at the end of this chapter. Our analysis of the burden of *S. pyogenes*–related diseases is organized as follows:

1. Superficial and locally invasive disease (pharyngitis, impetigo, pyoderma, cellulitis, erysipelas).
2. Immune-mediated disease (acute rheumatic fever and acute post-streptococcal glomerulonephritis).
3. Disease with direct sequelae (rheumatic heart disease).
4. Invasive and toxin mediated disease (bacteremia, streptococcal toxic shock syndrome, scarlet fever, necrotizing fasciitis, meningitis, osteomyelitis, pneumonia, puerperal sepsis).

Table 3: Population-based studies of the incidence of acute post-streptococcal glomerulonephritis (APSGN)

Reference	Place	Year of Study	Age in Years	APSGN Incidence (per 100,000 per year)	Population
Carapetis (Carapetis, Steer, Mulholland, & Weber, 2005b)	Australia	1993 to 1995	less than 15	239	Indigenous
Carapetis (Carapetis, Steer, Mulholland, & Weber, 2005b)	Australia	1993 to 1995	less than 15	6	Non-indigenous
Muscatello (Muscatello, O'Grady, Neville, & McAnulty, 2001)	Australia	1989 to 1998	less than 20	2.2	
Berrios (Berríos, et al., 2004)	Chile	1980 to 1989	less than 15	18.1	
Simon (Simon, et al., 1994)	France	1986 to 1990	all	0.15	
Becquet (Becquet, et al., 2010)	French Polynesia	2005 to 2007	less than 15	18	
Herrera (Herrera & Rodríguez-Iturbe , 2003)	India	1991 to 1998	all	2.9	Goajiro Indians
Coppo (Coppo, Gianoglio, Porcellini, & Maringhini, 1998)	Italy	1998	>60	0.09	
Coppo (Coppo, Gianoglio, Porcellini, & Maringhini, 1998)	Italy	1998	<60	0.04	
Khuffash (Khuffash, Sharda, & Majeed, 1986)	Kuwait	1980 to 1983	children	19.5	
Majeed (Majeed, et al., 1987)	Kuwait	1980 to 1983	children	17.8	
Baker (Baker, et al., 2012)	New Zealand	1988 to 1998	5 to 14	8.1	all
Baker (Baker, et al., 2012)	New Zealand	1988 to 1998	5 to 14	48	Maori

Table 3 continued from previous page.

Reference	Place	Year of Study	Age in Years	APSGN Incidence (per 100,000 per year)	Population
Baker (Baker, et al., 2012)	New Zealand	1988 to 1998	5 to 14	80	Pacific Islander
Lennon (Lennon, Martin, Wong, & Taylor, 1988)	New Zealand	1981 to 1984	children	50.5	Maori
Lennon (Lennon, Martin, Wong, & Taylor, 1988)	New Zealand	1981 to 1984	children	46.5	Pacific Islander
Lennon (Atatoa-Carr, Bell, & Lennon, 2008)	New Zealand	1981 to 1984	children	5.9	other
Wong (Wong, Lennon, Crone, Neutze, & Reed, 2013)	New Zealand	2007 to 2009	0 to 14	9.7	Pacific 45.5, Maori 15.7, European/other
Wong (Wong, Lennon, Crone, Neutze, & Reed, 2013)	New Zealand	2007 to 2009	0 to 14		2.6 and Asian 2.1/100 000
Eke (Eke & Eke, 1994)	Nigeria	1986 to 1991	less than 15	24.3	
Yap (Yap, et al., 1990)	Singapore	1985	less than 12	10.8	

Superficial and Locally Invasive Disease: Pharyngitis and *S. pyogenes* skin infections

The absolute numbers of cases of superficial *S. pyogenes* skin infections (impetigo, pyoderma and "skin sores") and pharyngitis are much greater than those of more serious *S. pyogenes* diseases. Although superficial *S. pyogenes* diseases may seem relatively benign, their economic, social, and health utilization burdens can be considerable. Their direct costs can include use of antibiotics and other medication for symptomatic relief, while their indirect costs can include time away from school and parental time off work. Superficial *S. pyogenes* diseases also have clinical and public health implications, because of their causal association with invasive *S. pyogenes* infections and post-streptococcal diseases (Carapetis, Steer, Mulholland, & Weber, 2005b) (Table 1).

The burden of *S. pyogenes* skin infection is associated with scabies infection through co-infection and shared risk factors. The 2010 Global Burden of Disease study estimated that there are 140,495,000 cases of impetigo and 100,625,000 cases of scabies globally each year. This infectious burden ranks impetigo and scabies among the 50 most common diseases worldwide (Hay, et al., 2014). *S. pyogenes* skin infections and scabies are more prevalent, and are a larger cause of morbidity in resource-limited settings. Scabies can affect more than 60% of people who live in overcrowded communities with poor sanitation (Hay, et al., 2014).

Variations in the prevalence of *S. pyogenes* skin infections are related to accessibility to appropriate housing and hygiene. *S. pyogenes* also shows seasonal variation, being more common in dry than wet seasons in monsoonal climates (McDonald, et al., 2008). Communities with high scabies rates have been shown to have an elevated prevalence of pyoderma (Andrews, McCarthy, Carapetis, & Currie, 2009a). In 2005, pyoderma prevalence was found to range from 1 to 20% among children in less-developed countries, but was found to be as high as 40–90% in some areas of Pacific (Carapetis, Steer, Mulholland, & Weber, 2005b; Steer, Adams, Carlin, Nolan, & Shann, 1999) and Indigenous Australian children (Ralph & Carapetis, 2013; Shelby-James, Leach, Carapetis, Currie, & Mathews, 2002).

The incidence of cellulitis and erysipelas reported from Minnesota in 2007 was 200 cases per 100,000 patient-years (McNamara, et al., 2007). At San Francisco General Hospital, skin and soft tissue infections, including cellulitis, were the leading cause of admission for medical or surgical treatment (The Centers for Disease Control and Prevention, 2001). Almost all erysipelas episodes and many cellulitis episodes are attributable to *S. pyogenes*.

However, microbiological confirmation is uncommon unless there is associated bacteremia (Ralph & Carapetis, 2013; Bisno & Stevens, 1996).

S. pyogenes pharyngitis is generally self-limiting, but can lead to a number of complications, which can include retropharyngeal abscess, ARF, and APSGN (Jackson, Steer, & Campbell, 2011). Prevalence rates of *S. pyogenes* pharyngitis are difficult to determine because there is a high rate of *S. pyogenes* throat colonization, which is reported to be as high as 15–20% in some studies (Henningham, Barnett, Maamary, & Walker, 2012).

Pharyngitis is a common cause of presentation to general practitioners in high-income settings. In a prospective, cohort-based study in Melbourne, Australia, the incidence of acute sore throat, group A streptococcal swab–positive pharyngitis, and serologically confirmed group A streptococcal pharyngitis was 33, 13, and 8 per 100 child-years, respectively, for school-aged children (5–12 years) and 60, 20, and 15 per 100 family-years, respectively (Danchin, et al., 2007).

Infection rates are best determined from studies that use serological testing to avoid the issue of misclassification of pharyngeal carriage as infection. It has been previously estimated that in high-resource settings, about 15% of school children and 4–10% of adults suffer an episode of symptomatic *S. pyogenes* pharyngitis per year, based on a number of population studies. In some low-resource settings, these rates may be 5–10 times higher (Carapetis, 2005; Ralph & Carapetis, 2013; Danchin, et al., 2004; Nandi, Kumar, Ray, Vohra, & Ganguly, 2001).

Table 1: Studies since 2005 that document the prevalence of scabies or pyoderma

Reference	Year of study	Place	Age in Years	Number surveyed	Prevalence pyoderma	Prevalence scabies
Amro (Amro & Hamarsheh, 2012)	2005 to 2010	West Bank, Palestinian Territory	all	1734		17
Andrews (Andrews, et al., 2009b)	2004 to 2007	Northern Territory, Australia	0 to 15	2329	35.5	13.4
dos Santos (dos Santos, et al., 2010)	2007	Timor Leste	all	1535	7	17.3
El Khateeb (El-Khateeb, Lotfi, Abd Elaziz, & El-Shiekh, 2014)	2011 to 2012	Egypt	6 to 12	6162	5.5	0.03
Feldmeier (Feldmeier, et al., 2009)	2003	Brazil	all	2002		9.8
Ferie (Ferié, et al., 2006)	2003	Tanzania	6 to 19	820	8.4	1.5
Grills (Grills, et al., 2012)	2010	India	all	1250		4.4
Hogewoning (Hogewoning, et al., 2013)	2004	Ghana	4 to 16	463	4.3	0
Hogewoning (Hogewoning, et al., 2013)	2007	Ghana	4 to 20	1394	5.8	0.1
Hogewoning (Hogewoning, et al., 2013)	2005	Gabon	4 to 20	454	1.5	0.7
Hogewoning (Hogewoning, et al., 2013)	2007	Rwanda	4 to 16	2528	1.3	0.04
Leekassa (Leekassa, Bizuneh, Alem, Fekadu, & Shibre, 2005)	1998	Ethiopia	16 and older	4697	5.6	30
Ogunbiyi (Ogunbiyi, Omigbodun, & Owoaje, 2009)	2009*	Nigeria	10 to 20	1415	0.6	1.1

Table 1 continued from previous page.

Reference	Year of study	Place	Age in Years	Number surveyed	Prevalence pyoderma	Prevalence scabies
Ogunbiyi (Ogunbiyi, Owoaje, & Ndahi, 2005)	2005*	Nigeria	4 to 15	1066		4.7
Steer (Steer A. C., et al., 2009)	2006 to 2007	Fiji	5 to 15	3462	25.6	18.5
Steer (Steer A. C., et al., 2009)	2006 to 2007	Fiji	infants	451	12.2	14
Kumar (Kumar, et al., 2009)	2000 to 2002	India	5 to 15	4249	0.7	

* indicates publication date when study date is unknown

Immune mediated: acute rheumatic fever, and acute post-streptococcal glomerulonephritis

Incidence of Acute Rheumatic Fever

Acute rheumatic fever (ARF) is the most common cause of acquired heart disease in children worldwide (Carapetis, Steer, Mulholland, & Weber, 2005b; Jackson, Steer, & Campbell, 2011). ARF has become considerably less common in middle and high-income settings over the last half-century (Land & Bisno, 1983; Gordis, 1985). Studies in low-resource settings show that ARF rates remain high, and the resultant RHD is a source of substantial morbidity and mortality (Carapetis, Steer, Mulholland, & Weber, 2005b; Ahmed, Mostafa Zaman, & Monzur Hassan, 2005). However, accurate estimations of ARF incidence are difficult. There is no diagnostic test for ARF, its clinical presentation may be subtle and overlooked, and few countries, particularly among low and middle-income countries, have robust ARF notification programs (Table 2).

In 2005, the global incidence of ARF in children aged 5–14 years was estimated to be roughly 300,000–350,000 cases per year in the global population (Carapetis, Steer, Mulholland, & Weber, 2005b; Carapetis, McDonald, & Wilson, 2005a; Tibazarwa, Volmink, & Mayosi, 2008). Most of the studies published since 2005 come from Australia and New Zealand, and continue to demonstrate very high incidences in Indigenous populations in those countries (Table 2). New studies from the Northern Mariana Islands, Fiji, and Samoa confirm that high rates of ARF persist throughout Pacific Island nations (Seckeler, Barton, & Brownstein, 2010; Viali, Saena, & Futi, 2011; Parks, Kado, Colquhoun, Carapetis, & Steer, 2009). Interestingly, two studies have shown that ARF still frequently occurs in Italy (Breda, et al., 2012; Pastore, et al., 2011), and a study from Israel shows a relatively low rate overall, but one that is still higher than in most affluent nations (Vinker, Zohar, Hoffman, & Elhayany, 2010).

Overall, ARF incidence estimates vary widely, from a low of 0.1 per 100,000 in Greece (Jackson, Steer, & Campbell, 2011) and 0.7 per 100,000 children per year in Slovenia in 1990–1991 (Cernay, Rusnák, & Raisová, 1993) to 120 per 100,000 in Bangladesh (Ahmed, Mostafa Zaman, & Monzur Hassan, 2005), 374–508/100,000 in selected Australian Aboriginal populations in the 1980s–1990s (Ralph & Carapetis, 2013; Carapetis, Currie, & Mathews, 2000; Richmond & Harris, 1998) and 826 per 100,000 in Sudan (Jackson, Steer, & Campbell, 2011). However, it should be noted that these studies were based on various age ranges.

A systematic review in 2008 aimed to summarize population-based studies of ARF incidence, but restricted the analysis to only the first ARF episode (Tibazarwa, Volmink, & Mayosi, 2008). Studies from 10 countries on all continents (except Africa) were reviewed. The mean incidence rate of first attack of ARF was 19 per 100,000 per population per year (95% confidence interval; 9–30/100,000), lowest rates (< 10/100,000) documented in America and Western Europe, and higher incidences (> 10/100,000) documented in Eastern Europe, Middle East, Asia, and Australasia (Omar, 1995).

ARF incidence rates reported from each country would be expected to correlate with that country's RHD prevalence, but such correlation is not always evident. In particular, although South Africa has some of the highest global prevalence of RHD (Carapetis, Steer, Mulholland, & Weber, 2005b), ARF incidence is only reported there at a rate of 13.4/100,000. This suggests either a substantial under-diagnosis of ARF (Ralph & Carapetis, 2013) or a lack of classical ARF manifestations in this population.

Aside from RHD, consequences of recurrent ARF include chorea which sometimes lasts up to 6 months, with rare cases lasting 2–3 years, and the need for 10 years or longer of secondary prophylaxis with monthly benzathine penicillin injections. In high-resource settings, where ARF rates can be high in Indigenous people, access to secondary- and tertiary-level care may result in lower mortality rates.

Table 2: Recent studies (since 2005) that document the incidence of acute rheumatic fever (ARF) in children and adolescents

Reference	Setting	Place	Year of Study	Age in years	ARF incidence (Infections per 100,000 per year)
Atatoa (Atatoa-Carr, Bell, & Lennon, 2008)	Established Market Economies	Waikato, New Zealand	1998 to 2004	4 to 32 5 to 14	3.3 12.9
Breda (Breda, et al., 2012)	Established Market Economies	Abruzzo, Italy	2000 to 2009	2 to 17	4.1
Jaine (Jaine, Baker, & Venugopal, 2008)	Established Market Economies	New Zealand	1996 to 2005	all	3.4
Milne (Milne, Lennon, Stewart, Vander Hoorn, & Scuffham, 2012)	Established Market Economies	New Zealand	2000 to 2009	5 to 14	17.2
Pastore (Pastore, et al., 2011)	Established Market Economies	Trieste, Italy	2007 to 2008	5 to 15	25
Robin (Robin, Mills, Tuck, & Lennon, 2013)	Established Market Economies	New Zealand	2002 to 2011	0 to 35	7.7
Siriett (Siriett, Crengle, Lennon, Stonehouse, & Cramp, 2012)	Established Market Economies	Tairawhiti/ Gisborne, New Zealand	1997 to 2009	all	7.6 (all) 1.1 (non-Maori)
Vinker (Vinker, Zohar, Hoffman, & Elhayany, 2010)	Established Market Economies	Israel	2000 to 2005	0 to 30	3.2
Nordet (Nordet, Lopez, Dueñas, & Sarmiento, 2008)	Latin America	Cuba	1996	5 to 25	2.5
Cuboni (Cuboni, Finau, & Cuboni, 2006)	Pacific and Indigenous Australia/ NZ	Fiji	1998 to 2000	3 to 14	2.3
Lawrence (Lawrence, Carapetis, Griffiths, Edwards, & Condon, 2013)	Pacific and Indigenous Australia/ NZ	Northern Territory, Australia	1997 to 2010	5 to 14	194 (Indigenous people only)
Robin (Robin, Mills, Tuck, & Lennon, 2013)	Pacific and Indigenous Australia/ NZ	New Zealand	2002 to 2011	5 to 14	78 (Northland Maori only)
Seckeler (Seckeler, Barton, & Brownstein, 2010)	Pacific and Indigenous Australia/ NZ	Northern Mariana Islands	1984 to 2006	5 to 14	85.8 167 (Chamorro or Carolinian ancestry only)
Siriett (Siriett, Crengle, Lennon, Stonehouse, & Cramp, 2012)	Pacific and Indigenous Australia/ NZ	Tairawhiti/ Gisborne, New Zealand	1997 to 2009		15.2 (Maori)

Table 2 continued from previous page.

Reference	Setting	Place	Year of Study	Age in years	ARF incidence (Infections per 100,000 per year)
Steer (Steer, et al., 2009b)	Pacific and Indigenous Australia/ NZ	Fiji	2005 to 2007	5 to 15	15.2
Viali (Viali, Saena, & Futi, 2011)	Pacific and Indigenous Australia/ NZ	Samoa	2000 to 2008	5 to 19	9.5 (in 2009)
Parks (Parks, Kado, Colquhoun, Carapetis, & Steer, 2009)	Pacific and Indigenous Australia/ NZ	Fiji	2003 to 2008	4 to 20	24.9
Ahmed (Ahmed, Mostafa Zaman, & Monzur Hassan, 2005)	South-Central Asia	Bangladesh	1991	5 to 15	120

Acute post-streptococcal Glomerulonephritis

Acute post-streptococcal glomerulonephritis (APSGN) is often considered to be a relatively benign disease. However, in some resource-limited populations, it is associated with a 5 to 6-fold increased risk of chronic renal disease (White, Hoy, & McCredie, 2001). Factors such as crowding, poor hygiene, and poverty are associated with APSGN outbreaks (Marshall, et al., 2011). In 2005, the median APSGN incidence in children in low-resource settings was estimated to be 24.3/100,000 per year (Carapetis, 2005), as compared to approximately 6/100,000 per year in high-resource settings (Lennon, Martin, Wong, & Taylor, 1988; Carapetis & Currie, 1998). In addition, 97% of deaths (complicating about 1% of cases) occur in low-resource countries (Carapetis, 2005). This low-resource mortality predominance is similar to those found with other *S. pyogenes* manifestations. In Australia's Northern Territory, annual incidence rates of APSGN from 1992–2007 were found to be very high, at 94.3 and 7.3 per 1,000 in the 0–14 and >14 year age groups, respectively (Marshall, et al., 2011) (Table 3).

Studies on APSGN demonstrate significant global variation, with the highest incidence of 239 per 100,000 in Indigenous Australians and the lowest incidence of 0.04 per 100,000 in an Italian study of people under the age of 60 (Jackson, Steer, & Campbell, 2011).

APSGN incidence has been shown to have fallen substantially in Europe (Jackson, Steer, & Campbell, 2011; Simon, et al., 1994), South America (Berríos, et al., 2004), Asia (Yap, et al., 1990; Zhang, Shen, Feld, & Stapleton, 1994), and the United States (Roy & Stapleton, 1990). While it is known that the incidence of APSGN has decreased considerably in resource-rich countries, APSGN causes a substantial burden in Indigenous communities and in resource-limited countries (Jackson, Steer, & Campbell, 2011; Currie & Brewster, 2001; Prakash, Saxena, Sharma, & Usha, 2001).

Post-streptococcal Glomerulonephritis Mortality and Morbidity

Jackson and colleagues reported on six cohort studies that reported case fatality rates from APSGN. Three of these studies showed a case fatality rate of zero, and two studies in India revealed case fatality rates of 1.4% and 2%. Applying the mean case fatality ratio from the two India studies (1.7%) to the 2005 WHO incidence estimates, the mortality of APSGN in India can be estimated at 0.4 per 100,000. Using the same principle, the mortality from APSGN in Turkey (with a case fatality rate 0.08%) is estimated as 0.02 per 100,000 (Jackson, Steer, & Campbell, 2011).

Although the incidence of APSGN is as high as 239 per 100,000 among Indigenous Australians, the overall mortality from APSGN is low (0.02–0.4 per 100,000). This mortality estimate is somewhat higher than the estimate by the WHO of 0.005 per 100,000 (Carapetis, 2005). This could be because of the conservative approach taken by the WHO, which was partially due to poorly documented mortality rates and the long-term sequelae of

APSGN. In addition, WHO estimates may underestimate the association between APSGN and chronic renal failure (Chugh, et al., 1987; Richmond & Doak, 1990; Bohle, et al., 1992) and thus the total disease burden. As much of the world has not been studied, current estimates are likely to underrepresent the true rates of APSGN mortality (Jackson, Steer, & Campbell, 2011).

Several of the studies reported considerable mortality from APSGN, and documented associated long-term morbidity. ASPGN morbidity data is limited and inconsistent. Hypertension has been seen in 8–43.6% of subjects at follow-up (Chugh, et al., 1987; D'Cruz, Samsudin, Hamid, & Abraham, 1990). Hypertensive encephalopathy was seen in 0.8–11.3% of subjects (D'Cruz, Samsudin, Hamid, & Abraham, 1990; Oner, Demircin, & Bulbul, 1995). Hematuria was found in 13.7% of patients (D'Cruz, Samsudin, Hamid, & Abraham, 1990), while proteinuria was found in 0.8% (Oner, Demircin, & Bulbul, 1995). Studies have described the development of rapidly progressive glomerulonephritis after APSGN in 2–4% of patients (Oner, Demircin, & Bulbul, 1995; Shiva, Far, & Behjati, 1994). One study found that 0.5% of patients required peritoneal dialysis (Khuffash, Sharda, & Majeed, 1986), while another showed that 2% of subjects needed hemodialysis (Shiva, Far, & Behjati, 1994). Pulmonary edema developed in 36.3% of subjects and uremia developed in 16.5% of subjects (D'Cruz, Samsudin, Hamid, & Abraham, 1990).

Direct sequelae: rheumatic heart disease

Prevalence of Rheumatic Heart Disease

(Tables 3a and 3b)

The most serious sequela of ARF is established RHD, which occurs in 42–60% of people with a history of prior ARF (Ralph & Carapetis, 2013; Carapetis, Currie, & Mathews, 2000; The Rheumatic Fever Working Party of the Medical Council of Great Britain; The Subcommittee of Principal Investigators of the American Council on Rheumatic Fever and Congenital Heart Disease, 1960). In 2005, it was estimated to cause at least 250,000 premature deaths per year (Carapetis, Steer, Mulholland, & Weber, 2005b). Recent estimates suggest that the disability burden associated with RHD is equivalent to one-quarter of disability from all forms of cancer combined (Mirabel, Narayanan, Jouven, & Marijon, 2014). RHD rates are the best documented of *S. pyogenes*-related diseases (Ralph & Carapetis, 2013). In 2005, 15.6 to 19. 6 million people worldwide were estimated to have RHD (Carapetis, Steer, Mulholland, & Weber, 2005b).

In 1990 the Global Burden of Disease (GBD) study published global population-based data that showed estimates of RHD prevalence, incidence, and mortality (Carapetis, Steer, Mulholland, & Weber, 2005b). The GBD 2010 study was undertaken to provide an updated estimate of global epidemiology with the objective of updating the estimates of RHD burden of disease using vital statistics data in 187 countries and 21 regions of the world from 1990 to 2010. Inclusion criteria were population-based studies published from 1990–2009. Data on RHD incidence, prevalence, and mortality were included. Incidence, prevalence, number of deaths, and Disability Adjusted Life Years (DALYs), were calculated using epidemiological modeling tools. The results showed that, in 1990, there were an estimated 29,172,383 cases of RHD globally, which increased to 34,232,795 in 2010 (Figure 2); the areas with the highest prevalence were Oceania (Pacific Islands region) and Eastern Europe (both 9.8 per 1,000). The estimated global number of incident RHD cases for 1990 and 2005 were 2,146,273 and 1,542,454 cases respectively. The estimates presented in the GBD 2010 Study suggest a higher burden of RHD than previously reported.

Historic studies of RHD prevalence from the 1950s onwards were based on population-based screening of school children through cardiac auscultation. Evidence from the echocardiographic era suggests that auscultation is insufficiently sensitive to detect early valvular lesions of subclinical RHD. Estimates of RHD prevalence based on clinical surveillance methods alone, without echocardiography, have been shown to result

in gross underestimates and detect less than 1 in 10 cases of latent disease (Marijon, et al., 2007; Roberts, Colquhoun, Steer, Reményi, & Carapetis, 2013).

Within the last decade, many studies have used echocardiography alone to screen for RHD in asymptomatic children. Data using echocardiography to primarily screen for RHD has led to a marked increase in the prevalence of reported RHD (Seckeler & Hoke, 2011). In 2012, the World Heart Federation (WHF) released guidelines for characterizing RHD lesions, which has now helped to standardize their diagnosis (Reményi, et al., 2012). Since these guidelines are relatively recent, most studies reported in this chapter do not adhere to these guidelines. The inclusion or exclusion of asymptomatic disease represents millions of patients and creates a broad range of plausible estimates of the global prevalence of RHD. Therefore, Table 4 provides separate estimates of the prevalence of RHD. Supplementary Table 1 is based on studies that employed auscultation, then echocardiographic confirmation of clinical disease, and Supplementary Table 2 summarizes studies that diagnosed RHD based on echocardiographic screening only (without prior auscultation).

The distribution of RHD varies between continents, within countries, and by neighborhood. The highest RHD rates are reported in slum dwellers, followed by rural, then urban populations (Carapetis, 2005). Sub-Saharan Africa and Indigenous populations in Australia have the highest documented prevalence (Carapetis, Steer, Mulholland, & Weber, 2005b; Tibazarwa, Volmink, & Mayosi, 2008; Nkomo, 2007; Longo-Mbenza, et al., 1998), with high rates also documented in North Africa (Ba-Saddik, et al., 2011; Rossi, Felici, & Banteyrga, 2014), Latin America (Paar, et al., 2010; Miranda, Camargos, Torres, & Meira, 2014), the Indian subcontinent (Sadiq, et al., 2009; Saxena, et al., 2011; Shrestha, et al., 2012), and Asia (Marijon, et al., 2007). The highest national rates recorded were in South Africa, with recorded RHD prevalence of 5.7 per 1,000 in the 5–14 year population. This South African rate was derived from 14 studies that used clinical or echocardiographic confirmation of RHD (Carapetis, 2005; Carapetis, Steer, Mulholland, & Weber, 2005b). However, it is likely that an even greater prevalence exists in areas of sub Saharan Africa that do not have active surveillance programs. There are also high rates documented in Pacific Island nations, as well as in Indigenous Australians and New Zealanders (Carapetis, 2005; Carapetis, Steer, Mulholland, & Weber, 2005b; Carapetis, Currie, & Mathews, 2000).

Uncertainty about the prognostic significance of subclinical RHD has complicated efforts to extrapolate prevalence from screening of school children to adult populations. The prevalence of clinical RHD in 20-50 year olds is known to be greater than in the adolescent period, which reflects the natural history of progressive symptomatic valve disease (Carapetis, Steer, Mulholland, & Weber, 2005b; Carapetis, McDonald, & Wilson, 2005a; Sliwa, Carrington, Mayosi, Zigiriadis, Myungi, & Stewart, 2010b). As expected, there are much higher estimated rates of prevalence if asymptomatic disease diagnosed by echocardiographic screening is included.

	1990	2005	2010
Prevalence	29,172,383	33,468,203	34,232,795
YLL	13,267,810	9,670,605	8,720,292
YLD	1,150,422	1,365,502	1,429,575
DALY	14,418,232	11,036,107	10,149,867
Deaths	462,579	363,864	345,110

Compared to previous 2005 publication:
15.6 million cases
233,000 deaths

Figure 2. RHD rates, as estimated by the 2010 Global Burden of Disease Study.

Table 4: Hospital-based and cause of death Studies that relate to rheumatic heart disease (RHD)

Reference	Setting	Place	Year of Study	Age in Years	Findings
Grabauskas (Grabauskas, Gaižauskienė, Sauliūnė, & Mišeikytė, 2011)	Northern Europe	Lithuania	2001 to 2008	5 to 64	Mortality rate from chronic RHD was 0.3 per 100,000 population
Millard-Bullock (Millard-Bullock, 2012)	Caribbean	Jamaica	1975 to 1995	unknown	54 - 55% of hospital survey with RF
Jang (Jang, et al., 2014)	Asia	Korea	2006 to 2011	all	Prevalence of rheumatic valve lesions did not change between 2006 and 2011
Seo (Seo, et al., 2013)	Asia	Korea	2008	all	economic burden of RHD estimated at $67.25 million in Korea in 2008
Miyake (Miyake, Gauvreau, Tani, Sundel, & Newburger, 2007)	Established Market Economies	USA	2000	0 to 21	503 ARF hospitalizations occurred, 14.8 per 100,000 hospitalized children
Sriharibabu (Sriharibabu, Himabindu, & Kabir, 2013)	Indian Subcontinent	India	2007 to 2012	15 +	Screening in primary health centers, RHD prevalence 9.7/ 1000 population based on clinical criteria
Abdul-Mohsen (Abdul-Mohsen & Lardhi, 2011)	ME and North Africa	Saudi Arabia	1999 to 2010	5 to 29	ARF in 12 of 100,000 admissions, compared to 45 in 1987-98 (decreasing)
Olgunturk (Olgunturk, Canter, Tunaoglu, & Kula, 2006)	ME and North Africa	Turkey	1982 to 2002	4 to 19	609 admissions for RF, no difference between decades
Ozer (Ozer, Davutoglu, Sari, Akkoyun, & Sucu, 2009)	ME and North Africa	Ankara, Turkey	1999 to 2002	6 to 21	129 admissions for ARF in 3 year study period
Cuboni (Cuboni, Finau, & Cuboni, 2006)	Pacific and Indig Aus/ NZ	Fiji	1998 to 2000	1 to 17	Average 18 ARF cases/ year, 173 RHD cases/ year

Table 4 continued from previous page.

Reference	Setting	Place	Year of Study	Age in Years	Findings
Davies (Davies, Hofer, & Reeve, 2014)	Pacific and Indig Aus/ NZ	Indigenous Australians Kimberley, Western Australia	1990 to 2010	all	35 RHD attributable deaths found, RHD annual death rate was 15.6 per 100,000 in Aboriginal people
Lawrence (Lawrence, Carapetis, Griffiths, Edwards, & Condon, 2013)	Pacific and Indig Aus/ NZ	Northern Territory of Australia	1997 to 2010	all	For Indigenous RHD patients, the relative survival rate was 88.4% at 10 years after diagnosis and the standard mortality ratio was 1.56 (95% confidence interval , 1.23 – 1.96).
Milne (Milne, Lennon, Stewart, Vander Hoorn, & Scuffham, 2012)	Pacific and Indig Aus/ NZ, also market economy (NZ)	NZ Maori and Pacific islanders	2000 to 2009	all	Average of 159 RHD deaths per year, with a mean annual mortality rate of 4.4 per 100,000. Age adjusted mortality was 5 to 10 fold higher for Maori and Pacific peoples.
Dubey (Dubey, Sharma, & Chaurasia, 2012)	South Central Asia	Nepal	2010 to 2012	11 to 95	RHD was found in 25.5% of admissions for heart failure
Adebayo (Adebayo, et al., 2013)	Sub Saharan Africa	Nigeria	2011 to 2013	10 to 76	No case of RHD among 168 consecutive referrals
Adekanmbi (Adekanmbi, Ogunlesi, Olowu, & Fetuga, 2007)	Sub Saharan Africa	Sagamu, Nigeria	2002 to 2003	0 to 14	1% of heart failure admissions were due to RHD
Bode-Thomas (Bode-Thomas, Ige, & Yilgwan, 2013)	Sub Saharan Africa	Nigeria		0 to 18	RHD leading cause of acquired heart disease in children (58%) in review of echocardiography records
Cilliers (Cilliers, 2014)	Sub Saharan Africa	Gauteng, South Africa	1993 to 2010	3 to 16	ARF and RF admissions declined from 64 in 1993 to 3 in 2010
Damasceno (Damasceno, et al., 2012)	Sub Saharan Africa	9 countries in southern, eastern, central, western regions of Sub Saharan Africa	2007 to 2010	12+	14.3% of acute heart failure admissions were due to RHD
Kennedy (Kennedy & Miller, 2013)	Sub Saharan Africa	Blantyre, Malawi	2009 to 2011	0 to 15	RHD accounted for 22.4% of patients attending pediatric cardiology clinic
Ogeng'o (Ogeng'o J. A., Gatonga, Olabu, Nyamweya, & Ong'era, 2013)	Sub Saharan Africa	Kenya	2006 to 2010	0 to 12	RHD accounts for 14.6% of CHF admissions
Ogeng'o (Ogeng'o, Gatonga, & Olabu, 2011)	Sub Saharan Africa	Kenya	2005 to 2009	22 to 79	RHD accounted for 6.7% of cardiac deaths
Akinwusi (Akinwusi, Peter, Oyedeji, & Odeyemi, 2013)	Sub Saharan Africa	Osogbo, Nigeria	2003 to 2011	14 to 40	Mortality from RHD was 9.1%. Heart failure was the most common complication (90%)
Onwuchekwa (Onwuchekwa & Asekomeh, 2009)	Sub Saharan Africa	south Nigeria	2001 to 2005	18 to 100	RHD accounted for 4.3% of admissions for CHF
Sani (Sani, Mohammed, Bapp, & Borodo, 2007)	Sub Saharan Africa	Nigeria	2002 to 2004	over 15	RHD in 11.2% of echos performed

Table 4 continued from previous page.

Reference	Setting	Place	Year of Study	Age in Years	Findings
Sliwa (Sliwa K. , et al., 2010a)	Sub Saharan Africa	South Africa	2006 to 2007	Over 14	344 new cases of RHD (36% of cardiology admissions). Incidence estimated a 23.5 cases / 100,000 per annum
Tantchou Tchoumi (Tantchou Tchoumi, et al., 2011)	Sub Saharan Africa	Cameroon	2002 to 2008	8 to 86	Rheumatic valvulopathies accounted for 14.6% of CCF admissions in ages 8-20
Tantchou Tchoumi (Tantchou Tchoumi & Butera, 2013)	Sub Saharan Africa	Cameroon	2009 to 2011	0 to 103	Post rheumatic valvulopathies are the main cause of CHF in teenagers and young adults. Valvulopathies account for 35% of CHF admissions.
Zuhlke (Zühlke, et al., 2015)(Remedy)	Africa, India, and Yemen	Ethiopia, Kenya, Malawi, Rwanda, Uganda, Zambia, Egypt, India, Mozambique, Nigeria, Sudan, Yemen, Namibia, South Africa	2010 to 2012		Registry enrolled 3343 patients (median age 28 years, 66.2% female) presenting with RHD at 25 hospitals. The majority (63.9%) had moderate-to-severe multivalvular disease complicated by congestive heart failure (33.4%), pulmonary hypertension (28.8%), atrial fibrillation (21.8%), stroke (7.1%), infective endocarditis (4%), and major bleeding (2.7%). One-quarter of adults and 5.3% of children had decreased left ventricular (LV) systolic function; 23% of adults and 14.1% of children had dilated LVs.

** ME = Middle East
** NZ = New Zealand

Clinical Screening

(Supplementary Table 1)

RHD Prevalence over Time

RHD prevalence is directly related to economic factors, as shown by consecutive studies conducted in the same place both before and after economic development. Some data from consecutive studies in certain low-resource settings imply that the prevalence of RHD is falling. These studies may reflect declining prevalence in areas of economic and social development. In a rural area of Tamil Nadu, India, the prevalence of RHD appears to have dropped from 646 per 100,000 in 199 (Agarwal, Yunus, Ahmad, & Khan, 1995) to 68 per 100,000 in 2001–2002 (by clinical screening) (Jose & Gomathi, 2003). While studies performed in the economically developing regions in Western India have shown a decrease in prevalence, the rates of prevalence in the more economically disadvantaged Eastern regions of the country do not appear to be decreasing. Similarly, economic diversity in South East Asia is reflected in a highly variable burden of RHD estimates (Carapetis, 2008). Rural Pakistan has a community prevalence as high as 12 per 1000 people (Rizvi, et al., 2004). In South and Central America, some regions have been found to have a lower prevalence than in previous studies (1.3 per 1000 school children by clinical screening) (Marijon, Mirabel, Celermajer, & Jouven, 2012; Carapetis, Steer, Mulholland, & Weber, 2005b), while economically deteriorating regions now have a higher prevalence than was previously reported.

Clinical Study Comparisons to 2005 Data

Only studies using clinical screening criteria were used to compare recent rates to those reported in 2005. Comparison data are limited because there are only 12 recent studies that primarily used clinical criteria with echocardiographic confirmation for diagnosis. In 2005, the prevalence in Sub-Saharan Africa was found to be 5.7 per 1000. Although there are many studies that used echocardiography for screening, we found only one clinical study in Sub-Saharan Africa published since 2005. This study was performed in Mozambique, and the RHD rate was found to be 2.3 per 1000 (Marijon, et al., 2007). Therefore, there are inadequate clinical studies from Sub-Saharan Africa to determine if the prevalence of RHD in that region has changed since 2005. A 2014 review published by Rothenbuhler et al. found that the pooled prevalence of RHD detected by cardiac auscultation was 2.9 per 1000 people (95% CI 1.7–5), and by echocardiography, it was 12.9 per 1000 people (8.9–18.6) (Rothenbühler, et al., 2014).

The prevalence of RHD in South Central Asia was reported to be 2.2 per 1000 in 2005, and 1.2 per 1000 in 2008 (Carapetis, 2008). We found 7 studies published from the region since 2005, with an overall estimated prevalence of 2.6 per 1000 (Supplementary Table 1). However, this calculation is dominated by one study that included almost 230,000 children and that found a low prevalence of RHD (Jose & Gomathi, 2003). If that study is excluded, the prevalence rises to 10.2 per 1000. Based on these data, it is likely that the prevalence in this region has increased. Again, data from this region are difficult to interpret because of the heterogeneity of the region: studies done in areas of economic growth will show the rate of prevalence to be decreasing, while studies done in poorer rural areas show the rate of prevalence to be increasing.

The Middle East and North Africa regions were found to have a prevalence of 1.8 per 1000 in 2005. We found one recent study from Yemen which showed a very high rate of 36.5 per 1000 (Ba-Saddik, et al., 2011). Again, little can be inferred by using just one study for comparison.

Pacific and Indigenous Australia and New Zealand were found to have a prevalence of 3.5 in the 2005 study. There was one clinical study included since 2005, which showed a rate of 8.4 per 1000 (Steer, et al., 2009a). There are no current clinical studies to compare the "Asia other," "Latin America," "Eastern Europe," "China," or "established market economies" data from 2005.

Disease Detected by Echocardiographic Screening

Echocardiography as a primary mode of screening has greatly increased the detection rate of RHD, although the significance of subclinical RHD is still unclear. Not surprisingly, in Cambodia, the case detection rate rose from 8 to 79 (2.2 to 21.5 cases per 1,000) when echocardiography was used for primary screening, rather than auscultation (Marijon, et al., 2007) (Supplementary Table 2).

In our search, 16 studies were found that reported on echocardiographic screening for RHD since 2005. These studies employed echocardiography as the primary mode of screening for RHD, as opposed to those studies mentioned above, which used auscultation with echocardiographic confirmation. One of the highest prevalence of RHD was found in Tonga (33.20 per 1,000) (Carapetis, et al., 2008), and one of the lowest was found in India (0.68 per 1,000) (Jackson, Steer, & Campbell, 2011).

Mortality from RHD

Accurate mortality rates from RHD are difficult to estimate. The GBD 2010 study, which aims to provide complete systematic assessments of the data on all diseases and injuries, estimated that there were 345,000 deaths from RHD alone in 2010 (Lozano, et al., 2012) (Figure 2). The GBD 2010 study methodology relied on vital statistics data (which was taken largely from wealthy countries where robust efficient data systems exist) to inform epidemiological modeling for resource-poor countries with few real data sources. The GBD 2010 study methodology captures only the primary cause of death; for example, people dying of stroke with RHD as an

underlying cause were not captured as RHD-related deaths. The GBD 2010 study RHD mortality estimate is higher than the 2005 estimate; however, it is likely to under-estimate the true burden of RHD in resource-poor countries. Mortality rates are higher in low-resource settings, where secondary prophylaxis programs are not always robust and medical and surgical management of RHD is limited. In low-resource settings, an average of 1.5% of RHD patients are estimated to die annually (Carapetis, 2005) (Table 4).

The highest mortality rates from RHD or ARF were found in Indigenous populations of northern Australia (23.8 per 100,000). Not surprisingly, mortality rates from high resource settings were lower. The USA reported a rate of 1.65 per 100,000 (Jackson, Steer, & Campbell, 2011). As data emerge from resource-poor settings, a frightening pattern of high mortality in the first year after diagnosis is emerging, which highlights the very late stage of presentation of RHD and the lack of capacity to provide care, including surgery, for severe RHD. A study done in rural Ethiopia reported an annual mortality rate of 12.5%, with 70% of cases dying before the age of 25 years (Marijon, Mirabel, Celermajer, & Jouven, 2012; Günther, Asmera, & Parry, 2006). The RHD mortality was 125.3 per 1000 person-years (CI 67.4- 232.9); however, the sample was small (n=43) and a large proportion of cases were lost to follow-up over the study period (Günther, Asmera, & Parry, 2006). A study using RHD register-based data (n=257) followed a cohort of patients from northern India, with 1263 person-years of follow-up (Kumar, Raizada, Aggarwal, & Ganguly, 2002), and found a RHD mortality of 32.5/1000 person-years, with a mean age at death of 24.4 years.

RHD during pregnancy can have a grave prognosis and is an important cause of maternal mortality in low-resource settings (Sawhney, et al., 2003), and of morbidity in high-resource settings (Sartain, Anderson, Barry, Boyd, & Howat, 2012). Maternal deaths caused by an exacerbation of pre-existing medical conditions are termed indirect maternal mortality. Indirect deaths account for 27.5% of maternal mortality worldwide, ranging from 28.6% of maternal deaths in Sub-Saharan Africa to 16.8% of maternal deaths in South East Asia (Say, et al., 2014). A large proportion of these indirect deaths are the result of cardiovascular disease, particularly RHD. For example, 41% of indirect obstetric deaths in South Africa were associated with heart disease, which was predominantly RHD (71-84%) (Diao, et al., 2011). Of 50 pregnant women with heart disease in Senegal, 46 were found to have RHD. This resulted in 17 maternal deaths, 6 fetal deaths, and 5 therapeutic abortions (Diao, et al., 2011).

Other RHD Complications—Cardiac failure, infective endocarditis, and stroke

Cardiac failure, infective endocarditis (IE), and stroke are devastating sequelae that add to the burden of RHD and ARF. In Australia, 28% of those diagnosed with RHD developed heart failure at some stage between diagnosis and the end of their 13-year study period (Lawrence, Carapetis, Griffiths, Edwards, & Condon, 2013).

A review of eight studies of stroke from resource-limited settings concluded that between 3 and 7.5% of all strokes are directly attributable to RHD (Carapetis, Steer, Mulholland, & Weber, 2005b). The Global Burden of Disease Study estimated that, during 2010, 11.5 million people suffered their first stroke in less developed countries (Krishnamurthi, et al., 2013). Based on the estimated percentage of strokes attributable to RHD in less developed countries, this would equate to 345,000 to 862,500 strokes per year that can be attributed to RHD.

RHD is the most common underlying heart lesion in IE in resource-limited settings (Yew & Murdoch, 2012) (Table 5). In a systematic review of 11 studies, RHD was the underlying cause of valve disease in 63% of cases of IE, and the endocarditis mortality rate in low-resource countries was 25% (Carapetis, Steer, Mulholland, & Weber, 2005b). A study performed in Brazil found the mortality rate to be even higher, at 31% (Nunes, Gelape, & Ferrari, 2010). Thus, RHD is a significant cause of IE-related morbidity and mortality in resource-limited settings.

The 2010 Global Burden of Disease study reported the incidence of IE to be between 1.5 to 11.6 cases per 100,000 people (Bin Abdulhak, et al., 2014). The only low or middle-income country that reported an IE incidence was Tunisia, with an incidence of 5.5 cases per 100,000 people. If we assume this incidence, with the median proportion of cases due to RHD (37.5%) and IE mortality (19%) from resource-limited countries, with an estimated population of 3,798,429,000 people over 5 years in resource-limited settings (The United Nations, 2012), this infers that there are 14,885 RHD-related IE deaths annually.

Table 5: Studies published since 2005 that document the association of rheumatic heart disease (RHD) and infective endocarditis

Reference	Setting	Country	Year of study	Age in Years	Proportion of IE with RHD as predisposing factor	Rank of RHD as predisposing factor	Notes / mortality
Assiri (Assiri, 2011)	ME and Northern Africa	Asseer, Saudi Arabia	2002 to 2007	13 to 65	71%	most common	
Correa de Sa (Correa de Sa, et al., 2010)	Established Market Economies	Minnesota, USA	1970 to 2006	adults	5% (in 2001 - 2006)	5th (tied)	proportion decreased from 31% in 1975 - 1979 to 5% in 2001-2006
Day (Day, Gauvreau, Shulman, & Newburger, 2009)	Established Market Economies	USA	2000 to 2002	0 to 21	5%	3rd	
Elbey (Elbey, et al., 2013)	ME and Northern Africa	Turkey	2005 to 2013	13 to 87	28%	1st	
Ferraris (Ferraris, et al., 2013)	Established Market Economies	Italy	2003 to 2010	43 to 72	5%	5th	RHD not associated with increased in hospital mortality
Garg (Garg, et al., 2005)	South Central Asia	India	1992 to 2001	4 to 68	47%	1st	21% in hospital mortality
Johnson (Johnson, Boyce, Cetta, Steckelberg, & Johnson, 2012)	Established Market Economies	USA	1980 to 2011	0 to 18	0%	n/a	8% (1950-1979)
Kucukates (Kucukates, Gultekin, & Bagdatli, 2013)	South Central Asia	Pakistan	2001 to 2010	47 +/- 18	55%	1st	operative mortality 18%
Lertsapcharoen (Lertsapcharoen, et al., 2005)	Asia	Thailand	1987 to 2004	0 to 15	12%	2nd	overall mortality 12%
Ma (Ma, Li, Que, & Lv, 2013)	Asia	China	2002 to 2011	35 to 61	5%	4th	20% mortality with RHD, 10% overall mortality
Nashmi (Nashmi & Memish, 2007)	ME and Northern Africa	Saudi Arabia	1993 to 2003	0 to 78	26%	1st	Hospital mortality 8.5%
Nakagawa (Nakagawa, et al., 2014)	Asia	Japan	1990 to 2009	all	0%	n/a	

Table 5 continued from previous page.

Reference	Setting	Country	Year of study	Age in Years	Proportion of IE with RHD as predisposing factor	Rank of RHD as predisposing factor	Notes / mortality
Nunes (Nunes, Gelape, & Ferrari, 2010)	Latin America	Brazil	2001 to 2008	15 to 76	39%	1st	31% overall in hospital mortality, RHD not associated with increased mortality
Pazdernik (Pazdernik, Baddour, & Pelouch, 2009)	Central Europe	Czech Republic	1998 to 2006	18 and older	17%	2nd	
Senthilkumar (Senthilkumar, Menon, & Subramanian, 2010)	South Central Asia	India	2008 to 2010	16 to 46	81%	1st	
Stockins (Stockins, Neira, Paredes, Castillo, & Troncoso, 2012)	Latin America	Chile	2003 to 2010	34 to 66	10%	1st	Overall hospital mortality 27%
Sucu (Sucu, Davutoğlu, Ozer, & Aksoy, 2010)	ME and Northern Africa	Turkey	2004 to 2007	18 to 80	36%	1st	15.3% hospital mortality
Trabelsi (Trabelsi, et al., 2008)	ME and Northern Africa	Tunisia	1997 to 2006	4 to 73	45.00%	1st	Overall mortality 19%
Tugcu (Tuğcu, et al., 2009)	ME and Northern Africa	Turkey	1997 to 2007	16 -88	40%	1st	Overall hospital mortality 25%
Walls (Walls, et al., 2014)	Established Market Economies	New Zealand	2000 to 2005	18 and older	4%	6th	6% overall mortality
Wang (Wang, et al., 2012)	Asia	China	2005 to 2012	all	6.80%	3rd	In Hospital overall mortality 6.7%
Watt (Watt, et al., 2014)	Asia	Thailand	2010 to 2012	16 to 85	28%	2nd	12.1% overall 1 month mortality
Webb (Webb, et al., 2014)	Established Market Economies and Maori NZ	New Zealand	1994 to 2012	0 to 16	11%	2nd	
Yiu (Yiu, et al., 2007)	Asia	Hong Kong	1995 to 2005	18 +	18%	2nd	major cardiovascular events in 28% of total with IE

Invasive Disease

S. pyogenes has the ability to penetrate epithelial surfaces and cause a wide array of invasive diseases, including bacteremia, cellulitis, and necrotizing fasciitis, all of which may also involve streptococcal toxic shock syndrome (STTS). Other, less common invasive *S. pyogenes* diseases include septic arthritis, puerperal sepsis, meningitis,

abscess, osteomyelitis, endocarditis, and peritonitis. Globally, in 2005 it was estimated that at least 663,000 cases of invasive *S. pyogenes* disease occurred each year, which resulted in 163,000 deaths (Carapetis, Steer, Mulholland, & Weber, 2005b) (Table 6).

It appears that the incidence of invasive *S. pyogenes* disease is increasing (Henningham, Barnett, Maamary, & Walker, 2012; Cleary, et al., 1992; Cole, Barnett, Nizet, & Walker, 2011). In 2005, the reported incidence of invasive *S. pyogenes* infections was 1.5–3.9 cases per 100,000 population per year in high-income countries (Carapetis, 2005); 6.4–10.2 per 100,000 in Australian non-Indigenous populations (Carapetis & Currie, 1999; Norton, et al., 2004); 13 per 100,000 in Kenyan children (Berkley, et al., 2005), and up to 82.5 per 100,000 in Australian Indigenous populations (Norton, et al., 2004). In Fiji, the incidence of *S. pyogenes* bacteremia in people over 5 years of age was found to be 11.6 per 100,000, with Indigenous Fijians disproportionately affected (Steer, et al., 2008).

The proportion of *S. pyogenes* positive blood cultures is highest in infants, and progressively falls during the first years of life. A WHO Young Infants Study found that *S. pyogenes* accounted for 29% of all bacteremia isolates in children under 90 days of age in four less developed countries (Papua New Guinea, Ethiopia, The Gambia, and the Philippines) (The WHO Young Infants Study Group, 1999). In Kenya, *S. pyogenes* is the fifth most common cause of community-acquired bacteremia in children less than 5 years of age, with a case fatality of 25% among all children (Berkley, et al., 2005).

Resource-limited environments have higher incidences of invasive *S. pyogenes*, but it carries a high mortality rate, no matter the setting. Overall, about 20% of patients with invasive *S. pyogenes* disease die within 7 days of infection (Henningham, Barnett, Maamary, & Walker, 2012; Lamagni, et al., 2008; O'Grady, et al., 2007; O'Loughlin, et al., 2007). Our literature review of population-based and multicenter hospital-based studies after 2005 revealed mortality rates of 4%–32% (Table 6), with high mortality rates in both resource-rich and resource-poor settings.

Table 6: Population-based studies of the incidence of invasive group A streptococcal infections

Reference	Year of Study	Place	Age in Years	Incidence (cases per 100,000 per year)	Mortality	Notes
Ekelund (Ekelund, Skinhøj, Madsen, & Konradsen, 2005)	2002	Denmark	all	3	23%	
Hollm-Delgado (Hollm-Delgado, Allard, & Pilon, 2005)	1995 to 2001	Montreal, Canada	All	2.4	14%	
Imohl (Imöhl, Reinert, Ocklenburg, & van der Linden, 2010)	2003 to 2007	Germany	All	0.7		
Kittang (Kittang, et al., 2011)	2006 to 2009	Norway	All	5	10% at 30 days	
Laupland (Laupland, Ross, Church, & Gregson, 2006)	1999 to 2004	Canada	All	4.3		
Le Hello (Le Hello, et al., 2010)	2006	New Caledonia	all	38		
LePoutre (Lepoutre, et al., 2011)	2006 to 2007	France	all	3.1	14%	case fatality higher amongst older people
Luca-Harari (Luca-Harari, et al., 2008)	2003 to 2004	Denmark	all	2.6	20%	case fatality higher amongst older people

Table 6 continued from previous page.

Reference	Year of Study	Place	Age in Years	Incidence (cases per 100,000 per year)	Mortality	Notes
Martin (Martin, Murchan, O'Flanagan, & Fitzpatrick, 2011)	2004 to 2010	Ireland	all	1.6	8%	incidence doubled from 2004 to 2009 and 2010, highest incidence under 5 and over 60
Meehan (Meehan, Murchan, Bergin, O'Flanagan, & Cunney, 2013)	2012 to 2013	Ireland	all	2.7	13%	incidence increased since 2010
O'Grady (O'Grady, et al., 2007)	2002 to 2004	Victoria, Australia	all	2.7	8%	Incidence highest in children < 5 and adults > 65
O'Loughlin (O'Loughlin, et al., 2007)	2000 to 2004	USA	all	3.5	14%	
Olafsdottir (Olafsdottir, et al., 2014)	1975 to 2012	Iceland	all	3.96	13% adults, 6% children	30 day mortality
Safar (Safar, et al., 2011)	2005 to 2006	New Zealand	all	8.1	10%	overall
Safar (Safar, et al., 2011)	2005 to 2006	New Zealand	all	20.4		Maori and Pacific Islanders
Steer (Steer A. C., et al., 2008)	2000 to 2005	Fiji	all	11.6	28%	Indigenous Fijians 4.6 times more likely to present with invasive BHS than other ethnicities
Steer (Steer A. C., et al., 2008)	2005 to 2007	Fiji	all	9.9	32%	Incidence rate ratio for indigenous Fijians 2.9, Highest rates in elderly and the young
Stockmann (Stockmann, et al., 2012)	2002 to 2010	Utah, USA	all	6.3	4%	higher than national rate of 3.5
Tyrrell (Tyrrell, Lovgren, Kress, & Grimsrud, 2005)	2000 to 2002	Alberta, Canada	all	3.8		incidence went from 5 to 3.8
Vlaminckx (Vlaminckx, et al., 2004; Whitehead, Smith, & Nourse, 2011)	1994 to 2003	the Netherlands		3.1 (2002)		incidence decreased since 1995
Whitehead (Whitehead, Smith, & Nourse, 2011)	2004 to 2009	Australia	0 to 18	3.5 (overall) 13.2 (indigenous)	4.1% (overall)	
Williamson (Williamson, et al., 2015)	2002 to 2012	New Zealand	all	7.9 (2012)	7.7 (7 days) 10.1 (30 days)	incidence increased from 3.9 in 2002

Challenges and the Way Forward

Even a decade after the first comprehensive review in 2005, quantifying the burden of *S. pyogenes* disease remains a global challenge. Although more data are emerging, our ability to confidently estimate the incidence and mortality from *S. pyogenes* remains limited because of the lack of high quality data from large parts of the

world, particularly from low-resource settings that appear to bear the greatest burden of disease. However, the REMEDY study is a recently published registry that has employed the systematic collection of RHD data from low and middle-income countries. This prospective registry enrolled 3343 patients (with a median age 28 years and that were 66.2% female) who presented with RHD at 25 hospitals in 12 African countries, India, and Yemen between January 2010 and November 2012. The study found that RHD patients were young, predominantly female, and had a high prevalence of major cardiovascular complications (Zühlke, et al., 2015). However, the available data on incidence and mortality from RHD, APSGN, and invasive disease, especially in children and young adults, support the conclusion that these diseases are significant causes of premature morbidity and mortality.

Establishing the true burden of *S. pyogenes* diseases is critically important as efforts to reduce morbidity and mortality progress. In particular, understanding the burden of *S. pyogenes* is an economic, public health, and advocacy prerequisite for further investment and development of an *S. pyogenes* vaccine. High-quality epidemiologic data will help assign an appropriate priority to these efforts and identify appropriate settings in which vaccine and other trials could be conducted (Jackson, Steer, & Campbell, 2011).

Non-vaccine strategies to reduce the burden of some *S. pyogenes* manifestations continue to progress. Developments in health systems and care delivery may provide opportunities to optimize primary and secondary prevention of ARF. In particular, secondary prevention with long-term antibiotics can prevent disease progression from ARF to RHD. Emerging data from developing countries indicate that clinical presentations with RHD occur only at a very advanced stage of diseases. Early echocardiography-based identification of asymptomatic RHD through active surveillance programs and the subsequent use of prophylactic antibiotics may help to prevent the devastating complications of RHD. It is critical that individuals with RHD that has been detected on echocardiographic screening are followed in rigorous research projects to identify the natural history of these lesions. Improved understanding of the natural history of RHD will determine which children identified through echocardiography screening are most appropriately included in burden of disease estimates. Finally, burden of disease estimates remain important for informing and monitoring essential efforts in primordial prevention. Understanding and addressing *S. pyogenes* disease risk factors including poverty, overcrowding, malnutrition, and maternal educational level and employment are essential elements of disease control programs (Marijon, Mirabel, Celermajer, & Jouven, 2012).

S. pyogenes is an important global pathogen with diverse clinical manifestations and limited epidemiologic data. Despite the paucity of high-quality studies, it is evident that resource-limited settings shoulder the majority of the burden of morbidity and mortality from *S. pyogenes*. As a result, there should be a focus on improving reporting systems and strengthening prevention and treatment programs in these parts of the world.

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Pharyngitis and Scarlet Fever

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Abstract

Pharyngitis, or sore throat, is the most common manifestation of infection with *Streptococcus pyogenes*. Sore throat is a frequently presenting complaint for outpatient medical visits; as a result, infection with *S. pyogenes* is diagnosed in 20 to 40% of pharyngitis cases in children, and in 5 to 15% in adults. Scarlet fever denotes a clinical syndrome that is characterized by the presence of a rash along with an *S. pyogenes* infection, usually pharyngitis. In this chapter, the topics covered include pathogenesis of pharyngitis, animal models of *S. pyogenes* pharyngitis, adherence of *S. pyogenes* to epithelial cells, intracellular survival and persistence in the pharynx, regulation of capsule production, *S. pyogenes* survival in the pharynx, and immunity to pharyngitis. Finally, there is an overview and discussion of clinical features, complications, diagnosis, and treatment of these diseases.

Pharyngitis, or sore throat, is the most common manifestation of infection with *Streptococcus pyogenes*. Sore throat is a frequent presenting complaint for outpatient medical visits, and infection with *S. pyogenes* is diagnosed in 20 to 40% of pharyngitis cases in children and in 5 to 15% in adults (Ebell, Smith, Barry, Ives, & Carey, 2000; Shaikh, Leonard, & Martin, 2010). The peak incidence of *S. pyogenes* pharyngitis occurs in children 5 to 15 years of age (Danchin, et al., 2007). Infection is more common during winter and spring in temperate climates. Outbreaks may occur in households, schools, military facilities, and other settings in which there is close human-to-human contact. There is no known environmental reservoir or natural animal host of *S. pyogenes*, apart from human beings; and as a result, direct or indirect contact with an infected person is the source of human infection. Transmission is thought to occur primarily by large droplets from respiratory secretions, although spread through contaminated objects and food are well-described alternate routes of transmission. Unpasteurized milk and contaminated food have also been sources of several well-documented *S. pyogenes* outbreaks (Dublin, Rogers, Perkins, & Graves, 1943; Kemble, et al., 2013).

Scarlet fever, or streptococcal pharyngitis associated with a characteristic rash, has been recognized for centuries. "Scarlatina" was clearly distinguished from other febrile rash illnesses (such as measles, in particular) by Sydenham in the 17th century. Epidemics of scarlet fever with high mortality occurred in cities in Europe and North America as late as the latter part of the 19th century (Rolleston, 1928). The historical aspects of scarlet fever are described in detail in Chapter 1. Since the advent of penicillin treatment, scarlet fever has become less common and fatal cases are extremely rare. However, some large outbreaks have been reported in the 21st century, most notably in China and Hong Kong (Yang, et al., 2013).

Pathogenesis

Todd and Lancefield observed that *S. pyogenes* freshly isolated from patients with tonsillitis or scarlet fever grew as "matt" colonies (those that are large, with an irregular surface), a characteristic that was associated with production of "M substance;" that is, the M protein (Todd & Lancefield, 1928). The matt (or matte) colony type was subsequently shown to be a later stage of the mucoid colony morphology, which occurs due to abundant production of the hyaluronic acid capsular polysaccharide (Wilson, 1959). In contrast to strains associated with symptomatic infection, isolates from individuals undergoing tonsillectomy for enlarged tonsils yielded a mixture

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of matte and glossy (small, smooth) colonies. In vitro passage of matte isolates often resulted in their conversion to the glossy morphology, with the latter colony type being deficient in both the M protein and capsule. These observations suggested that expression of the M protein and capsule were associated with symptomatic *S. pyogenes* pharyngitis, although not necessarily with asymptomatic pharyngeal carriage. More recent studies on clinical isolates and in experimental infection in non-human primates have supported these early insights (see below).

Animal models of *S. pyogenes* pharyngitis

Human beings are the only known natural hosts for *S. pyogenes* infection, and for pharyngitis in particular, it has been difficult to mimic human infection in animal models. Studies in mice have demonstrated colonization of the upper airway after intranasal challenges, but either the organisms are cleared over a few days, or the animals develop rapidly progressive pneumonia and systemic infections (Husmann, Dillehay, Jennings, & Scott, 1996; Wessels & Bronze, 1994). A study in rats showed the persistence of low numbers of *S. pyogenes* in throat cultures for several weeks in approximately 50% of animals (Hollingshead, Simecka, & Michalek, 1993). Models in non-human primates are thought to most closely resemble the features of human infection. A high rate of colonization has been demonstrated in rhesus and cynomolgous macaques and in baboons after intranasal inoculation or direct introduction of *S. pyogenes* into the oropharynx (Ashbaugh, et al., 2000; Skinner, et al., 2011; Virtaneva, et al., 2005; Watson, Rothbard, & Swift, 1946). Although one study in cynomolgous macaques reported pharyngeal erythema and swelling during infection, other studies have observed only minimal erythema or no clinical signs of infection, despite persistent colonization. In a baboon model, an M-type 3 invasive clinical isolate colonized the pharynx of all six animals after oral inoculation and persisted for at least 42 days (Ashbaugh, et al., 2000). In marked contrast, in the same study, an isogenic M protein-deficient mutant colonized only 2 of 6 animals after inoculation and was cleared from both after 14 days. A mutant that produced wild-type amounts of M protein but lacked the hyaluronic acid capsule colonized 5 of 6 animals, but was cleared by 28 days. These results suggest that M protein is required both for initial colonization of the pharynx and for persistence. The capsule appears to be unnecessary for colonization, but its presence enhanced persistence in this model.

S. pyogenes adherence to epithelial cells

In vitro studies have attempted to identify the molecular basis for the adherence of *S. pyogenes* to the oropharyngeal epithelium. Experiments that tested the attachment of *S. pyogenes* to various cell lines in vitro have identified multiple bacterial surface molecules that appear to mediate or modulate adherence. A two-step model of adhesion has been proposed, in which an initial weak interaction involves lipoteichoic acid, and a second phase of more avid binding is mediated by *S. pyogenes* surface proteins (Hasty, Ofek, Courtney, & Doyle, 1992). The participation and relative importance of particular adhesins varies widely and depends on bacterial strain, growth phase, target cell type, and the presence or absence of potential bridging molecules, such as fibronectin or fibrinogen. Certain M proteins contribute to *S. pyogenes* adherence to human cells, either directly or through binding with an intermediary integrin-binding protein, such as fibronectin. Types 6 and 24 M proteins mediated attachment to oropharyngeal keratinocytes in vitro, but type 18 did not (Schrager, Albertí, Cywes, Dougherty, & Wessels, 1998). On the other hand, type 1 M protein appears to inhibit adherence to oropharyngeal keratinocytes (Anderson, et al., 2014). The hyaluronic acid capsule can inhibit M protein-mediated adherence. However, the capsule itself mediates *S. pyogenes* attachment to CD44, a hyaluronic acid-binding glycoprotein that is expressed on many cell types, including oropharyngeal cells (Schrager, Albertí, Cywes, Dougherty, & Wessels, 1998). Eleven or more fibronectin-binding proteins are expressed by various strains of *S. pyogenes*. Binding of fibronectin can link surface proteins on the bacterial cell to fibronectin-binding $\alpha_5\beta_1$ integrins on human epithelial cells (Yamaguchi, Terao, & Kawabata, 2013). The level of expression of $\alpha_5\beta_1$ integrins on keratinocytes may be lower than on other cell types (Edwards, Potter, Meenan, Potts, & Massey,

2011), and the expression of fibronectin-binding protein F1 by *S. pyogenes* has been reported to direct bacterial binding to Langerhans cells in human skin tissue sections in vitro (Okada, Pentland, Falk, & Caparon, 1994).

Intracellular survival and persistence in the pharynx

Although *S. pyogenes* is considered an extracellular pathogen, multiple studies have demonstrated the internalization of the organism by human epithelial cells, including oropharyngeal keratinocytes, in vitro (LaPenta, Rubens, Chi, & Cleary, 1994; Molinari, et al., 1987; Ozeri, Rosenshine, Mosher, Fässler, & Hanski, 1998; Schrager, Rheinwald, & Wessels, 1996). Clinical isolates associated with pharyngitis or asymptomatic carriage appear to be internalized more efficiently than isolates associated with invasive infection, and it has been suggested that entry into epithelial cells may enhance persistent colonization by protecting intracellular bacteria from immune effectors and antibiotics (Molinari & Chhatwal, 1998). Conversely, strains that are associated with invasive infection tend to be internalized inefficiently, perhaps because they produce larger amounts of hyaluronic acid capsule and streptolysin O (SLO), both of which reduce *S. pyogenes* internalization by epithelial cells in vitro (Schrager, Rheinwald, & Wessels, 1996; Håkansson, Bentley, Shakhnovic, & Wessels, 2005). The production of SLO also influences the intracellular survival of *S. pyogenes* within epithelial cells. As they are both pore-forming toxins, SLO and streptolysin S (SLS) induce the formation of autophagosome-like compartments in epithelial cells (Nakagawa, et al., 2004; O'Seaghdha & Wessels, 2013). Streptococci are internalized into an early endosome; however, the production of SLO results in damage to the endosomal membrane and exposure of *S. pyogenes* to the cytosol, where they bind ubiquitin. Ubiquitination of the bacteria results in targeting of the autophagy pathway. In the absence of SLO, production of SLS can sufficiently damage the endosomal membrane to recruit galectin 8, a cytosolic lectin that binds to galactosides that are normally found on the interior surface of vacuolar compartments, but which become exposed upon SLS-mediated membrane injury (O'Seaghdha & Wessels, 2013). Galectin 8 binding to the endosome provides an additional means to traffic *S. pyogenes* to autophagosomes. Many *S. pyogenes* strains also produce NAD-glycohydrolase (NADase), an enzyme that is translocated into the cytosol of epithelial cells in an SLO-dependent fashion. NADase inhibits the fusion of lysosomes with *S. pyogenes*-containing autophagosomes, preventing their maturation into degradative autolysosomes and prolonging intracellular survival. In this way, SLO and NADase enhance the survival of *S. pyogenes* within epithelial cells and may contribute to its overall persistence in the pharynx.

Regulation of capsule production and *S. pyogenes* survival in the pharynx

The global two-component regulatory system CsrRS (or CovRS) appears to play a critical role in the adaptation of *S. pyogenes* for survival and persistence in the pharynx. Inactivating mutations are present in *csrS* or, less often, in *csrR* in approximately 40% of clinical isolates from patients with necrotizing fasciitis or streptococcal toxic shock. By contrast, such mutations are rarely observed among pharyngitis isolates—an observation that suggests CsrRS contributes to the survival of *S. pyogenes* in this host environment (Ikebe, et al., 2010; Shea, et al., 2011). Further support for this view comes from studies that show a competitive advantage of wild-type *S. pyogenes*, as compared to CsrRS mutants during growth in human saliva (Treviño, et al., 2009). Experiments using a mouse model of upper airway colonization also showed reduced colonization with CsrRS mutants (Alam, Turner, Smith, Wiles, & Sriskandan, 2013). Along with several other virulence factors, synthesis of the hyaluronic acid capsule is regulated by CsrRS (Levin & Wessels, 1998). The overproduction of capsule by CsrRS mutants favors resistance to phagocytosis and survival in blood or deep tissues, but may impair persistence in the pharynx. A study of serial throat isolates from monkeys with experimental *S. pyogenes* pharyngeal infection showed an accumulation of mutations over time that resulted in reduced capsule expression, which is consistent with this hypothesis (Shea, et al., 2011). In addition, clinical isolates from patients with pharyngitis revealed a similar pattern of mutations that reduced capsule production. These observations seem inconsistent with the finding discussed above, in which an acapsular mutant strain was more rapidly cleared from the pharynx in a

non-human primate model. These apparently contradictory results may be explained by the fact that the capsule not only impairs adherence to the pharyngeal mucosa and internalization by epithelial cells (thereby impairing colonization), but also helps to defend the bacteria against opsonophagocytic killing in the non-immune host (thereby resisting clearance). Highly encapsulated or mucoid strains of *S. pyogenes* have certainly been associated with outbreaks of pharyngitis and acute rheumatic fever (Marcon, et al., 1988; Veasy, et al., 1987; Westlake, Graham, & Edwards, 1990); however, the downregulation of capsule production may favor asymptomatic pharyngeal carriage.

Immunity to pharyngitis

Early studies in mice by Lancefield and others established that protective immunity to systemic *S. pyogenes* infection was conferred by opsonic serum antibodies against the M protein (Lancefield, 1962). Protective antibodies were type-specific, recognizing the antigenically variable amino terminal domain of M protein. Antibodies elicited from immunization with killed *S. pyogenes* bacteria protected against challenges by strains of the same, but not different, M types. Studies in non-human primates suggest that immunity to pharyngeal infection also is type-specific. Pharyngeal colonization of baboons with an M type 3 strain resulted in complete protection against subsequent rechallenge with the same strain, but no protection against infection with a type 1 strain (Ashbaugh, et al., 2000). The type-specificity of immunity in human pharyngitis is less well-defined. A prospective natural history study of the spread of several strains of *S. pyogenes* among families suggested that pre-existing type-specific serum antibodies did not prevent pharyngeal acquisition nor influence the duration of carriage (Guirguis, Fraser, Facklam, El Kholy, & Wannamaker, 1982). Fox et al. found that immunization with type 1 M protein had only a modest and statistically insignificant effect on pharyngeal colonization in volunteers after challenge with a type 1 strain, but that immunized subjects had a significantly lower rate of symptomatic infection, as compared to subjects in the control group (5% vs 47%) (Fox, Waldman, Wittner, Mauceri, & Dorfman, 1973). When taken together, these studies indicate that type-specific antibodies to M protein play an important role in protection against symptomatic infection. There is some evidence for type-specific immunity to pharyngeal colonization, although it is less clear whether such protection is mediated primarily or exclusively by type-specific serum antibodies. Local mucosal immunity induced by prior exposure or by a mucosal vaccine may also play a role in overall immunity to colonization.

Clinical features

The classical presentation of streptococcal pharyngitis begins with an abrupt onset of fever, malaise, and sore throat. Pain with swallowing and the presence of swollen, tender anterior cervical lymph nodes are typical features. Abdominal pain and vomiting is common, especially in younger children. Cough, rhinorrhea, hoarseness, conjunctival irritation, and diarrhea are notably absent in streptococcal pharyngitis, and the presence of these symptoms should suggest a non-streptococcal (usually viral) etiology. Physical findings include fever (often greater than 39 °C), erythema, and edema of the tonsils and posterior pharynx, which may be covered with a patchy white or yellowish exudate (Figure 1). Petechiae may be present on the soft palate. Anterior cervical lymph nodes typically are enlarged, firm, and tender. The presence of most or all of these characteristic clinical features is suggestive of, but not specific to, *S. pyogenes* pharyngitis. Without treatment, sore throat usually resolves in 3 to 6 days, and fever abates within 1 week. Despite the resolution of symptoms, throat cultures often remain positive for several weeks in the absence of antibiotic treatment (Catanzaro, et al., 1954). Infectious mononucleosis from the Epstein-Barr virus can have a similar presentation, as can infection with adenovirus or other respiratory viruses. Less commonly, a similar syndrome can be caused by various bacterial or viral pathogens. Conversely, culture-proven *S. pyogenes* pharyngeal infection may be associated with milder signs and symptoms than those described above. For these reasons, a diagnosis on clinical grounds alone is unreliable. Exudative pharyngitis is uncommon in children younger than 3 years of age, although *S. pyogenes* infection can occur in this age group and may be manifested through fever, lymphadenopathy, and mucopurulent rhinitis.

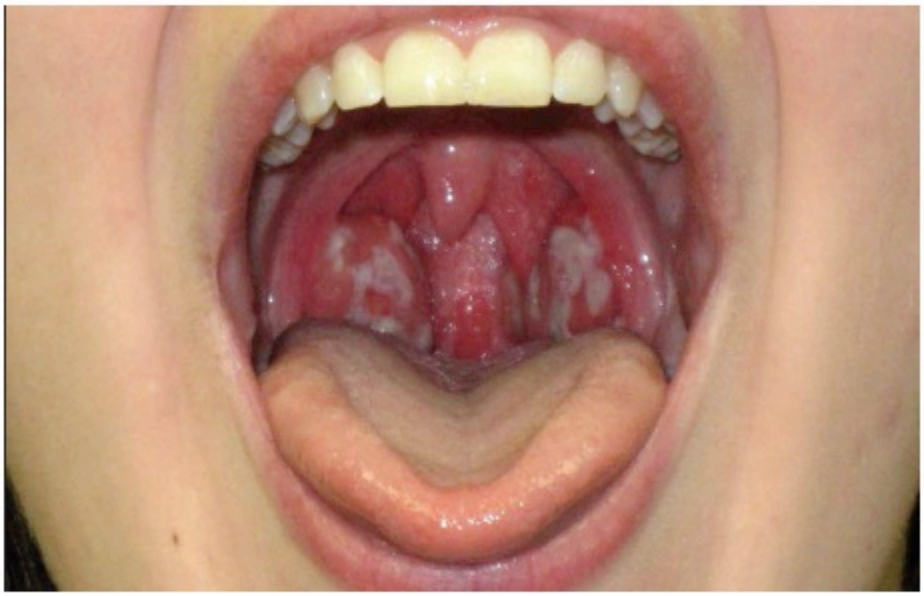

Figure 1. A 15-year-old girl with fever and exudative streptococcal pharyngitis (from (Block, 2014)).

Complications

Suppurative complications of streptococcal pharyngitis can arise from direct extension of pharyngeal infection to adjacent structures, or by hematogenous or lymphatic spread to more remote sites. Such complications include peritonsillar or retropharyngeal abscess, sinusitis, otitis media, cervical lymphadenitis, bacteremia, endocarditis, pneumonia, and meningitis. Local complications, such as peritonsillar or retropharyngeal abscess formation, should be considered in a patient with unusually severe or prolonged symptoms or localized pain associated with high fever and a toxic appearance. Non-infectious autoinflammatory complications can include acute rheumatic fever and post-streptococcal glomerulonephritis, both of which are thought to result from immune responses to streptococcal infection (see the chapters on PANDAS and poststreptococcal glomerulonephritis). Treatment of streptococcal pharyngitis with penicillin has been shown to reduce the likelihood of acute rheumatic fever, but not to reduce the likelihood of post-streptococcal glomerulonephritis.

Scarlet fever

Scarlet fever denotes a clinical syndrome characterized by the presence of a rash along with an *S. pyogenes* infection, usually pharyngitis. The rash typically begins on the first or second day of illness, initially on the trunk, and spreads to involve the extremities, sparing the palms and soles of the feet. The rash is often

accentuated in flexural creases, such as in the antecubital fossae and axillae (Pastia's lines). The cheeks are flushed, with sparing of the area around the mouth (circumoral pallor) (Figure 2). The rash is made up of minute papules, giving a characteristic "sandpaper" feel to the skin. Enlarged papillae may be seen on a coated tongue (strawberry tongue), which later may become denuded. The rash generally fades in 6–9 days, and is followed by desquamation of the palms and soles after several days, which typically begins on the fingertips at the free margin of the fingernails. The differential diagnosis of scarlet fever includes viral exanthems, Kawasaki disease, staphylococcal toxic shock syndrome, and allergic reactions.

The pathogenesis of scarlet fever is not completely understood. Studies in the 1920s by George and Gladys Dick and others implicated one or more secreted *S. pyogenes* proteins, which was previously called erythrogenic toxin, and which are now classified as pyrogenic exotoxins (Birkhaug, 1925; Dick & Dick, 1924; Dick & Dick, 1983). Injection of culture filtrates of scarlet fever-associated *S. pyogenes* evoked a rash in some naïve subjects, but not in those who had recovered from scarlet fever. Intradermal injection of antiserum to the scarlet fever strain caused blanching of the rash. Together, these observations suggested that the rash-associated illness was at least partially due to the effects of *S. pyogenes* products present in the culture supernatant. Subsequent research has identified erythrogenic toxin as a group of related streptococcal pyrogenic exotoxins (SPEs). Eleven such toxins have been identified, and an individual strain typically produces 4 to 6 of them (Spaulding, et al., 2013). No single toxin has been consistently implicated in scarlet fever, although SpeA, SpeC, and SSA, often in combination, have been associated with several outbreaks (Davies, et al., 2015; Silva-Costa, Carriço, Ramirez, & Melo-Cristino, 2014; Tyler, et al., 1992; Yu & Ferretti, 1989). The SPEs also appear to play an important role in the severe, systemic manifestations of streptococcal toxic shock syndrome through their activity as superantigens that are capable of stimulating cytokine secretion from a large population of T cells in an antigen-independent fashion (see the chapter on streptococcal superantigens).

Diagnosis

Scoring systems have been devised to improve the accuracy of clinical diagnoses of *S. pyogenes* pharyngitis. These systems assign points based on the presence or absence of suggestive clinical features, such as fever, absence of cough, presence of tonsillar exudates, and swollen, tender anterior cervical lymph nodes. The best-known clinical assessment tools are the Centor score and the very similar McIsaac score, which includes patient age as an additional criterion (Centor, Witherspoon, Dalton, Brody, & Link, 1981; McIsaac, White, Tannenbaum, & Low, 1998). A study analyzing data collected from more than 200,000 patients that were 3 years of age or older and were seen at a U.S. retail health chain found that the likelihood of a positive throat culture for *S. pyogenes* was 8% in individuals with a McIsaac score of 0, and rose to 55% in those with scores of 4 or 5 (Fine, Nizet, & Mandl, 2012). Algorithms based on such scoring systems have been used to limit the use of throat culture or rapid antigen detection tests in those patients in whom the probability of *S. pyogenes* infection is very low (McIsaac, Kellner, Aufricht, Vanjaka, & Low, 2004). For example, the clinical guidelines of the American College of Physicians-American Society of Internal Medicine and the U.S. Centers for Disease Control and Prevention recommend not testing for *S. pyogenes* in adults with a Centor score of 0 or 1 in the absence of special risk factors (Cooper, et al., 2001).

The "gold standard" for diagnosis of *S. pyogenes* pharyngitis is a positive culture from a properly collected throat swab specimen, i.e., a swab rubbed on both tonsillar pillars, avoiding the lips and tongue. Faster and more convenient rapid antigen tests are widely used in many clinical settings. These tests are highly specific, generally 95% or higher, so a positive result provides an immediate diagnosis and obviates the need for culture. In children and adolescents, a negative rapid test should be confirmed with a throat culture, as the sensitivity of the culture method is higher than that of the rapid antigen tests. Most clinical guidelines do not recommend the routine confirmation of a negative antigen test in adults, since the risk of rheumatic fever is extremely low in adults without a prior episode. Serologic assays for antibodies to *S. pyogenes* antigens, such as SLO or DNase B, are useful for retrospective diagnosis of an antecedent *S. pyogenes* infection in cases of suspected acute rheumatic

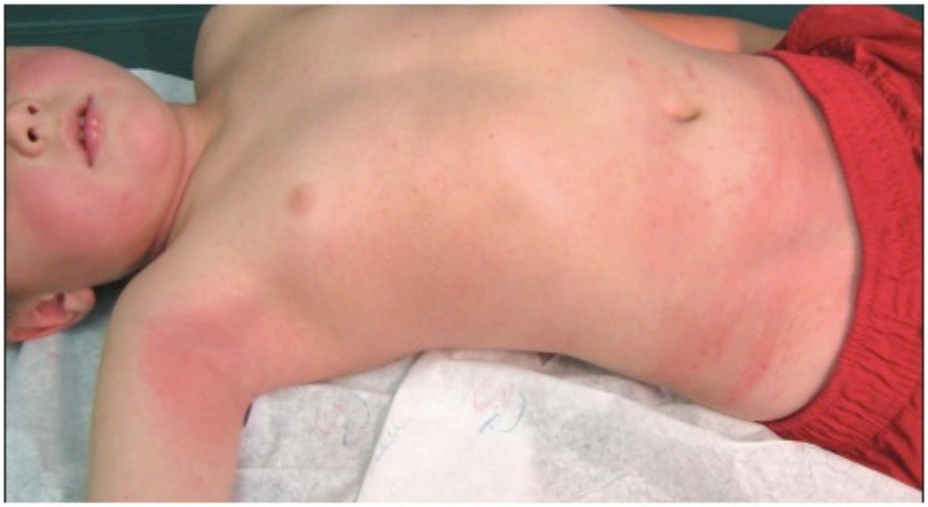

Figure 2. A 7-year-old boy with streptococcal pharyngitis and scarlet fever. Note the accentuation of the rash in skin folds and circumoral pallor (from (Block, 2014)).

fever or post-streptococcal glomerulonephritis, but these tests are not useful for the acute diagnosis of *S. pyogenes* pharyngitis, as a rise in specific antibodies only begins 7 to 14 days after the onset of infection, reaching maximum levels at 3 to 4 weeks.

Treatment

Streptococcal pharyngitis is almost always a self-limited illness, and many have questioned whether antibiotic treatment is warranted. However, such treatment can be justified for three reasons:

1. Treatment shortens the duration and severity of illness. Several studies suggest that specific therapy reduces the duration of fever and sore throat by approximately 1 day, on average.
2. Treatment prevents rheumatic fever. Acute rheumatic fever is a potential complication of *S. pyogenes* pharyngitis, and studies conducted primarily on the U.S. military in the mid-twentieth century demonstrated that penicillin treatment reduced the risk of subsequent rheumatic fever. While this rationale for treatment remains compelling in many resource-poor countries where the incidence of acute rheumatic fever is high, in industrialized countries, the relative risks and benefits no longer clearly support this treatment goal in routine cases.

3. Treatment prevents the suppurative complications of pharyngitis. Antibiotic treatment has been shown to reduce the incidence of secondary infectious complications, such as otitis media and sinusitis (Spinks, Glasziou, & Del Mar, 2013). An additional benefit of treatment is that it reduces the spread of infection to others—an important consideration for outbreak control. Clinical practice guidelines in the U.S. recommend treatment for children and adults with proven *S. pyogenes* pharyngitis (Gerber, et al., 2009; Shulman, et al., 2012). Guidelines from some European countries are similar, while other countries do not recommend specific diagnostic testing or treatment, since treatment has a small impact on the natural history of pharyngitis, and the incidence of suppurative and non-suppurative complications is low in these populations (Van Brusselen, et al., 2014).

Penicillin has been the mainstay of treatment for *S. pyogenes* pharyngitis for many years. Clinical isolates remain universally susceptible to penicillin and to many other beta-lactam antibiotics. Amoxicillin is similarly effective and is often preferred for its longer half-life, especially in children. Once-daily dosing of amoxicillin appears to have similar efficacy to a twice-daily dosing regimen (Clegg, et al., 2006; Lennon, Farrell, Martin, & Stewart, 2008). A cephalosporin may be used in patients with a history of allergy to penicillin or amoxicillin that is not of the immediate hypersensitivity type. Macrolide antibiotics are an additional alternative, but resistance to these is relatively common (Liu, et al., 2009; Tamayo, Pérez-Trallero, Gómez-Garcés, Alós, & Spanish Group for the Study of Infection, 2005; Tanz, et al., 2004). Table 1 summarizes antibiotic regimens recommended in guidelines from the Infectious Diseases Society of America.

Some have argued that clinical and/or bacteriological cure rates are lower with penicillin (or amoxicillin) than with alternate agents, including cephalosporins (Casey & Pichichero, 2004; Pichichero, et al., 2000). The counter-argument has been that studies showing superiority of alternative agents have inadvertently included *S. pyogenes* carriers, and that penicillin is inferior for the eradication of carriage, but is comparable to other agents for treatment of true infection (Bisno, 2004; Shulman & Gerber, 2004). Penicillin continues to be recommended as a first line treatment in clinical practice guidelines because of its well-established record of safety and efficacy, narrow spectrum, and low cost.

Table 1: Antibiotic Regimens Recommended for Streptococcal Pharyngitis (adapted from (Shulman, et al., 2012))

Drug, Route	Dose or Dosage	Duration or Quantity	Recommendation Strength, Quality	References
For individuals without penicillin allergies				
Penicillin V, oral	Children: 250 mg twice daily or 3 times daily; adolescents and adults: 250 mg 4 times daily or 500 mg twice daily	10 d	Strong, high	(Bass, Person, & Chan, 2000; Gerber, Spadaccini, Wright, Deutsch, & Kaplan, 1985)
Amoxicillin, oral	50 mg/kg once daily (max=1000 mg); alternate: 25 mg/kg (max=500 mg) twice daily	10 d	Strong, high	(Clegg, et al., 2006; Lennon, Farrell, Martin, & Stewart, 2008; Feder, Jr., Gerber, Randolph, Stelmach, & Kaplan, 1999; Gerber & Tanz, 2001; Shvartzman, Tabenkin, Rosentzwaig, & Dolginov, 1993)
Benzathine penicillin G, intramuscular	<27 kg: 600 000 U; ≥27 kg: 1 200 000 U	1 dose	Strong, high	(Bass, Person, & Chan, 2000; Bass, Crast, Knowles, & Onufer, 1976; Wannamaker, et al., 1951)

Table 1 continued from previous page.

Drug, Route	Dose or Dosage	Duration or Quantity	Recommendation Strength, Quality	References
For individuals with penicillin allergies				
Cephalexin,[a] oral	20 mg/kg/dose, twice daily (max=500 mg/dose)	10 d	Strong, high	(Disney, Breese, Green, Talpey, & Tobin, 1971; Disney, et al., 1992; Stillerman & Isenberg, 1970; Stillerman, Isenberg, & Moody, 1972)
Cefadroxil,[a] oral	30 mg/kg once daily (max 1 g)	10 d	Strong, high	(Gerber, et al., 1986)
Clindamycin, oral	7 mg/kg/dose 3 times daily (max=300 mg/dose)	10 d	Strong, moderate	(Jackson, 1973)
Azithromycin,[b] oral	12 mg/kg once daily (max 500 mg)	5 d	Strong, moderate	(Hooton, 1991)
Clarithromycin,[b] oral	7.5 mg/kg/dose twice daily (max=250 mg/dose)	10 d	Strong, moderate	(Kafetzis, et al., 2004)

Abbreviation: Max, maximum.
[a] Avoid in individuals with immediate type hypersensitivity to penicillin.
[b] Resistance of *S. pyogenes* to these agents is well-known and varies both geographically and temporally.

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Impetigo, Erysipelas and Cellulitis

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Introduction

Streptococcus pyogenes (group A *Streptococcus*) is one of the most important bacterial causes of skin and soft tissue infections (SSTIs) worldwide. In addition, no other pathogen causes as many diverse clinical entities as *S. pyogenes*. Specifically, this organism causes infections in the superficial keratin layer (impetigo), the superficial epidermis (erysipelas), the subcutaneous tissue (cellulitis), the fascia (necrotizing fasciitis), or muscle (myositis and myonecrosis). It is also the etiologic agent of scarlet fever and Streptococcal Toxic Shock Syndrome (StrepTSS). Impetigo is a non-life-threatening infection, but can result in post-streptococcal acute glomerulonephritis (AGN). Cellulitis and erysipelas can be mild or moderately severe, while necrotizing fasciitis, myonecrosis and StrepTSS are life-threatening. This chapter focuses on the clinical and epidemiological features of these infections, as well as treatment options, and includes a discussion of bacterial pathogenesis.

Anatomical Relationship of Skin and Skin Structure Infections with *S. pyogenes*

An understanding of the anatomy of the skin layers is crucial for the clinician to establish a correct diagnosis of streptococcal skin and soft tissue infections. Figure 1 illustrates the relationship between different anatomical structures and the types of *S. pyogenes* infections that affect specific layers of the skin and deeper tissues (from (Stevens, 2015)). Note that impetigo involves the outer keratin layer of the skin, which results in crusty lesions, whereas erysipelas affects the superficial epidermis, which results in well-demarcated borders of infection and a brilliant red skin color. Cellulitis occurs in the deeper subcutaneous tissues and has a pinkish hue with less defined edges. Necrotizing fasciitis caused by *S. pyogenes* has been defined by surgeons as an infection that involves the superficial or deep fascia, in association with destruction of tissue. In reality, necrotizing fasciitis in its later stages involves all layers of the skin and muscle as well. Muscle infection by *S. pyogenes* can take on two forms, myositis or myonecrosis. Myositis is not usually associated with necrosis and is most commonly caused by *Staphylococcus aureus*. Myonecrosis due to *S. pyogenes* is an aggressive, often life-threatening infection that can develop in any open wound or by hematogenous seeding of muscle that has been injured by a simple strain or other type of non-penetrating trauma (such as a contusion). The latter infection has been called cryptogenic or spontaneous myonecrosis and is associated with mortality as high as 80%. Note the intimate relationship of blood supply to the muscle layer. As myonecrosis progresses, the blood supply becomes compromised, resulting in the appearance of purple violaceous bullae, hemorrhage into the skin and rapid progression of tissue destruction. See the chapter on invasive *S. pyogenes* infections for further discussion of the pathogenesis of necrotizing fasciitis and myonecrosis.

Figure 2 shows a treatment algorithm prepared for the 2014 Infectious Diseases Society of America Guidelines for the Diagnosis and Management of Skin and Soft Tissue Infections (SSTI) (Stevens, et al., 2014). Purulent soft tissue infections are most commonly caused by staphylococcal species, whereas group A streptococcal cellulitis, erysipelas, necrotizing fasciitis, and myonecrosis are non-purulent. Once the clinician has made this distinction, the next decision is to determine whether the patient's signs, symptoms, and laboratory tests indicate mild,

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moderate, or severe infection. Appropriate diagnostic and therapeutic approaches are then provided for each category.

Streptococcal pyoderma

Pyoderma, impetigo, and impetigo contagiosa are terms used synonymously to describe discrete purulent lesions that are primary infections of the skin and that are extremely prevalent in many parts of the world. In the great majority of cases, pyoderma is caused by β-hemolytic streptococci, *Staphylococcus aureus*, or both.

Epidemiology

The number of existing cases of streptococcal pyoderma in children less than 15 years of age is estimated to be 111 million (Carapetis, Steer, Mulholland, & Weber, 2005). Notably, this figure excludes cases in adults and those in adults and children in developed countries. The incidence of this disease is markedly influenced by several factors, the most important of which appear to be climate and hygiene—both of which parallel global socio-economic inequities between developed countries (northern latitudes, temperate climates) and developing countries (equatorial latitudes, tropical/sub-tropical climates). Thus, the greatest global burden of this infection is found in economically disadvantaged children in tropical or subtropical climates, though it is also prevalent in northern climates during the summer months (Ferrieri, Dajani, Wannamaker, & Chapman, 1972). The peak incidence of impetigo is in children aged 2 to 5 years, but may also occur among older children and adults whose recreational activities or occupation results in cutaneous cuts or abrasions (Adams, 2002; Fehrs, et al., 1987; Wasserzug, et al., 2009). There is no particular gender predilection, and all races appear to be susceptible.

Meticulous prospective studies of streptococcal impetigo have demonstrated that the responsible microorganisms initially colonize the unbroken skin (Ferrieri, Dajani, Wannamaker, & Chapman, 1972), an observation that probably explains the influence of personal hygiene on the incidence of disease. Development of skin colonization with a given streptococcal strain precedes the development of impetiginous lesions by an average interval of 10 days. The mechanism of production of skin lesions is unproven, but it is most likely caused by intradermal inoculation of surface organisms by abrasions, minor trauma, or insect bites. Frequently, there is a transfer of the streptococcal strains from the skin and/or pyoderma lesions to the upper respiratory tract. The interval between colonization of the skin and colonization of the nose and/or throat averages 2 to 3 weeks.

Scabies is also highly prevalent in socially disadvantaged communities, such as indigenous populations and in developing countries. Its association with group A streptococcal pyoderma is well established, and treatment of scabies clearly decreases the prevalence of subsequent bacterial infection. However, such infestations may do more than simply provide a portal of bacterial entry into the skin. A recent molecular study by Mika and colleagues demonstrated that the intestinal compounds excreted from the scabies mite have potent complement-inhibitory functions that promote the growth of group A streptococcus, even in the presence of type-specific antibodies (Mika, et al., 2012). These findings provide the first molecular mechanism to account for the well-recognized association between scabies and streptococcal pyoderma and suggest new targets for intervention.

Bacteriology and Immunology

Streptococci isolated from pyodermal lesions are primarily group A, but occasionally other serogroups are responsible, such as C and G. *S. pyogenes* strains that cause impetigo differ in several respects from those usually associated with tonsillitis and pharyngitis. Skin strains belong to different M serotypes and to different *emm* genotype patterns than the classic throat strains (reviewed in (Bessen, 2009)). For example, among the 3 main *emm* pattern genotypes, (namely, AC, D and E), the *emm* pattern AC group has a strong predilection to cause infection of the throat, and *emm* pattern D strains have a strong tendency to cause impetigo (Bessen, 2009). In contrast, the pattern E strains are designated generalists that readily cause infections at both tissue sites (Anthony, Kaplan, Wannamaker, & Chapman, 1976). Thus, *S. pyogenes* strains can be divided into 3 clinically and ecologically relevant groups, based on their *emm* pattern genotype. Aside from differences in *emm*, throat

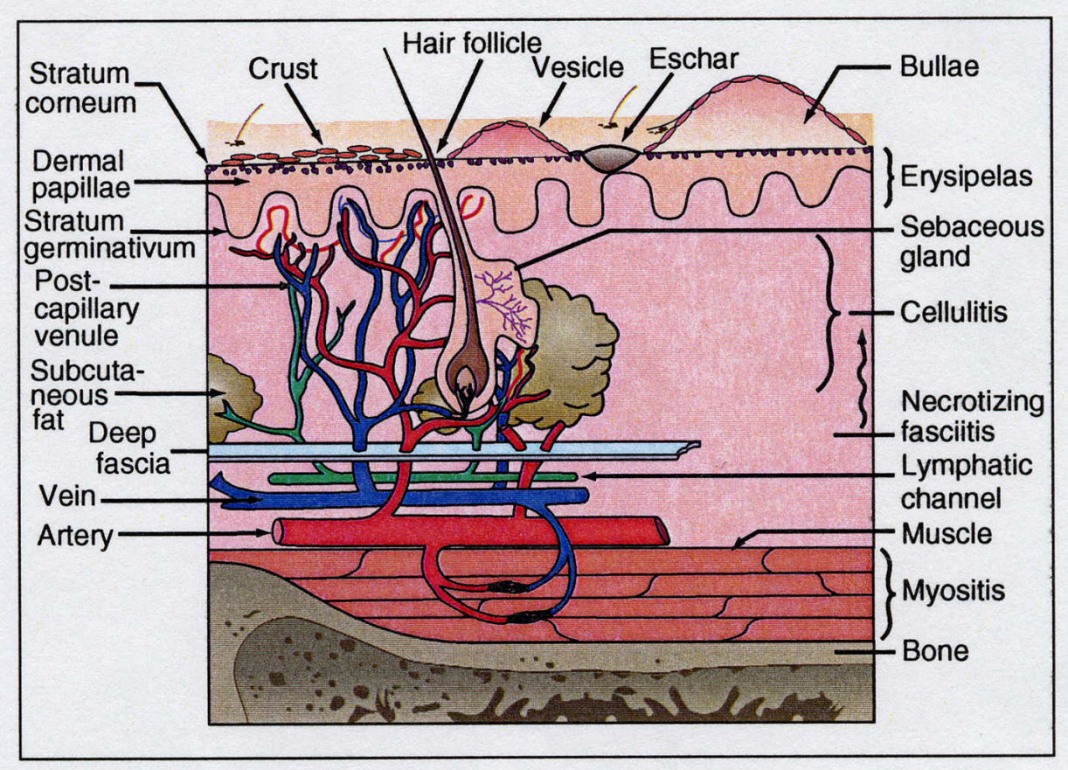

Figure 1. Anatomical relationship between skin and skin structures and different *S. pyogenes* infections. From Harrison's Principals of Internal Medicine, 19th edition, and used with permission (Stevens, 2015).

and skin strains can also be differentiated by other genetic markers (Fiorentino, Beall, Mshar, & Bessen, 1997) and functional traits, including differences in their binding avidity for keratinocytes. Because most skin strains have been identified more recently, they tend to be comprised of the higher numbered M types. The classic skin strain associated with impetigo and post-streptococcal AGN is the M-49 strain, which was isolated in Red Lake, Minnesota.

Clinical manifestations and diagnosis

The lesion of streptococcal pyoderma begins as a papule that rapidly evolves into a vesicle, surrounded by an area of erythema. The vesicular lesions are evanescent and rarely clinically recognized; they give rise to pustules that gradually enlarge and then break down over a period of 4 to 6 days to form characteristic thick crusts. The lesions heal slowly and leave depigmented areas. A deeply ulcerated form of impetigo is known as ecthyma.

Streptococcal impetigo occurs on exposed areas of the body, most frequently on the lower extremities or face. The lesions remain well localized, but frequently appear in multiples. Although regional lymphadenitis may occur, systemic symptoms are not ordinarily present.

In the past, the lesions previously described could be confidently diagnosed as streptococcal. This was the predominant form of impetigo, which could be distinguished from bullous impetigo caused by phage group II *S. aureus.* Although bullous impetigo remains almost exclusively caused by staphylococcus, the bacteriology of non-bullous impetigo has changed (Barnett & Frieden, 1992). *S. aureus*, either alone or in combination with *S. pyogenes*, is now the predominant causative agent (Gonzalez, et al., 1989). Almost all such staphylococci are penicillinase producers. Therefore, treatment with penicillin, which had previously been highly effective against non-bullous impetigo, even when both streptococci and staphylococci were isolated from the lesions, now frequently fails (Demidovich, Wittler, Ruff, Bass, & Browning, 1990).

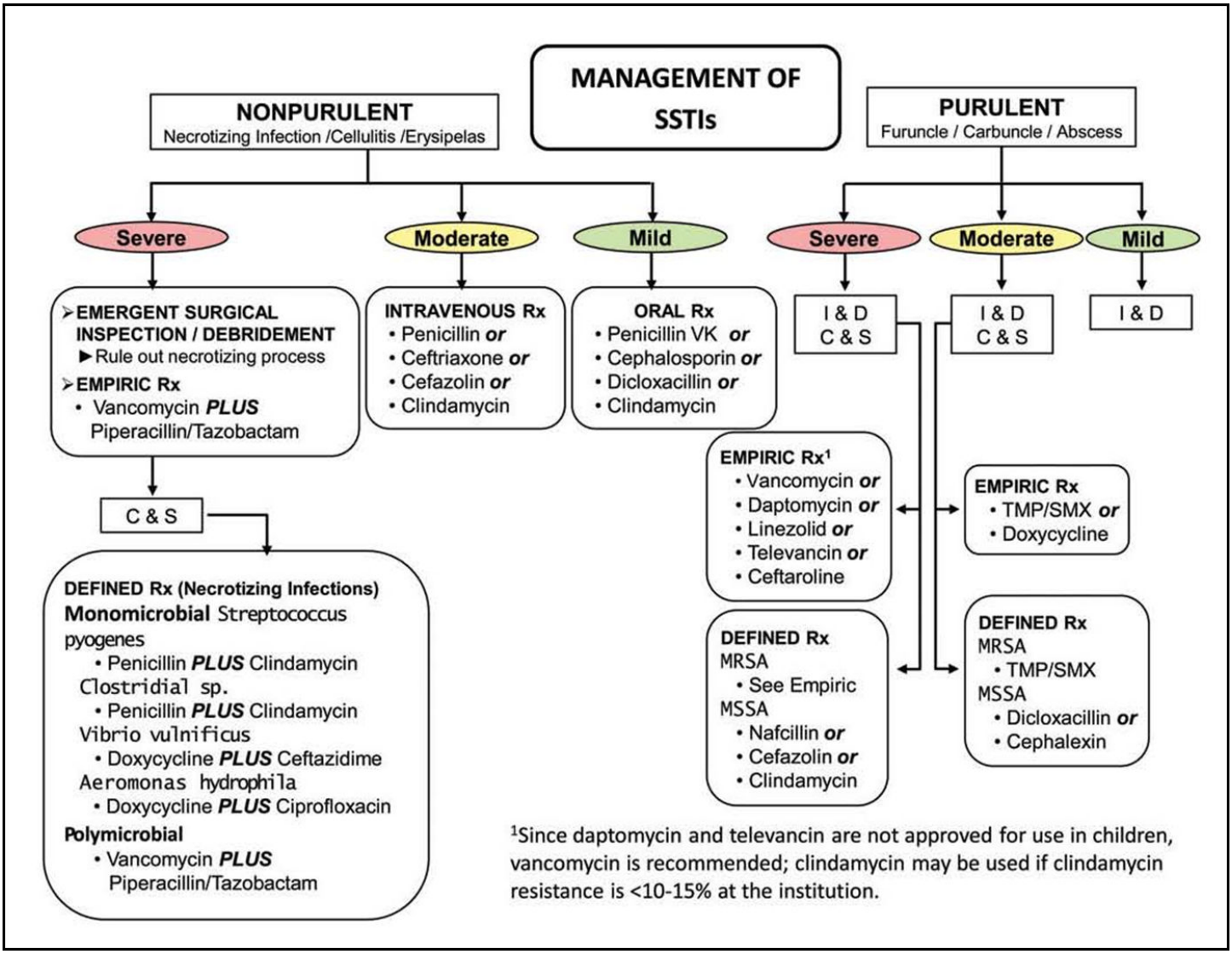

Figure 2. Algorithm for the Management of Skin and Soft Tissue Infections. From Clinical Infectious Diseases, 2014, and used with permission (Stevens, et al., 2014).

Assays of anti-streptolysin O (ASO) antibodies are of no value in the diagnosis and management of impetigo, because the ASO response is weak in patients with streptococcal impetigo (Kaplan, Anthony, Chapman, Ayoub, & Wannamaker, 1970; Bisno, Nelson, Waytz, & Brunt, 1973), presumably because the activity of streptolysin O is inhibited by skin lipids (cholesterol) (Kaplan & Wannamaker, 1976). In contrast, anti–DNase B levels are elevated (Kaplan, Anthony, Chapman, Ayoub, & Wannamaker, 1970; Bisno, Nelson, Waytz, & Brunt, 1973) and thus provide helpful supporting evidence of recent streptococcal infection in patients who are suspected of having post-streptococcal glomerulonephritis.

Treatment and prevention

Because of the current frequency of isolation of *S. aureus* from non-bullous impetigo lesions and concomitant reports of penicillin failures (Demidovich, Wittler, Ruff, Bass, & Browning, 1990; Dagan & Bar-David, 1989; Barton & Friedman, 1987), penicillinase-resistant penicillins or first-generation cephalosporins are preferred (Demidovich, Wittler, Ruff, Bass, & Browning, 1990). Erythromycin has long been a mainstay of pyoderma therapy, but its use is contraindicated in areas in which erythromycin-resistant strains of *S. aureus* or, more recently, *S. pyogenes* are prevalent. Topical therapy with mupirocin is equivalent to oral systemic antimicrobial

agents (Barton, Friedman, Sharkey, Schneller, & Swierkosz, 1989; Britton, Fajardo, & Krafte-Jacobs, 1990), and may be used when lesions are limited in number. However, it is expensive, and some strains of staphylococci may be resistant (Yun, et al., 2003). Retapamulin, a novel pleuromutilin antibacterial, has recently been approved by the FDA for treatment of bullous and non-bullous impetigo caused by *S. pyogenes* and methicillin-susceptible strains of *S. aureus* in children 9 months of age or older (Yang & Keam, 2008). In vitro data have suggested that it may be more effective than mupirocin against methicillin-resistant *Staphylococcus aureus* (MRSA).

Improved living conditions, improved personal hygiene and topical treatment of impetiginous lesions can prevent the spread of impetigo to susceptible individuals. In addition, a recent molecular study provided evidence that scabies mite proteins act as complement inhibitors and enhance *S. pyogenes* growth in whole blood assays, presumably by the inhibition of host innate immunity. Based on this groundwork data, we hypothesize that the complement-inhibitory functions of excreted gut molecules promote the growth of bacterial pathogens in the microenvironment of the epidermal burrows.

Complications

Suppurative complications are uncommon, but cutaneous infections with nephritogenic strains of *S. pyogenes* predispose patients to post-streptococcal AGN in many areas of the world. As yet, there are no conclusive data to indicate that treatment of an individual case of pyoderma prevents the subsequent occurrence of nephritis in these patients. Such therapy is nevertheless important as an epidemiologic measure in eradicating nephritogenic strains from the environment. For as-yet unexplained reasons, rheumatic fever has never been shown to occur after streptococcal pyoderma.

Erysipelas

Streptococci were first demonstrated in cases of erysipelas and wound infections by Billroth in 1874 and in the blood of a patient with puerperal sepsis by Pasteur in 1879. In 1883, Fehleisen showed that hemolytic streptococci fulfilled Koch's postulates when he isolated chain-forming organisms in pure culture from erysipelas lesions and then demonstrated that these organisms could induce typical erysipelas in humans. Rosenbach applied the designation *Streptococcus pyogenes* to these organisms in 1884. In 1938, Lancefield gave them the distinction of group A streptococcus.

Erysipelas is a superficial cutaneous process that is usually restricted to the dermis, but with prominent lymphatic involvement. It is distinguished clinically from other forms of cutaneous infection by three features: the lesions are raised above the level of the surrounding skin, there is a clear line of demarcation between involved and uninvolved tissue, and the lesions are a brilliant salmon-red color. This disorder is more common in infants, young children, and older adults, and is almost always caused by β-hemolytic streptococci. In most cases, the infecting agent is *S. pyogenes*, but similar lesions can be caused by groups C or G streptococci. Rarely, group B streptococci or *S. aureus* may be the culprits. In older reports, erysipelas was described as characteristically involving the butterfly area of the face, but at present, the lower extremities are more frequently involved. In patients with facial erysipelas, there is frequently a history of preceding streptococcal sore throat, although the exact mode of spread to the skin is unknown. When erysipelas involves the extremities, breaks in the cutaneous barrier serve as portals of entry; these include surgical incisions, trauma or abrasions, dermatologic diseases (such as psoriasis), or local fungal infections.

The cutaneous lesion begins as a localized area of erythema and swelling and then spreads rapidly with advancing red margins, which are raised and well demarcated from adjacent normal tissue. There is marked edema, often with bleb formation, and in facial erysipelas, the eyes are frequently swollen shut. The lesion may demonstrate central resolution while continuing to extend on the periphery. The cutaneous inflammation is accompanied by chills, fever, and toxicity.

The differential diagnosis is limited. Early on, the lesions of facial herpes zoster, contact dermatitis, or giant urticaria may be confused with erysipelas. Lesions that resemble erysipelas may occur in patients with familial Mediterranean fever. Cutaneous lesions similar in appearance to those of erysipelas may occur on the hands of patients who sustain cuts or abrasions while handling fish or meats. This entity, which is known as erysipeloid of Rosenbach and caused by *Erysipelothrix rhusiopathiae*, is usually unaccompanied by fever or systemic symptoms.

With early diagnosis and treatment, the prognosis is excellent. Rarely, however, the process may spread to deeper levels of the skin and soft tissues. Penicillin, either parenterally or orally, depending on clinical severity, is the treatment of choice. If a staphylococcal infection is suspected, a penicillinase-resistant semisynthetic penicillin or cephalosporin should be selected. In a randomized, prospective multicenter trial (Bernard, et al., 1992), roxithromycin, a macrolide antimicrobial, was equivalent to penicillin. However, increased levels of macrolide resistance among *S. pyogenes* have been detected in certain areas of the United States (Martin, Green, Barbadora, & Wald, 2002; York, Gibbs, Perdreau-Remington, & Brooks, 1999).

Streptococcal cellulitis

Streptococcal cellulitis, an acute spreading inflammation of the skin and subcutaneous tissues, usually results from infection of burns, wounds, or surgical incisions, but may also follow mild trauma. Clinical findings include local pain, tenderness, swelling, and erythema. The process may rapidly extend to involve large areas of skin. Systemic manifestations include fever, chills, and malaise, and there may be associated lymphangitis, bacteremia, or both. In contrast to erysipelas, the lesion is not raised, the demarcation between involved and uninvolved skin is indistinct, and lesions are more pink than salmon-red in color. Often, however, the clinical differentiation between these entities is not clear-cut.

Two predisposing causes of streptococcal cellulitis deserve special mention. One is the parenteral injection of illicit drugs (Lentnek, Giger, & O'Rourke, 1990; Barg, Kish, Kauffman, & Supena, 1985). These cases are often associated with bacteremia and deep tissue infections, such as septic thrombophlebitis, suppurative arthritis, osteomyelitis, and occasionally infective endocarditis. Second, patients who have impaired lymphatic drainage from upper or lower extremities are prone to recurrent episodes of streptococcal cellulitis. Examples include individuals with filariasis and women who have undergone radical mastectomy with axillary node dissection (Simon & Cody, 1992). It is speculated that repetitive infection further damages local lymphatics and worsens lymphatic stasis (de Godoy, de Godoy, Valente, Camacho, & Paiva, 2000).

Recurrent episodes of severe cellulitis have also been reported in certain patients who have undergone coronary artery bypass grafting (Baddour & Bisno, 1982). The lesion invariably occurs in the extremity from which the saphenous vein was removed, and at times, may exhibit features of erysipelas. Patients with tinea pedis of the venectomy limb appear to be particularly at risk (Baddour & Bisno, 1984; Semel & Goldin, 1996). As with other forms of cellulitis, pathogenic bacteria are difficult to recover during these episodes, but the appearance of the lesions and the response to penicillin therapy suggest a streptococcal cause. The few β-hemolytic streptococci that have been recovered and characterized often belong to serogroups other than group A (Baddour & Bisno, 1985).

Disruption of the cutaneous barrier (leg ulcers, wounds, dermatophytosis) is a risk factor for the development of cutaneous streptococcal infection (Dupuy, et al., 1999). There is suggestive evidence that local dermatophyte infection (such as athlete's foot) may serve as a reservoir for β-hemolytic streptococci that initiate episodes of erysipelas or cellulitis of the lower extremities (Semel & Goldin, 1996; Roldan, Mata-Essayag, & Hartung, 2000). Thus, care should be taken to eradicate such fungal infections in patients who experience recurrent bouts of erysipelas or cellulitis. Another potential reservoir is anal streptococcal colonization (Eriksson B. K., 1999). Other risk factors include venous insufficiency, edema, and obesity (Dupuy, et al., 1999). An increased risk of recurrent cellulitis has been associated with a lack of foot hygiene, and has also been reported in homeless persons (Lewis, Peter, Gómez-Marín, & Bisno, 2006).

Cellulitis may be caused by infection with a variety of bacterial pathogens, but most cases are caused by *S. pyogenes* (or, occasionally, streptococci of groups B, C, or G) or by *S. aureus*. *S. aureus* infection of the skin is usually associated with a pyogenic, fluctuant focus with surrounding erythema and has been referred to as "purulent cellulitis;" however, a better term would be purulent skin and soft tissue infection with surrounding erythema. In these cases, the erythema is entirely due to the host response to the purulent focus. The reddened skin is not itself infected, since simple drainage and no antibiotics are usually enough to resolve the infection. In the absence of positive blood cultures, which are present in only 5% of cases of non-purulent cellulitis, a specific microbiologic diagnosis is often not possible. Aspirate or biopsy samples from sites of active cellulitis are helpful when positive on smear or culture, but unfortunately, such specimens are usually negative in adult patients (Hook, et al., 1986; Howe, Eduardo Fajardo, & Orcutt, 1987; Newell & Norden, 1988).

Treatment

In some cases, it may be difficult to differentiate streptococcal from staphylococcal skin and soft tissue infection on initial presentation. In this case, a semi-synthetic penicillinase-resistant penicillin should be used. In penicillin-allergic patients, a first-generation cephalosporin may be used if the hypersensitivity is not of the immediate type. Clindamycin, linezolid, or vancomycin may be used in patients who manifest anaphylactic hypersensitivity to β-lactam antibiotics, and these agents should be administered later if there is reason to suspect infection with MRSA strains. Patients with milder cases of streptococcal cellulitis may be switched to oral medications after an initial favorable response to parenteral therapy.

The role of continuous antimicrobial prophylaxis (Wang, et al., 1997; Sjöblom, Eriksson, Jorup-Rönström, Karkkonen, & Lindqvist, 1993; Kremer, Zuckerman, Avraham, & Raz, 1991) in patients prone to frequent recurrences remains unsettled. At present, such prophylaxis seems justified only for patients with very frequent or severe episodes, and an optimal regimen has not been established.

Other Streptococcal Infections with Skin Manifestations

Suppurative soft-tissue complications following streptococcal pharyngitis

Inflammation in the facial area induced by acute streptococcal infection may affect structures that are directly contiguous to the pharynx or that drain that site. Such relatively rare complications include peritonsillar cellulitis, peritonsillar abscess, retropharyngeal abscess, and suppurative cervical lymphadenitis, as well as mastoiditis, acute sinusitis, and otitis media (Dajani, Taubert, Ferrieri, Peter, & Shulman, 1995). However, peritonsillar or retropharyngeal abscesses, frequently contain a variety of other oral flora, including anaerobes, with or without *S. pyogenes*. *S. pyogenes* are responsible for only a small minority of cases of otitis media or sinusitis.

Scarlet fever

Scarlet fever results from infection with a streptococcal strain that elaborates streptococcal pyrogenic exotoxins (erythrogenic toxins). Although this disease is usually associated with pharyngeal infections, it may follow streptococcal infections at other sites, such as wound infections or puerperal sepsis. The clinical syndrome is similar in most respects to that associated with non-toxigenic strains, except for the characteristic scarlatina-type rash. The latter must be differentiated from those of viral exanthems, drug eruptions, staphylococcal toxic shock syndrome, and Kawasaki disease.

The rash usually appears on the second day of clinical illness as a diffuse red blush, with many points of deeper red that blanch on pressure. It is often first noted over the upper part of the chest, and then spreads to the remainder of the trunk, neck, and extremities. The palms, soles, and usually the face are spared. Skin folds in the neck, axillae, groin, elbows, and knees appear as lines of deeper red (Pastia's lines). There are scattered petechiae,

and the Rumpel-Leeds test of capillary fragility is positive. Occlusion of sweat glands imparts a sandpaper texture to the skin—a particularly helpful finding in dark-skinned patients.

The face appears flushed, except for marked circumoral pallor. In addition to findings of exudative pharyngitis and tonsillitis, patients display an exanthem that is characterized by small, red, hemorrhagic spots on the hard and soft palates. The tongue is initially covered with a yellowish-white coating, through which may be seen the red papillae (white strawberry tongue). Later, the coating disappears, and the tongue is beefy red in appearance (red strawberry tongue). The skin rash fades over the course of one week and is followed by extensive desquamation that lasts for several weeks. A modest eosinophilia may be present early in the course of the illness.

Severe forms of scarlet fever, associated with either local or hematogenous spread of the organism (septic scarlet fever) or with profound toxemia (toxic scarlet fever), are characterized by a high fever and marked systemic toxicity. The course may be complicated by arthritis, jaundice, and, very rarely, hydrops of the gallbladder. Such severe forms of the disease are infrequent in the antibiotic era. However, in the late 1800s, scarlet fever was associated with mortalities of 20% in Chicago, New York and Scandinavia. During the American Civil War, epidemics of surgical scarlet fever, also called "hospital gangrene," developed among soldiers after amputation for wounds. Recently, an epidemic of 900 cases of scarlet fever occurred in China in 2011 between January and July that was associated with *emm*-12 strains of *S. pyogenes* (Luk, et al., 2012).

Lymphangitis

Lymphangitis may accompany cellulitis or may occur after clinically minor or inapparent skin infection. Lymphangitis is readily recognized by the presence of red, tender, linear streaks directed toward enlarged, tender, regional lymph nodes. It is accompanied by systemic symptoms, such as chills, fever, malaise, and headache.

Puerperal sepsis and infection of the genitalia

Puerperal sepsis follows abortion or natural childbirth when streptococci that colonize the vaginal vault or are transmitted from medical personnel invade the endometrium and surrounding structures, lymphatics, and bloodstream. The resulting endometritis and septicemia may be complicated by pelvic cellulitis, septic pelvic thrombophlebitis, peritonitis, or pelvic abscess. This disease was associated with high mortality in the pre-antibiotic era. Vaginal wall tears during delivery or episiotomy sites can also be infected with *S. pyogenes* and can cause cellulitis of the perineum or necrotizing fasciitis. Clostridial species can also cause necrotizing infection under these conditions, but can be distinguished from *S. pyogenes*, because the latter does not produce gas.

S. pyogenes perianal cellulitis and vulvo-vaginitis are symptomatic but benign disorders that primarily affect children (Petersen, Kaltoft, Misfeldt, Schumacher, & Schønheyder, 2003; Mogielnicki, Schwartzman, & Elliott, 2000). Asymptomatic carriage of *S. pyogenes* in the vagina, anus, scalp or, rarely, the upper respiratory tract of adults has, however, been the source of some outbreaks of nosocomial streptococcal infection (Mastro, et al., 1990).

Streptococcal Virulence Factors Associated with SSTI

Adhesins

A number of somatic streptococcal components play critical roles in the first step of colonization, namely, adherence to the surface of human epithelial cells. At least 17 adhesin candidates have been described (Courtney, Hasty, & Dale, 2002; Hasty, Itzhak, Courtney, & Doyle, 1992), but the most extensively studied have been lipoteichoic acid (LTA), M protein, and fibronectin-binding proteins. Through hydrophobic interactions, LTA serves as a "first-step" adhesin, which brings the organisms into close contact with host cells and then allows other adhesins to promote high-affinity binding (Hasty, Itzhak, Courtney, & Doyle, 1992). Although M protein

does not appear to promote adhesion to human buccal or tonsillar epithelial cells (Ofek, Beachey, Jefferson, & Campbell, 1975; Caparon, Stephens, Olsén, & Scott, 1991), it does mediate the adherence to skin keratinocytes via the attachment of the C repeat region to keratinocyte membrane cofactor CD46 (Okada, Pentland, Falk, & Caparon, 1994; Okada, Liszewski, Atkinson, & Caparon, 1995). *S. pyogenes* surface proteins that bind fibronectin have been studied extensively and are important in adherence to both throat and skin. These include protein F1 (PrtF1) (Hanski & Caparon, 1992), also known as SfbI (streptococcal fibronectin-binding protein I) (Talay, Valentin-Weigand, Jerlström, Timmis, & Chhatwal, 1992); and related proteins known as SbfII (Kreikemeyer, Talay, & Chhatwal, 1995), FBP54 (Courtney, Dale, & Hasty, 1996), protein F2 (Jaffe, Natanson-Yaron, Caparon, & Hanski, 1996), and PFBB (Rocha & Fischetti, 1999).

Moreover, the expression of these adhesins has been reported to be environmentally regulated (Gibson, et al., 1995). Expression of protein F1 is enhanced in an O_2-rich environment, whereas that of M protein is greater at higher partial pressures of CO_2 (Caparon, Geist, Perez-Casal, & Scott, 1992). Thus, teleologically, it might be postulated that the organism displays protein F1 on its surface when it seeks to adhere to the cutaneous surface, but expresses M protein in the deeper tissues, where it is more likely to encounter phagocytic cells.

Exotoxins

During the course of growth either in vitro or in vivo, *S. pyogenes* elaborates numerous extracellular products, only a limited number of which have been well characterized. Two distinct hemolysins have been described. The first is streptolysin O (SLO)—a member of the cholesterol-binding family of cytolysins (reviewed in (Alouf & Geoffroy, 1999)). Like several of its family members, SLO derives its name from its oxygen lability. It is reversibly inhibited by oxygen and irreversibly inhibited by cholesterol. In addition to its ability to lyse erythrocytes, it is toxic to a variety of cells and membrane-bound cell fractions, including leukocytes, platelets, endothelial cells, dermal fibroblasts, lysosomes, and isolated mammalian and amphibian cardiomyocytes. SLO is produced by virtually all strains of *S. pyogenes* (as well as many group C and G streptococci) and is antigenic. Measurement of anti-SLO (ASO) antibodies in human sera is useful as an indicator of recent streptococcal infection, but is of less value in patients with impetigo.

Other *S. pyogenes* extracellular products may facilitate the liquefaction of pus and the spreading of streptococci through tissue planes characteristic of streptococcal cellulitis and necrotizing fasciitis. These include: (1) four antigenically distinct deoxyribonucleic acid-degrading enzymes (DNases A, B, C, and D); (2) hyaluronidase, which degrades hyaluronic acid found in the ground substance of connective tissue; (3) streptokinase, which promotes the dissolution of clots by catalyzing the conversion of plasminogen to plasmin; (4) streptococcal pyrogenic exotoxin B (SpeB), which is a potent protease; and (5) C5a peptidase, which specifically cleaves the human chemotaxin C5a at the PMNL binding site (Ji, McLandsborough, Kondagunta, & Cleary, 1996; Wexler, Chenoweth, & Cleary, 1985). SpeB also cleaves IgG bound to *S. pyogenes*, thus interfering with ingestion and killing by phagocytes (Eriksson & Norgren, 2003).

Several members of the streptococcal pyrogenic exotoxin (Spe) family have been associated with StrepTSS, necrotizing fasciitis, and other severe *S. pyogenes* infections. This family of superantigens includes the bacteriophage-encoded SpeA and SpeC (historically known as the scarlatina toxins), as well as the cysteine protease SpeB and several of the more recently identified pyrogenic exotoxins, such as mitogenic factor (MF and SpeF), and streptococcal superantigen (SSA), as reviewed in (Bisno, Brito, & Collins, 2003). Superantigens are potent immunostimulators that cause the clonal proliferation of T cells and watershed production of pro-inflammatory cytokines that mediate tissue destruction, shock, and organ failure.

Genetic Control of Virulence Factors

Control of virulence factor gene expression over time and under diverse environmental conditions depends on multiple complex and interrelated genetic systems (reviewed in (Bisno, Brito, & Collins, 2003). Of the known

transcriptional regulators in *S. pyogenes*, the most intensively studied include Mga (**m**ultiple virulence gene regulator of **g**roup **A** streptococcus) (McIver, 2009), Mry (**M** protein **R**NA **y**ield) (Perez-Casal, Caparon, & Scott, 1991), and a two-component regulatory system known as CsrRS (**c**apsule **s**ynthesis **r**egulator, **R**esponse and **S**ensor components) (Levin & Wessels, 1998) or, alternatively, as CovRS (**c**ontrol **o**f **v**irulence genes) (Federle, McIver, & Scott, 1999), which represses the synthesis of the hyaluronic acid capsule and several exotoxins. The **r**egulator of **p**roteinase **B** (RopB) has also been shown to have multiple polymorphisms that control the virulence of *S. pyogenes* (Carroll, et al., 2011).

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Severe Group A Streptococcal Infections

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Introduction

Life-threatening infections caused by *Streptococcus pyogenes* (group A streptococcus) include scarlet fever, bacteremia, pneumonia, necrotizing fasciitis, myonecrosis and Streptococcal Toxic Shock Syndrome (StrepTSS). This chapter focuses on the clinical and epidemiological features of these infections, as well as treatment options and bacterial pathogenesis. In brief, such invasive infections can simply be defined as any infection in which *S. pyogenes* is isolated from a normally sterile body site. Patients with invasive *S. pyogenes* infections have a relatively low mortality rate, unless they meet the established criteria for StrepTSS.

Patient mortality is also influenced by the site of infection and the patient's presenting history. For example, in patients with *S. pyogenes* necrotizing fasciitis/myonecrosis that lacks a discernable portal of bacterial entry, classical cutaneous signs of a necrotizing process are not initially apparent. In the absence of such clinical clues, the correct diagnosis is often missed or delayed until the patient manifests systemic shock and organ failure. This delay results in high morbidity and mortality rates. Interestingly, patients in this "no portal" category frequently develop soft-tissue infection at a site of relatively minor antecedent soft tissue injury (strain, bruise) that did not break the skin. Pain at the time of presentation is often out of proportion to the injury itself, and is an important clinical clue. A molecular mechanism that links injury to secondary *S. pyogenes* infection has been recently proposed, and is discussed later in this chapter.

Development of severe invasive infections is associated with strains that produce streptococcal pyrogenic exotoxins (Spe)—a family of bacterial superantigens that includes the classical scarlatina toxins SpeA and SpeC, the cysteine proteinase SpeB, and a number of more recently described superantigens (such as mitogenic factor [MF, SpeF] and streptococcal superantigen [SSA]). Superantigens are potent immunostimulators that cause clonal proliferation of T cells and watershed production of pro-inflammatory cytokines that mediate shock and organ failure. This concept, as well as the molecular biology of streptococcal virulence, colonization, and tissue invasion, is discussed in more detail in the section on the pathogenesis of StrepTSS.

Invasive Streptococcal Infections of Skin and Soft Tissues

In the mid-1980s, outbreaks of invasive streptococcal infections, of a frequency and severity not seen in the preceding decades, began to be reported both in the United States and abroad (Hoge, et al., 1993; Martin & Høiby, 1990; Strömberg, Romanus, & Burman, 1991; Demers, et al., 1993). Although certain *S. pyogenes* M-types (M 1, 3, 11, 12, and 28) account for 50% of the associated isolates (O'Brien, et al., 2002), there has been a definite and consistent tendency for M-types 1 and 3 to be associated with the more life-threatening infections (reviewed in (Wong & Stevens, 2013)). A high proportion of these cases have occurred in adults, and the portal of entry is frequently the skin, mucous membranes, or soft tissues. Invasive infections are often associated with shock and multi-organ failure, features similar to those of severe staphylococcal toxic shock syndrome (StaphTSS) (Silversides, Lappin, & Ferguson, 2010). Thus, the entity attributed to *S. pyogenes* has been termed *StrepTSS*. Clinical features of serious streptococcal skin and soft tissue infections and StrepTSS are described below.

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Necrotizing Fasciitis (Streptococcal Gangrene)

Necrotizing fasciitis (NF) is an infection of the deeper subcutaneous tissues and fascia that is characterized by extensive and rapidly spreading necrosis (gangrene) of the skin and underlying structures. While necrotizing soft-tissue infections may be caused by multiple aerobic and anaerobic microorganisms and may vary in their clinical manifestations, the present discussion is limited to necrotizing fasciitis caused by *S. pyogenes* (Bisno & Stevens, 1996), and as described by Meleney in 1924 as hemolytic streptococcal gangrene (Meleney, 1924). Characteristically, streptococcal gangrene begins at a site of trivial or even unapparent trauma or in an operative incision. The initial lesion may appear only as an area of mild erythema, but undergoes a rapid evolution over the next 24–72 hours. The inflammation becomes more pronounced and extensive, the skin becomes dusky and then purplish, and bullae containing yellow or hemorrhagic fluid appear. Bacteremia is frequently present, and metastatic abscesses may occur. By the fourth to fifth day, frank gangrenous changes are evident in the affected skin, followed by extensive sloughing. The process may march inexorably over large body areas unless measures are taken to contain it. The patient with streptococcal gangrene appears perilously ill, with high fever and extreme prostration. Mortality rates are high, even with appropriate treatment (Stevens, 1992).

In modern times, the course of *S. pyogenes* necrotizing fasciitis appears to be much more fulminant than that described by Meleney. Specifically, ecchymoses and bullae may appear within 2-3 days and deep muscle involvement is more common. In addition, the mortality rate in 1924 was only 20%, despite the lack of antibiotics, IV fluids, ventilators, and dialysis. In contrast, mortality rates of as high as 70–80% have been reported in the current era (reviewed in (Wong & Stevens, 2013)). Given the destruction of multiple layers of soft tissue (epidermis, dermis, subcutaneous tissue, fascia, muscle) in today's infections, this author believes that "necrotizing soft-tissue infection" is a more accurate term to describe the modern disease.

Successful management of necrotizing fasciitis is dependent on early recognition, yet patients may initially present with cutaneous findings that appear relatively benign (Bisno, Cockerill, & Bermudez, 2000). Fever, when present (Bisno, Cockerill, & Bermudez, 2000), and severe pain are often the earliest manifestations of disease and are important clinical clues that should not be dismissed. In those patients with a defined portal of entry, such as a surgical incision, burn, insect bite or varicella lesion, there is redness of the skin, pain, and swelling. However, in the 50% of patients who develop necrotizing fasciitis/myonecrosis without a defined portal of entry, the infection begins deep in the tissues, frequently at the site of a hematoma, muscle strain, or traumatic joint injury. In these patients, the classical cutaneous signs of inflammation and infection are absent until late in the course. The most important clinical clue in this setting is crescendo pain.

Routine radiographs, computed tomography (CT) scanning, and magnetic resonance imaging (MRI) may show localized swelling of the deep structures, but characteristically do not show frank abscess formation or gas in the tissue—and *thus are not definitive procedures.* This is particularly problematic in the "no portal" patients who report antecedent trauma, since this history complicates the interpretation of imaging studies, in that clinicians cannot easily distinguish the cause of the deep swelling. In these cases, such studies often delay, rather than facilitate, a diagnosis. Further, patients with prior injury or surgery may have taken non-steroidal anti-inflammatory drugs (NSAIDs) that mask fever and reduce pain. Unexplained tachycardia, a marked left shift, and an elevated creatine phosphokinase level are also important clues to the diagnosis of necrotizing soft-tissue infections, and their presence should prompt surgical inspection of the deep tissues. Gram stains of aspirated fluid will reveal chains of Gram-positive cocci and few, if any, white blood cells. Similarly, a biopsy with frozen section may aid in the diagnosis of NF (Stamenkovic & Lew, 1984; Majeski & Majeski, 1997).

Myositis and Myonecrosis

Strictly speaking, myositis is a localized purulent infection of muscle. Most cases occur in tropical regions where *S. aureus* is the predominant causative agent; myositis due to *S. pyogenes* is rare. In contrast, non-purulent soft tissue infection due to *S. pyogenes* is common in patients with necrotizing fasciitis, myonecrosis, and StrepTSS.

Many of these cases occur at sites of blunt, non-penetrating trauma, or arise spontaneously in the soft tissues. Organisms are likely hematogenously translocated from the throat to the deep soft tissues, though antecedent or concomitant streptococcal pharyngitis is not a prerequisite for this infection. Systemic toxicity is also common, and mortality as high as 80% has been reported (Adams, et al., 1985). The destruction of tissue is poorly understood, but infection within the confined muscle compartment may result in pressures that exceed arterial pressure, which necessitate emergent fasciotomy and debridement. In addition, bacterial toxin-induced formation of intravascular aggregates of platelets and leukocytes could obstruct blood flow, which leads to the ischemic necrosis of tissue (Bryant, et al., 2005).

As previously mentioned, there is a great deal of overlap in the clinical features of necrotizing fasciitis and myonecrosis (Stevens, 1992; Adams, Gudmundsson, Yocum, Haselby, Craig, & Sundstrom, 1985), since later in the disease course, both infections frequently destroy all layers of the soft tissues, including muscle. Differentiation of these entities can be made by surgical inspection or biopsy; however, treatment recommendations are the same for both (Stevens, et al., 2014).

Streptococcal Toxic Shock Syndrome

StrepTSS is more fully defined in Table 1 (The Working Group on Severe Streptococcal Infections, 1993), but, simply stated, is any streptococcal infection that is associated with the sudden onset of shock and organ failure. Definite cases are those in which *S. pyogenes* is isolated from a normally sterile body site. Such cases were first described in the United States and Europe during the mid- to late 1980s (Martin & Høiby, 1990; Stevens, et al., 1989; Francis & Warren, 1988). Since then, reports of StrepTSS in adults and children have emerged worldwide. Most cases have occurred sporadically, though some clusters have been reported. The highest incidence of invasive streptococcal disease occurred in a small Minnesota community, where 26 cases/100,000 population were recorded (Cockerill, et al., 1997). In addition, outbreaks have occurred in closed environments, such as nursing homes (Thigpen, et al., 2007; Hohenboken, Anderson, & Kaplan, 1994; Jordan, Richards, Burton, Thigpen, & Van Beneden, 2007; Harkness, Bentley, Mottley, & Lee, 1992; Ruben, Norden, Heisler, & Korica, 1984) and hospitals (DiPersio, et al., 1996). Secondary cases of StrepTSS are unusual, but transmission to family members (DiPersio, et al., 1996; Gamba, et al., 1997) or health care workers (DiPersio, et al., 1996; Valenzuela, Hooton, Kaplan, & Schlievert, 1991) has been well documented by demonstrating identical pulsed-field gel electrophoresis patterns from cross-infecting strains. Although many of the initial reports described StrepTSS in adults, children are also affected (Cockerill, et al., 1997; Wheeler, Roe, Kaplan, Schlievert, & Todd, 1991; Kiska, et al., 1997; Givner, Abramson, & Wasilauskas, 1991; Brogan, Nizet, Waldhausen, Rubens, & Clarke, 1995; Stockmann, et al., 2012). In 2010, the incidence of invasive infection in children in Utah reached 14 cases/100,000 population (Stockmann, et al., 2012). Thus, persons of all ages can be afflicted and, although some have underlying medical conditions such as diabetes and alcoholism (Francis & Warren, 1988; Wheeler, Roe, Kaplan, Schlievert, & Todd, 1991; Schwartz, Facklam, & Breiman, 1990; Barnham, 1989; Braunstein, 1991; Holm, Norrby, Bergholm, & Norgren, 1992), many have no predisposing medical condition and are not immunocompromised. This contrasts sharply with reviews of *S. pyogenes* bacteremia from several decades ago (Francis & Warren, 1988; Barnham, 1989; Braunstein, 1991), which found that the disease occurred primarily among the very young, the very old, or patients with predisposing conditions, such as cancer, renal failure, leukemia, severe burns, or iatrogenic immunosuppression.

The common portals of entry for streptococci include the vagina, pharynx, mucosa, and skin (Stevens, et al., 1989). In other cases, surgical procedures such as suction lipectomy, hysterectomy, vaginal delivery, bunionectomy, reduction mammoplasty, hernia repair, bone pinning, and vasectomy have provided portals for entry. StrepTSS rarely occurs secondary to streptococcal pharyngitis (Herold, 1990; Bradley, Schlievert, & Peterson, 1991; Chapnick, et al., 1992), while viral infections, such as varicella and influenza, have provided portals of entry in other cases (Stevens, et al., 1989; Kiska, et al., 1997; Herold, 1990; Lesko, O'Brien, Schwartz, Vezina, & Mitchell, 2001).

Additional factors increase the risk of invasive *S. pyogenes* infections. Three studies have demonstrated that a high or increasing prevalence of M-types 1 or 3 strains among throat isolates may signal an increased incidence of StrepTSS in a community (Kiska, et al., 1997; Holm, Norrby, Bergholm, & Norgren, 1992; Sellers, Woods, Morris, & Saffle, 1996). The use of NSAIDs for pain associated with muscle strain, trauma, chickenpox or childbirth may mask the early signs and symptoms of streptococcal infection or possibly predispose patients to more severe infection, such as necrotizing fasciitis or StrepTSS (Stevens, et al., 1989; Stockmann, et al., 2012; Stevens, 1995a; Barnham, 1997).

Table 1. Case Definition for the Streptococcal Toxic Shock Syndrome*

I	Isolation of group A streptococci (*Streptococcus pyogenes*)
A	From a normally sterile site (e.g., blood, cerebrospinal, pleural, or peritoneal fluid, tissue biopsy, surgical wound)
B	From a nonsterile site (e.g., throat, sputum, vagina, superficial skin lesion)
II	Clinical signs of severity
A	Hypotension: systolic blood pressure ≤ 90 mm Hg in adults or below fifth percentile for age in children
And	
B. Two or more of the following signs:	
1	Renal impairment: creatinine ≥ 177 μmol/L (≥2 mg/dL) for adults or ≥2× the upper limit of normal for age. In patients with preexisting renal disease, a twofold or greater elevation over the baseline level
2	Coagulopathy: platelets ≤ 100×10^9/L (≤ 100,000/mm^3) or disseminated intravascular coagulation defined by prolonged clotting times, low fibrinogen level, and the presence of fibrin degradation products
3	Liver involvement: serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), or total bilirubin levels ≥2× the upper limit of normal for age. In patients with preexisting liver disease, a twofold or greater elevation over the baseline level.
4	Adult respiratory distress syndrome defined by acute onset of diffuse pulmonary infiltrates and hypoxemia in the absence of cardiac failure, or evidence of diffuse capillary leak manifested by acute onset of generalized edema, or pleural or peritoneal effusions with hypoalbuminemia
5	A generalized erythematous macular rash that may desquamate
6	Soft tissue necrosis, including necrotizing fasciitis or myositis, or gangrene

* An illness fulfilling criteria IA and II (A and B) can be defined as a definite case. An illness fulfilling criteria IB and II (A and B) can be defined as a probable case if no other cause for the illness is identified.
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Pathogenesis of StrepTSS

Colonization and Translocation

The entry of *S. pyogenes* into the bloodstream and deeper tissues may occur as a result of a breach of an epithelial barrier, or the organism itself may penetrate intact membranes, such as the pharyngeal mucosa. Although bacteremia rarely follows streptococcal pharyngitis, transient bacteremia likely occurs in ~50% of patients who develop invasive infections without a portal of entry. The organism adeptly avoids destruction by the host's immune system largely because of the anti-phagocytic properties of the M protein (Lancefield, 1933). Adherence of *S. pyogenes* to pharyngeal mucosal cells is a prerequisite to colonization or infection, and has been related to surface structures, such as lipoteichoic acid and fibronectin-binding proteins. Penetration or translocation of the organism through respiratory epithelial cells has been demonstrated for M-type 1 *S. pyogenes*. Some have suggested that M-1 strains possessing an invasin (*inv*+) gene penetrate more efficiently (LaPenta, Rubens, Chi, & Cleary, 1994). If penetration of mucosal barriers occurs readily with these strains, it generally does not result in clinically detectable bacteremia in the vast majority of cases, since the incidence of invasive infection remains

generally very low (~3.5 cases/100,000 population) (O'Brien, et al., 2002). Thus, the clearance of *S. pyogenes* by the human immune system must be highly efficient.

Recent studies have suggested that, following the colonization of mucosa or skin, SpeB attenuates the local host response and limits bacterial exotoxin functionality through its proteolytic activity. Later in the course of the disease, production of SpeB is curtailed by in vivo selection of strains that harbor mutations in *covS* (**c**ontrol **o**f **v**irulence), the sensory component of a key 2-component regulatory system, CovRS (Aziz, et al., 2004; Kansal, et al., 2010). This results in a stable phase-shift to a SpeB negative phenotype, which allows these particular strains to bind plasminogen, evade the immune system, and switch to an invasive phenotype (Walker, et al., 2007). In addition, streptolysin O (SLO) likely disrupts the local tissue inflammatory response in much the same way as the related toxin, perfringolysin O from *Clostridium perfringens,* inhibits leukocytosis in gas gangrene (Stevens, Tweten, Awad, Rood, & Bryant, 1997b).

S. pyogenes produces many surface-bound and extracellular virulence factors that contribute to pathogenesis in unique ways (reviewed in (Stevens & Kaplan, 2000)). Temporal and environmental control of virulence factor gene expression depends on multiple complex and interrelated stand-alone or two-component regulatory systems. The most intensively studied of these include Mga (multiple virulence gene regulator of group A streptococcus) (McIver, 2009), Mry (M protein RNA yield) (Perez-Casal & Caparon, 1991), and the two-component regulator CsrRS (capsule synthesis regulator, Response and Sensor components (Levin & Wessels, 1998)) which is alternately known as CovRS (control of virulence (Federle, McIver, & Scott, 1999)). The regulator of proteinase B (RopB) has also been shown to have multiple polymorphisms that control the virulence of *S. pyogenes* (Carroll, et al., 2011).

The Role of Antecedent Soft-Tissue Injury

A critical role for antecedent soft-tissue injury has been well established for some bacterial infections, such as clostridial myonecrosis, where a deep, penetrating injury interrupts the blood supply and directly introduces organisms (or spores) into devitalized tissues. Though the rate at which *S. pyogenes* myonecrosis progresses is comparable to that of clostridial gangrene (inches per hour), the types of predisposing injuries are distinctly different. With *S. pyogenes* infection, a minor muscle strain, sprain, or bruise is often the rule (Adams, et al., 1985; Stevens, et al., 1989). For instance, in our initial report of 20 cases of invasive streptococcal infection, one had a superficial bruise to the hand, and the portal of entry was entirely unknown in the other 7 patients (Stevens, et al., 1989). Thus, 8 of 20 patients (40%) had no known portal of entry, and overall mortality was 30% (Stevens, et al., 1989). Similarly, Adams et al. documented 21 cases of life-threatening *S. pyogenes* infection, 19 of which lacked an obvious portal of entry and 18 (85.7%) died (Adams, et al., 1985). Finally, a recent case-controlled study found that non-penetrating trauma was significantly associated with *S. pyogenes* necrotizing fasciitis (Nuwayhid, Aronoff, & Mulla, 2007). In these "no portal" cryptic infections, the correct diagnosis is often delayed until after shock and organ failure manifest (Bisno & Stevens, 1996), which often causes mortality to exceed 70% (Adams, et al., 1985). Survivors undergo emergent amputation or extensive surgical debridement and prolonged hospitalization (Bisno & Stevens, 1996; Stevens, et al., 1989; Schurr, Engelhardt, & Helgerson, 1998). Such findings have prompted several authors to conclude that non-penetrating muscle injury may be a prerequisite for *S. pyogenes* necrotizing fasciitis or myonecrosis (Adams, et al., 1985; Nuwayhid, Aronoff, & Mulla, 2007).

Our initial studies of this process demonstrated that injury of cultured human skeletal muscle cells increased the binding of *S. pyogenes* (Bryant, Bayer, Huntington, & Stevens, 2006). While *S. pyogenes* bind host proteins such as fibronectin, collagen, and laminin (reviewed in (Courtney, Hasty, & Dale, 2002)), multiple lines of evidence suggest that these did not contribute to the initial *S. pyogenes*/skeletal muscle interaction within the first 24–48 hrs after injury (reviewed in (Bryant, Bayer, Aldape, & Stevens, 2015)), but could contribute at later times in the disease course. Instead, our findings demonstrated that the ubiquitous intermediate filament protein, vimentin, was the principal *S. pyogenes* adhesin on injured muscle cells (Bryant, Bayer, Huntington, & Stevens, 2006).

Though classically an intracellular cytoskeletal protein (reviewed in (Fuchs & Weber, 1994)), our studies clearly demonstrated that injured muscle cells in culture also display vimentin on their surface (Bryant, Bayer, Huntington, & Stevens, 2006)—adding to other reports that describe a cell-surface form of vimentin in platelets, endothelial cells, and lymphocytes (Xu, et al., 2004; Podor, et al., 2002; Boilard, Bourgoin, Bernatchez, & Surette, 2003). Further, *S. pyogenes*, but not *S. aureus*, bound soluble vimentin in vitro (authors' unpublished data) and was associated with vimentin-positive necrotic muscle in a human case of *S. pyogenes* NF (Bryant, Bayer, Huntington, & Stevens, 2006).

In a murine model of injury-associated cryptogenic *S. pyogenes* infection (Hamilton, Bayer, Stevens, Lieber, & Bryant, 2008), vimentin expression was significantly increased by 6 hrs, peaked at 48 hrs, and remained elevated over 72 hrs after injury (Hamilton, Bayer, Stevens, Lieber, & Bryant, 2008). Intravenous infusion of M-type 3 *S. pyogenes* at the peak of vimentin expression resulted in the homing of the organism to the injured site (Hamilton S. M., Bayer, Stevens, Lieber, & Bryant, 2008). Since regenerating muscle cell precursors (satellite cells), but not mature healthy myofibers, express vimentin (Vaittinen, et al., 2001), these results provided the first molecular mechanism to explain the development of severe *S. pyogenes* soft tissue infections precisely at sites of prior minor muscle trauma.

Mechanisms of Shock and Organ Failure: Cytokine Induction

Within the deeper tissues and bloodstream, the induction of cytokine synthesis plays a critically important role in the production of shock and organ failure. Like the staphylococcal enterotoxins and TSST-1, multiple *S. pyogenes* exotoxins (such as streptococcal pyrogenic exotoxins [Spe] A, B and C, MF, and SSA (Norrby-Teglund, et al., 1998)) and potentially M protein fragments (Kotb, et al., 1993) act as superantigens to stimulate T-cell responses, through their ability to bind to both the MHC class II complex of antigen-presenting cells and specific Vβ regions of the T-cell receptor (Mollick & Rich, 1991). The net effect is a watershed induction of both monocyte- and lymphocyte-derived cytokines (tumor necrosis factor [TNF] -α, interleukin [IL]-1β, IL-6 and TNF-β, IL-2, interferon-γ, respectively) (Norrby-Teglund, et al., 1998; Kotb, et al., 1993; Hackett & Stevens, 1993; Fast, Schlievert, & Nelson, 1989; Norrby-Teglund, Newton, Kotb, Holm, & Norgren, 1994a; Norrby-Teglund, Norgren, Holm, Andersson, & Andersson, 1994b). Superantigens drive the clonal proliferation of specific Vβ T-cells, in part through induction of IL-2. Thus, it would be expected to find the expansion of superantigen-specific Vβ T-cell clones in an infected individual. However, studies in patients with StrepTSS demonstrate depletion, rather than expansion, of superantigen-specific T-cell subsets (Watanabe-Ohnishi, et al., 1995). This enigma remains to be reconciled.

Among the 4 alleles of SpeA, alleles 2 and 3 are most common and have the highest affinity for MHC class II on antigen presenting cells (Kline & Collins, 1996). Some clinical studies have suggested that variations in human leukocyte antigen (HLA) haplotype may result in a predisposition to worse outcomes in some patients with StrepTSS (Kotb, et al., 2002). Finally, a lack of anti-SpeA antibodies is a predisposing factor for development of StrepTSS (Mascini, et al., 2000).

Other streptococcal virulence factors can also induce mononuclear cell pro-inflammatory cytokine production. Specifically, SpeB releases active IL-1β from preformed intracellular pools (Kapur, Majesky, Li, Black, & Musser, 1993). SLO also stimulates mononuclear cells to produce TNF-α and IL-1β and, in the presence of SpeA, has synergistic effects on IL-1β production (Hackett & Stevens, 1992). Heat-killed *S. pyogenes*, as well as isolated peptidoglycan and lipoteichoic acid, are also potent inducers of TNF-α and IL-1β (Hackett, Ferretti, & Stevens, 1994; Müller-Alouf, et al., 1994).

Recent evidence suggests that cardiomyocyte-derived cytokines are produced following direct *S. pyogenes* stimulation and after exposure to *S. pyogenes*-activated inflammatory cells (Li, et al., 2011). In addition, viable *S. pyogenes* induced the production of cardiomyocyte-derived stimulator/s that boosts macrophage production of matrix metalloproteinase-9, pro-inflammatory cytokines (IL-1β, IL-6) and cardiodepressant factors (iNOS) (Li,

Bryant, Parimon, & Stevens, 2012). These locally produced, cardiomyocyte-derived cytokines (termed "cardiokines") may mediate cardiac contractile dysfunction observed in some patients with StrepTSS who develop a unique and reversible form of cardiomyopathy that is characterized by global hypokinesia and reduced cardiac index (Stevens, Shelly, Stiller, Villasenor-Sierra, & Bryant, 2008).

Other non-cytokine-mediated mechanisms of shock may also play a role in this process. For example, SpeB has been shown to release bradykinin from a high-molecular-weight kininogen (Herwald, Collin, Müller-Esterl, & Björck, 1996). Bradykinin is a potent vasodilator of systemic and pulmonary vasculature and could be at least partially responsible for the early hypotension observed in StrepTSS (Stevens, et al., 1996). Finally, recent studies demonstrated that SLO, through its ability to form membrane pores, is the major *S. pyogenes* exotoxin responsible for direct cardiomyocyte contractile dysfunction (Bolz, et al., 2015). Within minutes of exposure, SLO disrupted the normal contraction response of isolated murine cardiac cells to electrical pacing. Later, SLO induced spontaneous, non-paced contractions that were characterized by hyper-augmented contractile force. These effects were mediated by an influx of calcium through SLO-induced membrane pores. Upon removal of SLO, normal electrical pacing resumed, which suggests that membrane lesions were repaired and normal intracellular calcium levels were restored. These observations are consistent with the clinical observation that cardiomyopathy is a reversible condition in patients who survive StrepTSS (Stevens, Shelly, Stiller, Villasenor-Sierra, & Bryant, 2008).

There are likely many streptococcal and host factors that contribute to the shock and organ failure characteristic of StrepTSS. Experimental evidence suggests that TNF plays a central role in this process. Specifically, high levels of TNFα were observed in a baboon model of *S. pyogenes* bacteremia when profound hypotension was manifest (Stevens, et al., 1996); administration of a neutralizing anti-TNFα antibody restored normal blood pressure and reduced mortality by 50% (Stevens, et al., 1996). Diffuse capillary leaking also contributes to hypotension in StrepTSS and is likely attributable to cytokines and other mediators, though it may also be related to circulating M protein-fibrinogen complexes (Herwald, et al., 2004).

NSAIDs and Severe S. pyogenes Infection

In 1985, a report by Brun-Buisson and colleagues suggested a possible association between NSAID use and development of severe *S. pyogenes* necrotizing fasciitis (Brun-Buisson, et al., 1985). These authors identified 6 previously healthy individuals with no underlying conditions in whom NF either developed spontaneously (2/6) or following minor non-penetrating trauma (4/6). All had received at least one NSAID in the 4–10 days prior to hospitalization. One patient died; survivors underwent multiple surgeries and prolonged hospitalization. The authors concluded that NSAIDs contributed to the development and/or extension of the disease process. Following this report, other case series and retrospective studies appeared in the literature concerning this possible association (reviewed in (Bryant, Bayer, Aldape, & Stevens, 2015)).

In 1995, Stevens proposed that NSAIDs, through their ability to interrupt the negative feedback loop that limits production of TNFα, may predispose individuals to more severe *S. pyogenes* infections (Stevens, 1995a). Others argued that NSAIDs merely mask the signs and symptoms of developing infections, such that diagnosis and antibiotic treatment are delayed. In an effort to examine a potential cause/effect relationship, Aronoff and Bloch reviewed the available published reports through 2002 (Aronoff & Bloch, 2003) and concluded that because most studies lacked appropriate control groups or had other significant limitations, the data did not support a causal role for NSAIDs in the development of *S. pyogenes* NF or to a worsening of the infection once established. However, their work suggested that further investigations were warranted.

Since then, additional reports have emerged that lend support to an association between NSAIDs and *S. pyogenes*, including one from the United Kingdom that demonstrated that NSAID use was independently associated with a 3-fold increased risk for development of StrepTSS (Lamagni, et al., 2008), a second multicentre prospective study in France (Dubos, Hue, Grandbastien, Catteau, & Martinot, 2008), and a nested case-

controlled study in the UK by Mikaeloff et al. (Mikaeloff, Kezouh, & Suissa, 2008) that each found that NSAID use was independently associated with severe secondary complications in children with varicella infections.

Experimental evidence that directly addresses this issue is limited. Guibal et al. challenged rabbits with a combination of viable *S. pyogenes* plus *S. aureus* alpha toxin and treated them immediately thereafter with diclofenac (Guibal, et al., 1998). This relatively COX-2-selective NSAID (Süleyman, Demircan, & Karagöz, 2007) limited the extent of necrotizing fasciitis and lowered the bacterial numbers in the tissues (Guibal, et al., 1998). The authors concluded that any NSAID-induced increase in severity of NF in humans is likely due to the therapeutic delay induced by the misleading clinical effects of the NSAID, and not to any inhibition of antibacterial defenses (Guibal, et al., 1998). Goldmann et al. showed that a highly COX-2-selective NSAID (NS-398), delivered 2 hr before and again 2 hr after intravenous *S. pyogenes* challenge, significantly but transiently delayed the mortality of mice (Goldmann, et al., 2010). Our studies of NSAIDs in experimental *S. pyogenes* myonecrosis demonstrated that a highly COX-2-selective NSAID (SC-236), given 1 hr after IM challenge and continuing every 12 hr for 3 days, showed no benefit in either survival or disease severity (Hamilton, Bayer, Stevens, & Bryant, 2014). Together, these limited data suggest that COX-2-selective NSAIDs provide little to no benefit in *S. pyogenes* infections.

In striking contrast to the results with highly COX-selective NSAIDs, our studies of experimental *S. pyogenes* myonecrosis (Hamilton, Bayer, Stevens, & Bryant, 2014) and those of Weng et al. (Weng, Chen, Toh, & Tang, 2011) clearly demonstrate that different non-selective NSAIDs each accelerated the disease course, worsened outcomes, and reduced antibiotic efficacy. Of note, one non-selective NSAID, ketorolac tromethamine, also significantly augmented *S. pyogenes* infection of experimentally injured muscles in the above-mentioned murine model of cryptogenic *S. pyogenes* infection (Hamilton, Bayer, Stevens, Lieber, & Bryant, 2008).

As a result, a preponderance of clinical evidence and some experimental data suggest that non-selective NSAIDs do more than merely mask the signs and symptoms of developing *S. pyogenes* infection.

Clinical Manifestations and Stages of Infection

The first phase of StrepTSS begins with an influenza-like prodrome that is characterized by fever, chills, myalgias, nausea, vomiting, and diarrhea that precedes hypotension by 24–48 hours (Stevens, et al., 1989). Confusion and/or combativeness is present in 55% of patients. Where there is a defined portal of entry, early cutaneous evidence of streptococcal infection may be present. In contrast, in patients without a portal of entry (~50% of cases) and who subsequently develop necrotizing infection, increasingly severe pain is the most common symptom. Such pain is so severe as to prompt patients to seek medical care and, interestingly, often precedes cutaneous evidence of localized infection by 12-24 hours (Stevens, et al., 1989). In both children (Kiska, et al., 1997) and adults (Stevens, et al., 1989), the soft tissues are the most common primary site of infection. In the remaining cases, pneumonia, meningitis, endophthalmitis, peritonitis, myocarditis, joint infection, and intrauterine infection have been described.

Phase 2 of StrepTSS is characterized by tachycardia, tachypnea, increasing pain and persistent fever (Stevens, 1995b). In children with varicella infection, toxicity or persistence of fever longer than 4 days should also prompt careful evaluation. Many patients are seen in emergency departments at this stage and frequently sent home on one or two occasions with mistaken diagnoses, such as deep vein thrombophlebitis, muscle strain, viral gastroenteritis, dehydration, or sprained ankle (Bisno, Cockerill, & Bermudez, 2000). High fever and excruciating pain, particularly in individuals with no risk factors for deep vein thrombosis, should arouse suspicion of a deep-seated infection. The laboratory tests described later are helpful, and CT and MRI may be useful to define the level of tissue involvement but are not specific.

In Phase 3 of StrepTSS, the sudden onset of shock and organ failure are manifested. Many patients are in florid shock at the time of admission or within hours thereafter. Clinical evidence of necrotizing fasciitis is frequently a

late finding, often occurring after hypotension is present. The appearance of purple bullae and dusky-appearing skin is a bad prognostic sign and should prompt emergent surgical exploration. In modern cases, the progression of necrotizing fasciitis from red skin to purple bullae may occur within a 24-hour period, whereas that described by Meleney in 1924 took 7-10 days (Meleney, 1924). In addition, the rapidity with which shock and multi-organ failure can progress is impressive, and many patients die within 24-48 hours of hospitalization (Stevens, et al., 1989).

Laboratory tests should be performed in patients with aggressive soft tissue infections or patients with severe pain and fever who appear toxic. The serum creatinine measurement is particularly useful because renal impairment (creatinine level more than twice normal) is apparent even during phase 2, before hypotension is apparent. In addition, creatine phosphokinase levels in serum are markedly elevated in those with necrotizing fasciitis and myonecrosis. The white blood count is usually normal or modestly elevated at admission but with a profound left shift that includes myelocytes and metamyelocytes. Finally, serum albumin and calcium levels are usually low on admission and drop precipitously as a diffuse capillary leak syndrome develops. Thrombocytopenia does not develop until later in the course but is the earliest sign of disseminated coagulopathy. Profound metabolic acidosis develops early in phase 3, and serum bicarbonate, lactate, and blood gas pH determinations are crucial tests to follow therapeutic progress. Because the acute respiratory distress syndrome (ARDS) develops in 55% of patients with StrepTSS, pulse oximetry and, later, blood gas levels are necessary to evaluate the need for intubation and ventilation.

Management

Source Control

Prompt and aggressive surgical exploration and debridement of suspected deep-seated streptococcal infection are mandatory. Emergent surgical consultation should be sought in patients with extreme pain and fever or who are toxic. Surgical inspection provides samples for etiologic determination and allows assessment of the extent of necrosis. CT and MRI are helpful to locate the primary site of infection, but because *S. pyogenes* do not elaborate gas in the tissues or form frank abscesses, radiologist interpretations are frequently not definitive. Once necrosis is established, extensive debridement is necessary, since shock and organ failure continue to progress if devitalized tissue remains. While necrosis of the fascia may be present, it is important to recognize that global necrosis of muscle, skin, fascia and sub-cutaneous tissue is commonly present.

Fluid Resuscitation

Because of intractable hypotension and diffuse capillary leak, massive amounts of IV fluids (10 to 20 L/day) in an adult may be required. If several liters of crystalloid intravenous fluid challenge do not rapidly improve blood pressure (mean arterial pressure to more than 60 mm Hg) or tissue perfusion, then invasive monitoring or echocardiography is indicated. If despite adequate crystalloid administration, hypotension persists, the serum albumin concentration and hematocrit should be checked because capillary leak contributes to profoundly low albumin levels (< 2 g/dL) and because hemolysins produced by *S. pyogenes* can cause dramatic drops in circulating red cell mass. Thus, transfusion with packed red blood cells, with or without albumin, may be useful to improve blood pressure and preserve tissue perfusion.

Antimicrobial Treatment

Prompt antimicrobial therapy is mandatory, and empirical broad-spectrum coverage for septic shock should be initially instituted. Once the etiology of *S. pyogenes* is confirmed, high-dose penicillin and clindamycin should be given (Stevens, et al., 2014). This recommendation is based on the following: (1) all strains of *S. pyogenes* remain sensitive to penicillin; (2) resistance to clindamycin has only rarely been reported and erythromycin resistance among *S. pyogenes* is currently <5% in the United States, though some locales have reported higher rates; (3)

clindamycin is more efficacious in experimental models of necrotizing fasciitis and myonecrosis; (4) penicillin-binding proteins are not expressed during stationary-phase growth of *S. pyogenes*, and thus penicillin is ineffective in severe deep infections in which large numbers of bacteria are present; (5) clindamycin suppresses *S. pyogenes* exotoxin and M protein production; (6) clindamycin has a much longer half-life and post-antibiotic effect; (7) no antagonistic effects between penicillin and clindamycin were found when used together in vitro at clinically relevant concentrations (Stevens, Madaras-Kelly, & Richards, 1998); and (8) clindamycin suppresses pro-inflammatory cytokine production by human mononuclear cells (Stevens, Bryant, & Hackett, 1995; Stevens, Hackett, & Bryant, 1997a). When combined, these facts have resulted in the current (2014) recommendation by the Infectious Disease Society of America to use clindamycin as the main antibiotic to treat invasive *S. pyogenes* infections (Stevens, et al., 2014); penicillin is included in this recommendation, largely due to the potential for clindamycin resistance.

Management in the Intensive Care Unit

In patients with persistent hypotension, monitoring of cardiac outputs, pulmonary artery occlusion pressure, and mean arterial pressure is important. Intubation and ventilator support are usually required because of the high incidence of ARDS (55%) in patients with StrepTSS. Vasopressors such as dopamine are used frequently, although no controlled trials have been performed in StrepTSS. In patients with intractable hypotension, high doses of dopamine, epinephrine, or phenylephrine have been used, but caution should be exercised in those with evidence of disseminated intravascular coagulation (DIC) and in particular in those with cold, cyanotic digits. Symmetrical gangrene involving all fingers and toes, the tip of the nose, and the breast areola has been described. In addition, amputation of 1–4 extremities has been observed (Stevens & Bryant, 2015). In these cases, both excessive vasopressors and DIC are likely to contribute to symmetrical gangrene.

Dialysis and Hemoperfusion

Dialysis and/or hemoperfusion may be necessary because more than 50% of patients develop acute renal failure. Both techniques may also non-specifically reduce the concentrations of circulating toxins. A Swedish study of severe *S. pyogenes* infection suggested that plasma exchange might be a beneficial adjunct to treatment of patients who fail conventional treatment (Stegmayr, et al., 1992). Finally, a polystyrene superantigen absorbing device (SAAD) was developed in Japan and was shown to be highly efficacious in absorbing both streptococcal pyrogenic exotoxin A and staphylococcal toxic shock syndrome toxin 1 (TSST-1) from plasma and, when used extra-corporeally in animals infused with TSST-1 and lipopolysaccharide (LPS), mortality was reduced from 100% to 50% (Miwa, Fukuyama, Ida, Igarashi, & Uchiyama, 2003).

Intravenous Immune Globulin

The rationale for the use of intravenous immune globulin (IVIG) in the treatment of StrepTSS is based on the data implicating extracellular toxins as mediators of shock and organ failure. This concept was demonstrated as early as 1924, when George and Gladys Dick showed that convalescent sera from scarlet fever patients neutralized scarlatina toxins in vitro and, when passively administered, attenuated the course of severe scarlet fever in humans (Dick & Dick, 1925). Anti-scarlatina toxin horse serum became commercially available in the United States shortly thereafter, but because of the new widespread availability of penicillin and the decline in the severity of scarlet fever, it was never used.

Several reports have described the successful use of IVIG in patients with StrepTSS (Lamothe, D'Amico, Ghosn, Tremblay, Braidy, & Patenaude, 1995; Barry, Hudgins, Donta, & Pesanti, 1992; Stevens, 1998). The largest treatment study (15 patients) showed a significant reduction in mortality with IVIG, as compared with matched historical controls (Kaul, et al., 1999). However, the mortality rate of 70% in the control group was among the highest ever reported, whereas mortality in the IVIG group (30%) was similar to that of some series that did not use IVIG (Stevens, et al., 1989). A double-blind clinical trial was undertaken in northern Europe that compared

IVIG with albumin in patients with StrepTSS. All patients received clindamycin. The mortality rate in the IVIG group was 16%, whereas that in the albumin group was 32% (Darenberg, et al., 2003). Unfortunately, the study was stopped because of low enrollment, and only seven or eight patients with proven *S. pyogenes* infections were in each group. Thus, the differences were not significant. A retrospective study in patients with StrepTSS has also shown no benefit of IVIG on mortality (Beaulieu, McGeer, & Muller, 2008). It is hoped that further double-blind studies with sufficient numbers of cases will resolve the continuing dilemma regarding the potential efficacy of IVIG (Stevens, 2003). It is clear that if IVIG were to be used, it should be given early and probably more than one dose should be given, because batches of IVIG have variable neutralizing activity against streptococcal exotoxins (Norrby-Teglund, et al., 1998; Norrby-Teglund, et al., 1996).

Hyperbaric Oxygen

There have been no comparative trials describing the efficacy of hyperbaric oxygen treatment in StrepTSS, although some state that such treatment reduces mortality and the need for further debridements (Riseman, et al., 1990). Certainly, use of this modality should not delay (or be used in preference to) surgical debridement, when the latter is indicated.

Bacteremia

Group A streptococcal bacteremia has been relatively uncommon in the antibiotic era (Weinstein, Reller, Murphy, & Lichtenstein, 1983). Before the mid-1980s, bacteremia predominantly occurred at the extremes of life and was usually community-acquired. Occasional cases were seen in young and middle-aged adults, and were associated with surgical wound infections and endometritis.

During the past decade, however, there has been an increase in the number of reported cases of *S. pyogenes* bacteremia, which reflects the changing epidemiology and clinical patterns of invasive streptococcal infection, as noted earlier. Many of the patients were previously healthy adults between the ages of 20 and 50 years. There has also been an apparent increase in cases associated with parenteral injection of illicit drugs (Stevens, et al., 1989; Braunstein, 1991), as well as nosocomial outbreaks in nursing homes (Thigpen, et al., 2007; Hohenboken, Anderson, & Kaplan, 1994; Jordan, Richards, Burton, Thigpen, & Van Beneden, 2007; Harkness, Bentley, Mottley, & Lee, 1992; Ruben, Norden, Heisler, & Korica, 1984).

Bacteremia in children may emanate from an upper respiratory infection, but it is more commonly associated with cutaneous foci, including burns and varicella (Valenzuela, Hooton, Kaplan, & Schlievert, 1991). Older patients with streptococcal bacteremia present with a variety of chronic illnesses; their relation to the bacteremia is often unclear. Diabetes mellitus and peripheral vascular disease do appear to be predisposing factors in older adults, and, as in children, the portal of entry is usually the skin. Malignancy and immunosuppression are risk factors in both age groups (Stevens, 1992; Duma, Weinberg, Medrek, & Kunz, 1969). Although *S. pyogenes* bacteremia may be transient and relatively benign at times (Dan, Maximova, Siegman-Igra, Gutman, & Rotmensch, 1990), it is more often fulminant. The onset is abrupt, with chills, high fever, and prostration. Rarely, patients may present with acute abdominal pain (Dan, Maximova, Siegman-Igra, Gutman, & Rotmensch, 1990; Ispahani, Donald, & Aveline, 1988). Mortality in five modern series (Francis & Warren, 1988; Dan, Maximova, Siegman-Igra, Gutman, & Rotmensch, 1990; Ispahani, Donald, & Aveline, 1988; Bucher, et al., 1992; Burkert & Watanakunakorn, 1992) has ranged from 27% to 38%.

Other Streptococcal Infections

Infection Associated with Pregnancy

S. pyogenes infection associated with pregnancy (also referred to as puerperal sepsis, childbed fever, or postpartum infection) was first described by Semmelweis in the 1850s and remains an important cause of

maternal and infant mortality worldwide (reviewed in (Hamilton, Stevens, & Bryant, 2013)). Unlike in Semmelweis' era, only 14% of modern-day *S. pyogenes* postpartum infections in developed countries are nosocomially acquired. Rather, the majority occur after hospital discharge. Nearly 20 percent of cases of *S. pyogenes* infection occur during the third trimester, prior to the onset of labor or rupture of membranes (Hamilton, Stevens, & Bryant, 2013).

Patients with *S. pyogenes* puerperal sepsis typically present with fever, abdominal pain, and hypotension without tachycardia or leukocytosis. About 220 cases occur annually in the United States, for an overall rate of 6 cases per 100,000 live births (Chuang, Van Beneden, Beall, & Schuchat, 2002). In 2002, the case fatality rate was about 3.5 percent. Maternal mortality is highest when infection develops within four days of delivery or during the late third trimester (Hamilton, Stevens, & Bryant, 2013).

Meningitis and Endocarditis

Although endocarditis caused by *S. pyogenes* was relatively common in the pre-antibiotic era, it is now rarely seen (Ramirez, Naragi, & McCulley, 1984; Baddour, 1998). Meningitis caused by *S. pyogenes* usually follows upper respiratory infection, including sinusitis or otitis (van de Beek, et al., 2002), or neurosurgical conditions (Sommer, et al., 1999). It is clinically indistinguishable from other forms of acute pyogenic meningeal infection (Murphy, 1983).

Pneumonia

Pneumonia caused by *S. pyogenes* is frequently associated with antecedent viral infections such as influenza, measles, or varicella, or with chronic pulmonary disease. Numerous epidemics have been described in military recruit populations (Basiliere, Bistrong, & Spence, 1968; Crum, et al., 2005). An increased number of cases has been reported over the past few years in association with the resurgence of invasive streptococcal infections. In one-third or fewer of the cases, there was a history of preceding streptococcal upper respiratory infection. The onset is typically abrupt and the disease is characterized by chills, fever, dyspnea, cough productive of blood-streaked sputum, pleuritic chest pain, and, in more severe cases, cyanosis. The pulmonary picture is that of bronchopneumonia, with consolidation being uncommon. Empyema develops in 30–40% of cases, tends to appear early in the disease, and typically consists of copious amounts of thin serosanguinus fluid. Bacteremia occurs in 10-15% of cases. Complications include mediastinitis, pericarditis, pneumothorax, and bronchiectasis, and the clinical course of the disease is often prolonged. Mortality has generally been low with penicillin therapy and adequate drainage of empyema, which perhaps reflects its occurrence in healthy military recruits. However, in a recent Canadian report of 222 cases of community-acquired pneumonia among adults (with a median age of 56 years), the case-fatality rate was 38% (Muller, et al., 2003). Interestingly, a recent review of the 1918 pandemic of influenza has demonstrated that the major cause of death was secondary bacterial pneumonia (Morens, Taubenberger, & Fauci, 2008). While *S. pneumoniae* was the most common etiologic agent, *S. pyogenes* was second, followed by *S. aureus*. Among patients with pneumonia with empyema, *S. pyogenes* was first. Investigators have demonstrated in a mouse model that a non-lethal influenza infection greatly enhanced the severity and mortality of secondary respiratory infection with *S. pyogenes* (Okamoto, et al., 2003).

Prophylaxis for, and Risk of, Secondary StrepTSS

StrepTSS is most commonly community-acquired and sporadic in nature, yet clusters of invasive cases have been described in nursing homes (Thigpen, et al., 2007; Hohenboken, Anderson, & Kaplan, 1994; Jordan, Richards, Burton, Thigpen, & Van Beneden, 2007; Harkness, Bentley, Mottley, & Lee, 1992; Ruben, Norden, Heisler, & Korica, 1984), families (DiPersio, et al., 1996; Gamba, et al., 1997), and hospital workers (Valenzuela, Hooton, Kaplan, & Schlievert, 1991; Kakis, et al., 2002). In San Francisco, 23 hospital workers became colonized or infected with *S. pyogenes* as a result of contact from a single case of StrepTSS (Kakis, et al., 2002). This example, as well as many historical studies in schools, military posts, and nursing homes, has taught us that *S.*

pyogenes is highly contagious. Fortunately, mere contact or colonization is usually not sufficient to cause a secondary case of invasive *S. pyogenes* infection. Epidemiologic studies by the Centers for Disease Control and Prevention found one secondary case of invasive infection among more than 1500 contacts (Prevention of Invasive Group A Streptococcal Infections Workshop Participants, 2002). This would extrapolate to 66/100,000 population/year for secondary cases (Robinson, et al., 2003). As noted, the current incidence of primary cases of invasive *S. pyogenes* infections in the United States is 3.5/100,000 population/year. Thus, the risk to contacts is roughly 20 times greater than that for the general population, but still remains very low. Given the relative infrequency of these infections and the lack of a clearly effective chemoprophylactic regimen, routine screening for and prophylaxis against streptococcal infection are not recommended for household contacts of index patients (Prevention of Invasive Group A Streptococcal Infections Workshop Participants, 2002). In deciding who should receive prophylaxis, the clinician needs to consider the duration and intimacy of contact and underlying host factors of individual contacts. Specifically, contacts with open wounds, who have had recent surgery or childbirth, who have concurrent viral infections such as varicella or influenza, or those with immunodeficiency diseases should receive prophylaxis. In a multicenter study of adults aged 18–45 years, human immunodeficiency virus infection and injecting drug use were independently associated with an increased risk of invasive *S. pyogenes* disease. In those aged 45 years of age or older, diabetes, cardiac disease, cancer, and corticosteroid use were significant risk factors (Factor, et al., 2003). Moreover, persons aged 65 years or older are at an increased risk of mortality, should they contract invasive disease. Thus, it may be prudent to initiate prophylaxis in households with older adults or those with the above-mentioned risk factors.

As there is a present lack of firm data on which to base antimicrobial prophylaxis, it seems reasonable to choose those agents that have achieved highest rates of pharyngeal eradication in asymptomatic individuals; among these are penicillin, clindamycin, and azithromycin. Specific regimens have been published elsewhere (Prevention of Invasive Group A Streptococcal Infections Workshop Participants, 2002).

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Acute Rheumatic Fever and Rheumatic Heart Disease

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Introduction

Acute rheumatic fever (ARF) results from the body's autoimmune response to a throat infection caused by Streptococcus pyogenes, also known as the group A Streptococcus bacteria. Rheumatic heart disease (RHD) refers to the long-term cardiac damage caused by either a single severe episode or multiple recurrent episodes of ARF. It is RHD that remains a significant worldwide cause of morbidity and mortality, particularly in resource-poor settings. While ARF and RHD were once common across all populations, improved living conditions and widespread treatment of superficial S. pyogenes infections have caused these diseases to become comparatively rare in wealthy areas (Carapetis, 2007). Currently, these diseases mainly affect those in low- and middle-income nations, as well as in indigenous populations in wealthy nations where initial S. pyogenes infections may not be treated, which allows for the development of harmful post-infectious sequelae (Carapetis, 2007).

The development of ARF occurs approximately two weeks after S. pyogenes infection (Gewitz, et al., 2015). The clinical manifestations and symptoms of ARF can be severe and are described in the Revised Jones Criteria (Gewitz, et al., 2015). Symptoms of ARF can include polyarthritis, carditis, chorea, the appearance of subcutaneous nodules, and erythema marginatum or a rash associated with ARF (Gewitz, et al., 2015; Martin, et al., 2015). These symptoms usually require patients to be hospitalized for two to three weeks, during which time the outward symptoms resolve, but the resultant cardiac damage may persist. With repeated S. pyogenes pharyngitis infections, ARF can recur and cause cumulative damage to the heart valves (Martin, et al., 2015).

This chapter will briefly cover the epidemiology and pathophysiology of ARF and RHD, and will also outline the clinical manifestations, diagnostic considerations, and recommended treatment and management options for both conditions. Finally this chapter will also highlight prevention strategies for ARF and RHD and will discuss current vaccination efforts against S. pyogenes.

Epidemiology of ARF and RHD

Burden of Disease

The global burden of ARF and RHD is significant, and is predominantly found in populations living in low-resource settings (Carapetis, Steer, Mulholland, & Weber, 2005). Incidence rates of ARF are poorly documented in most low- and middle-income countries, including in populations with a high prevalence of RHD, where it is presumed that a high incidence of ARF also occurs. This relates both to the lack of infrastructure for disease surveillance in those settings, but also to a paucity of ARF cases that are presented for clinical care. It is not known if the latter issue is a result of health-seeking behavior (people with ARF who choose not to seek health care), or due to inadequate diagnosis of ARF by health staff. The latter may in turn be due to true misdiagnosis as a result of problems with training, a lack of access to diagnostic facilities (such as electrocardiography,

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streptococcal serology, acute phase reactant testing, and echocardiography); or possibly because many cases of ARF may be milder, or even sub-clinical, in highly endemic settings (Bishop, Currie, Carapetis, & Kilburn, 1996).

ARF incidence rates have been reported to be as high as 155 per 100,000 children aged 5 to 14 years in indigenous populations in North Queensland, Australia (Gray, Brown, & Thomson, 2012) with rates in the Northern Territory reported at 380.1 per 100,000 children in 2002 (Parnaby & Carapetis, 2010). In New Zealand, ARF affects mainly children and teens aged 4–19 years who are predominantly of Māori and Pasifika descent and are living in low socioeconomic regions of the North Island (Jack, et al., 2015). Between 1993 and 2009, the average incidence rates for ARF based on hospitalization data for children 5–14 years were 81.2 per 100,000 for Pasifika children, as compared with 40.2 per 100,000 for Māori children and 2.1 per 100,000 for non-Māori, non-Pasifika children (Milne, Lennon, Stewart, Vander Hoorn, & Scuffham, 2012a).

Global disease estimates in 2005 reported 471,000 ARF cases annually, which largely occurred in children and teens aged 5–15 years, with the prevalence of RHD cases ranging between 15.6–19.6 million (Carapetis, Steer, Mulholland, & Weber, 2005). Approximately 350,000 deaths occurred each year due to either ARF or RHD (Carapetis, Steer, Mulholland, & Weber, 2005). The number of new cases of RHD diagnosed was estimated at 282,000 per year, with approximately 233,000 deaths annually (Carapetis, Steer, Mulholland, & Weber, 2005). Global burden of disease estimates performed in 2010 calculated the number of individuals living with RHD was at least 34.2 million, with 10.1 million disability-adjusted life years lost (de Dassel, Ralph, & Carapetis, 2015). There are challenges in obtaining precise global figures concerning ARF and RHD, with one example being that the diagnosis of ARF remains difficult and problematic across many settings. Improved diagnostic tools and measures are vital, and such efforts would support enhanced global disease estimation efforts (Sheel, Moreland, Fraser, & Carapetis, 2016). It should also be noted that these figures concerning incidence and prevalence are likely to be underestimated, due to variable and insufficient data collection in resource-poor settings, where the rates of ARF and RHD are often highest (Zühlke, et al., 2014). More accurate estimates of RHD prevalence may result from the increased availability of echocardiography, which can detect cardiac damage that is due to RHD more accurately than auscultation (Roberts, et al., 2015).

Risk Factors

Risk factors for ARF and RHD include age, gender, and various environmental factors (Carapetis, et al., 2016). In terms of age, ARF largely affects children between the ages of 5 and 14 years, and initial cases of ARF can affect children even younger than this (Lawrence, Carapetis, Griffiths, Edwards, & Condon, 2013; Parnaby & Carapetis, 2010).· Recurrent episodes generally affect older children and can occur into young adulthood. Because RHD often results from cumulative damage, the peak prevalence of RHD occurs in an individual's twenties and thirties, though the burden of RHD in children and adolescents remains substantial (Lawrence, Carapetis, Griffiths, Edwards, & Condon, 2013).

While ARF is equally common in both males and females, RHD tends to be more common in females (Lawrence, Carapetis, Griffiths, Edwards, & Condon, 2013; Parnaby & Carapetis, 2010). It is unclear whether this difference in RHD prevalence is due to greater susceptibility to developing autoimmune responses following *S. pyogenes* infection, or whether social factors such as involvement in child-raising, which may cause increased susceptibility and likelihood of *S. pyogenes* infections, may combine with reduced access to primary and secondary prevention regimens (Carapetis, et al., 2016). Furthermore, RHD often becomes apparent during pregnancy, because of its associated higher cardiac burden (Carapetis, et al., 2016).

Environmental factors affect the prevalence of ARF by increasing exposure to *S. pyogenes* infections. A major environmental factor that increases the likelihood of ARF is household crowding, which facilitates the spread of *S. pyogenes* infections (Quinn, 1982). In addition, it has been shown that ARF and RHD are more prevalent in rural and remote areas as well as in urban slums, but this likely reflects other risk factors, such as greater

household crowding due to low socioeconomic status or limited access to medical resources (Carapetis, et al., 2016). There is also a potential link between insufficient nutrition in childhood and susceptibility to ARF, but it is unclear whether this occurs because insufficient nutrition can increase susceptibility to developing aggressive autoimmune responses to *S. pyogenes* infection, or whether poor nutrition is connected to household overcrowding and other factors associated with poverty that increase susceptibility to *S. pyogenes* infection (Steer, Carapetis, Nolan, & Shann, 2002).

Pathophysiology

The pathogenic mechanisms of ARF are not completely understood. Studies of the pathogenesis of ARF have been constrained by the lack of a highly suitable animal model, although a Lewis rat model of valvulitis and chorea has been used for some time (Quinn, Kosanke, Fischetti, Factor, & Cunningham, 2001; Brimberg, et al., 2012). In order for ARF to occur, it appears that a pharyngeal infection caused by *S. pyogenes* must occur in a host with a genetic susceptibility to the disease (Denny, Wannamaker, Brink, Rammelkamp, & Custer, 1950; Bryant, Robins-Browne, Carapetis, & Curtis, 2009).

Activation of the innate immune system begins with a pharyngeal infection that leads to the presentation of *S. pyogenes* antigens to T and B cells. $CD4^+$ T cells are activated and production of specific IgG and IgM antibody by B cells ensues (Cunningham, Pathogenesis of group A streptococcal infections, 2000). Tissue injury is mediated through an immune-mediated mechanism that is initiated via molecular mimicry (Guilherme, Kalil, & Cunningham, 2006). Structural similarity between the infectious agent and human proteins leads to the cross-activation of antibodies and/or T cells directed against human proteins (Cunningham, 2000).·In ARF, this cross-reactive immune response results in the clinical features of rheumatic fever, including carditis, due to antibody binding and infiltration of T cells; transient arthritis, due to the formation of immune complexes; chorea, due to the binding of antibodies to basal ganglia; and skin manifestations, due to a delayed hypersensitivity reaction (Figure 1; Carapetis, et al., 2016).

Molecular mimicry

There are a number of lines of evidence that suggest molecular mimicry plays a role in the development of carditis by stimulating both humoral and cellular cross-reactive immune responses (Cunningham, 2000; Guilherme, Kalil, & Cunningham, 2006; Cunningham, et al., 1992). The alpha-helical protein structures found in M protein and N-acetyl-beta-D-glucosamine (the carbohydrate antigen of *S. pyogenes*) share epitopes with myosin, and antibodies against both of these antigens cross-react against human tissues (Galvin, Hemric, & Cunningham, 2000). Monoclonal antibodies generated from tonsillar or peripheral blood lymphocytes of patients infected with *S. pyogenes* cross-react with myosin (Cunningham, 2000; Cunningham, et al., 1988). Monoclonal antibodies directed against myosin and N-acetyl-beta-D-glucosamine isolated react against human valvular endothelium in patients with rheumatic fever (Cunningham, et al., 1988). In a Lewis rat model, immunization with recombinant streptococcal M protein type 6 led to development valvulitis (Quinn, Kosanke, Fischetti, Factor, & Cunningham, 2001).

Human heart intralesional T cell clones react against cardiac tissues, including myosin and valve-derived proteins (Faé, et al., 2006). Autoreactive T cells appear to play an important role in granulomatous inflammation in cardiac valves. Vascular cell adhesion molecule 1 may be the link between humoral and cellular immunity at the valve surface (Figure 2; Roberts, Kosanke, Terrence Dunn, Jankelow, Duran, & Cunningham, 2001). Vascular cell adhesion molecule 1 is upregulated at the valve endothelium surface as a result of binding of cross-reactive antibodies. This leads to adherence of $CD4^+$ T cells to the endothelium, with subsequent infiltration of these cells into the valve. The T-cells initiate a predominantly TH1 response with the release of γ -IFN. Inflammation leads to neovascularization, which allows further recruitment of T-cells. Epitope spreading may occur in the

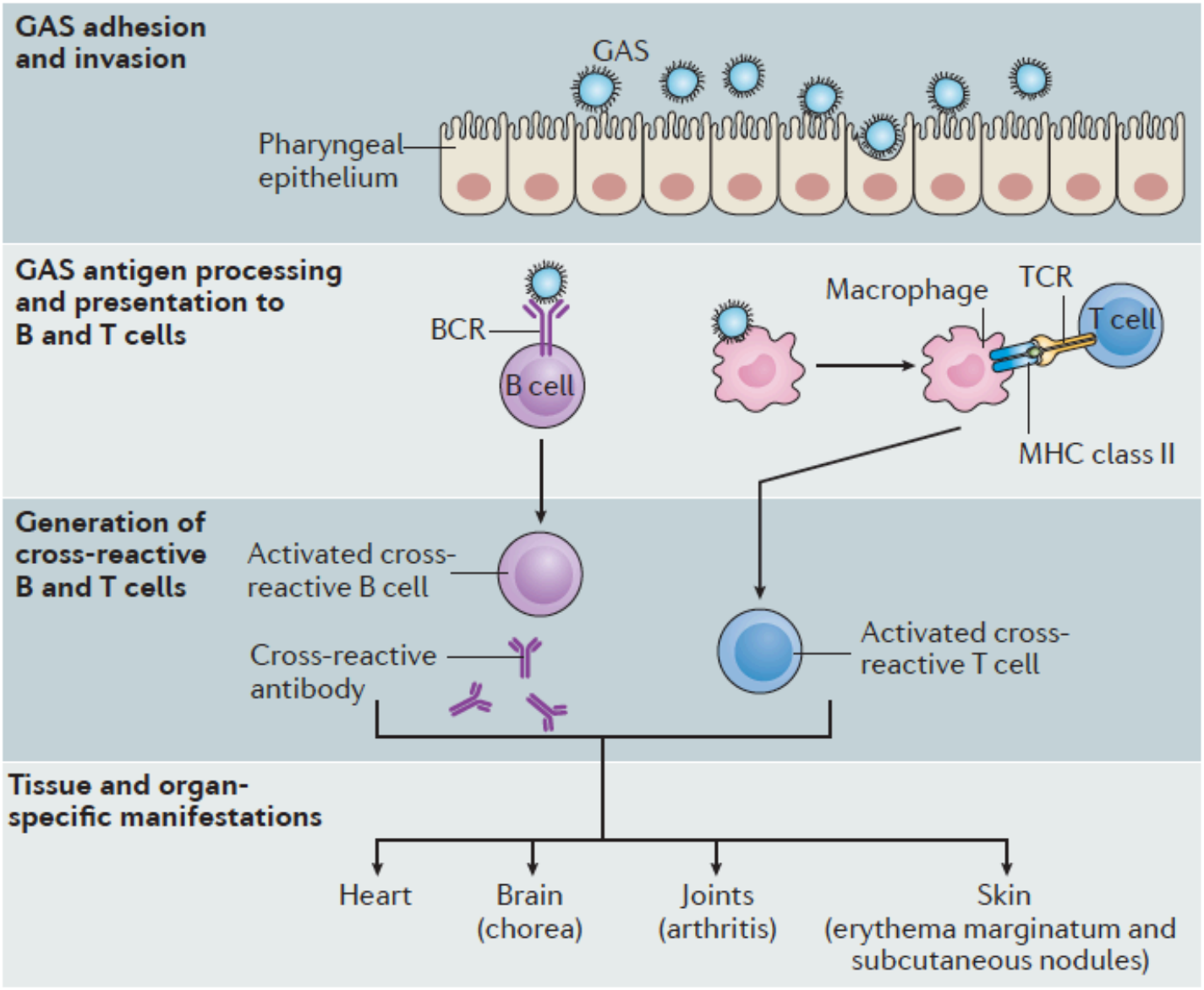

Figure 1. Overview of the pathogenesis of acute rheumatic fever (GAS: group A Streptococcus; BCR: B cell receptor; TCR: T cell receptor) Figure reproduced with permission from (Carapetis, et al., 2016).

valve, where T-cells respond against other cardiac proteins such as vimentin and tropomyosin and lead to the formation of granulomatous lesions underneath the endocardium (Aschoff bodies) (Roberts, et al., 2001).

Genetic susceptibility

Acute rheumatic fever is a highly heritable disease, with frequent cases observed in family members, including twins (Bryant, Robins-Browne, Carapetis, & Curtis, 2009). A meta-analysis of twin studies found that the pooled probandwise concordance risk was 44% and 12% in monozygotic and dizygotic twins respectively, and the association between zygosity and concordance was strong, with an odds ratio of 6.4 (95% CI 3.4 to 12.1) (Engel, Stander, Vogel, Adeyemo, & Mayosi, 2011).

It is most likely that susceptibility to ARF is polygenic. Polymorphisms in several genes coding for immune proteins have been associated with ARF susceptibility. Several studies have reported genetic associations related to class II human leukocyte (HLA) molecules (Anastasiou-Nana, Anderson, Carlquist, & Nanas, 1986; Ayoub,

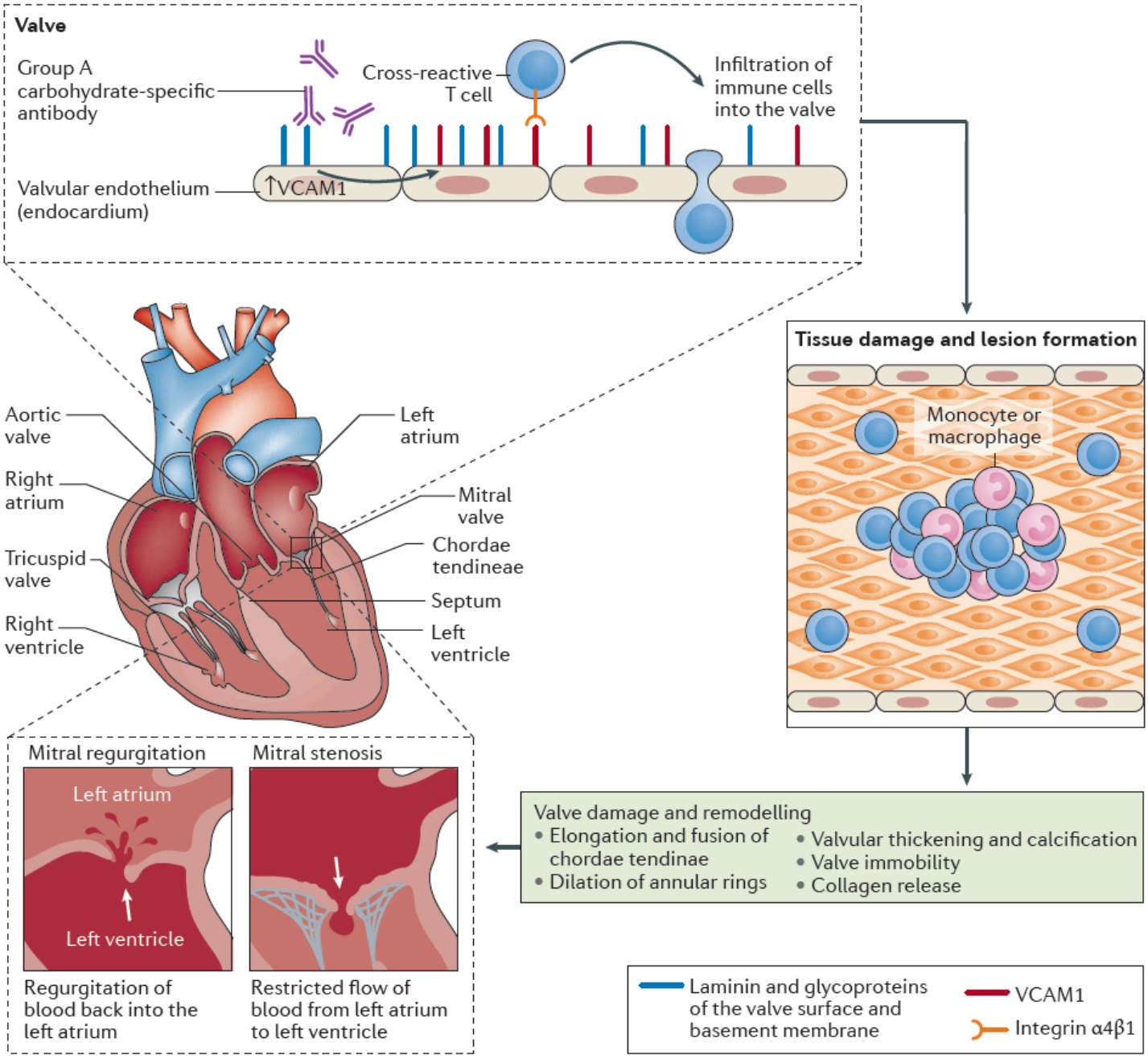

Figure 2. Pathogenesis of carditis in acute rheumatic fever (VCAM1: Vascular cell adhesion molecule 1). Figure reproduced with permission from (Carapetis, et al., 2016).

Barrett, Maclaren, & Krischer, 1986; Carlquist, et al., 1995; Hafez, et al., 1985), while others have reported associations with non-HLA related immune proteins (Bryant, Robins-Browne, Carapetis, & Curtis, 2009). Large-scale genome wide association studies of rheumatic heart disease in multiple populations in over 20 countries, including in Africa, the Pacific, and northern Australia are currently underway.

Acute Rheumatic Fever

Clinical Manifestations

ARF can present with several different clinical manifestations in the weeks following an episode of *S. pyogenes* pharyngitis (Special Writing Group of the Committee on Rheumatic Fever, Endocarditis, and Kawasaki Disease of the Council on Cardiovascular Disease in the Young of the American Heart Association, 1992; Gewitz, et al., 2015). The most common presenting features of ARF are fever (>90% of patients) and arthritis (75% of patients). The most serious manifestation is carditis (>50% of patients) because it can lead to chronic rheumatic heart disease—while all other clinical features fully resolve, often within weeks.

Arthritis

There are a large number of differential diagnoses of the arthritis of ARF, and as a result, it is the most diagnostically challenging manifestation (World Health Organisation, 2004). The arthritis of ARF most commonly affects the large joints, especially the knees, ankles, elbows, and wrists. Multiple joints are frequently involved, with the onset of arthritis in different joints either separated in time or overlapping, giving rise to the description of a "migratory" or "additive" polyarthritis (Jansen, Janssen, de Jong, & Jeurissen, 1999; Congeni, Rizzo, Congeni, & Sreenivasan, 1987; Veasy, Tani, & Hill, 1994). Each joint is affected for a few days to a week, with the entire episode resolving without treatment within one month. The joint pain can be quite severe, especially in older children and adolescents, and is often out of keeping with the clinical signs of inflammation (Feuer & Speira, 1997; Wallace, Garst, Papadimos, & Oldfield, 1989).

An important feature of the arthritis of ARF is its rapid response to anti-inflammatory therapy. If joint symptoms do not respond to aspirin or other nonsteroidal anti-inflammatory drugs (NSAIDs) or glucocorticoid treatment within 48 hours, then the diagnosis of ARF should be reconsidered (ARF/RHD Writing Group, 2012). In patients presenting with a monoarthritis in whom ARF is suspected, some experts recommend withholding NSAID therapy and treating with acetaminophen or paracetamol until a second joint is affected, thus declaring the diagnosis of ARF. Although patients with ARF may present with only a monoarthritis (Carapetis & Currie, 2001; Wilson, Wilson, Voss, Morreau, & Lennon, 2007b), a consideration of septic arthritis as a differential diagnosis is needed in these patients, especially in younger children. Joint aspiration with synovial fluid white cell count and culture is indicated in these patients. The synovial fluid is sterile, with a lymphocyte predominance in patients with ARF (Harlan, Tani, & Byington, 2006; Mataika, Carapetis, Kado, & Steer, 200). The recently updated Jones criteria highlight that joint manifestations may include aseptic monoarthritis or polyarthralgia as major manifestations in high-risk populations, while in low-risk populations, only polyarthritis is acceptable as a major manifestation (Gewitz, et al., 2015).

Carditis

ARF can cause a pancarditis that involves the pericardium, epicardium, myocardium, and endocardium (Gewitz, et al., 2015). However, the main clinical manifestation of ARF carditis reflects involvement of the endocardium, which presents as valvulitis of the mitral valve (mitral regurgitation) and, less frequently, of the aortic valve (aortic regurgitation) (Abernethy, et al., 1994; Vasan, et al., 1996; Veasy, et al., 1987). In patients with mitral regurgitation, auscultation reveals the characteristic pansystolic murmur of mitral regurgitation, and if the mitral regurgitation is severe, then an additional diastolic murmur may be present (Carey-Coombs murmur). Cardiomegaly may occur when there is more severe valvular regurgitation. A pericardial rub may be heard when there is extensive involvement of the pericardium (Special Writing Group of the Committee on Rheumatic Fever, Endocarditis, and Kawasaki Disease of the Council on Cardiovascular Disease in the Young of the American Heart Association, 1992).

Patients with suspected ARF should have an echocardiogram to confirm clinical findings and to grade severity of valvular regurgitation, to evaluate cardiac function and to diagnose any subclinical involvement (Gewitz, et al., 2015). Subclinical carditis refers to evidence of regurgitation on echocardiography in the absence of ausculatory findings (Tubridy-Clark & Carapetis, 2007; Minich, Tani, Pagotto, Shaddy, & Veasy, 1997; Figueroa, et al., 2001). As specific criteria for diagnosing sub-clinical carditis are lacking, the 2015 Jones Criteria suggest that the WHF criteria may be used to distinguish physiological from pathological regurgitation (Gewitz, et al., 2015), as shown in Table 2. Both clinical and subclinical carditis are considered a major manifestation of ARF in both low- and high-risk populations (Gewitz, et al., 2015).

Table 2: WHF Criteria for the Echocardiographic Diagnosis of RHD. Content reproduced and used with permission from (Reményi, et al., 2012).

2012 WHF Criteria for the Echocardiographic Diagnosis of RHD	
Definite (>20 years): Either A, B, C, or D	
A Pathologic MR and at least two morphologic features of RHD of the mitral valve	
B Mitral stenosis with mean gradient ≥4 mmHg*	
C Pathologic AR and at least two morphologic features of RHD of the aortic valve✚	
D (Only in individuals <35 years)	
E D. Pathological AR and at least two morphological features of RHD of the mitral valve	
Definite (≤ 20 years): Either A, B, C, or D	
A Pathologic MR and at least two morphologic features of RHD of the mitral valve.	
B Mitral stenosis with mean gradient ≥4 mmHg*	
C Pathologic AR and at least two morphologic features of RHD of the aortic valve✚	
D Borderline disease of both the aortic and mitral valves	
Borderline (≤ 20 years): Either A, B, or C	
A At least two morphologic features of RHD of the mitral valve	
B Pathologic MR	
C Pathologic AR	
Pathologic MR (all criteria must be met)	**Pathologic AR (all criteria must be met)**
Seen in two views	Seen in two views
Jet length ≥2 cm** (in at least one view)	Jet length ≥1 cm** (in at least one view)
Velocity ≥3 m/s for one complete envelope	Velocity ≥3 m/s for one complete envelope
Pansystolic jet in at least one envelope	Pandiastolic jet in at least one envelope
Morphologic features of the mitral valve	**Morphologic features of the aortic valve**
Anterior leaflet thickening ≥3 mm	Irregular or focal thickening
Chordal thickening	Coaptation defect
Restricted leaflet motion	Restricted leaflet motion
Excessive leaflet tip motion during systole	Prolapse

MR: mitral regurgitation; AR: Aortic regurgitation
*Must exclude congenital mitral valve anomalies and in adults non-rheumatic mitral annular calcification
✚Bicuspid aortic valve, dilated aortic root, and hypertension must be excluded
Combined MR and AR in high prevalence regions and in the absence of congenital heart disease is regarded as rheumatic

Chorea

The chorea of ARF, also referred to as Sydenham's chorea or St. Vitus's dance, occurs in up to 30% of patients with ARF. It is characterized by involuntary, non-rhythmic, and purposeless movements of the trunk and limbs, which are often more pronounced on one side of the body (al-Eissa, 1993). Rheumatic chorea frequently affects the face and is characterized by grimaces, grins, and frowns. Note that chorea disappears with sleep. Emotional lability is also a feature of rheumatic chorea, especially in older children and adolescents, and is characterized by restlessness and outbursts of inappropriate behavior, including crying (Wilcox & Nasrallah, 1988; Asbahr, et al., 1998).

Chorea may present on its own, without other features of ARF and without evidence of a recent streptococcal infection, because chorea can occur many months after the inciting streptococcal infection. If chorea has an isolated presentation, it is important to exclude other causes of chorea, such as systemic lupus erythematosus, Wilson disease, and drug reactions (World Health Organisation, 2004). In all cases of suspected rheumatic chorea, a careful cardiac examination and echocardiogram must be performed, because chorea is strongly associated with carditis (Elevli, Celebi, Tombul, & Gökalp, 1999).

Skin findings: erythema marginatum and subcutaneous nodules

The skin manifestations of ARF occur in less than 10% of patients, and rarely occur as the sole manifestation of ARF (Gewitz, et al., 2015). Erythema marginatum, also referred to as "erythema annulare," is characterized by bright pink, blanching, non-pruritic macules or papules that spread outwards in a serpiginous pattern, usually on the trunk and proximal limbs. Subcutaneous nodules are small (0.5–2 cm), painless, round nodules that develop over bony prominences (especially the elbows) or extensor tendons, and are usually symmetric. There are usually three to four nodules, and they are generally present for one to two weeks.

Other clinical features

There are four other clinical features that are considered minor manifestations of ARF: fever, arthralgia, elevated acute phase reactants, and prolonged PR interval on electrocardiogram (Gewitz, et al., 2015). The fever of ARF is usually ≥ 38.5 degrees Celsius, but may be lower grade (≥ 38°C) in high-risk populations (Carapetis & Currie, 2001). Arthralgia usually involves several joints in a pattern similar to that of polyarthritis (Cherian, 1979). The erythrocyte sedimentation rate (ESR) is often above >60 mm/hour, and levels of C reactive protein (CRP) are usually above >3.0 mg/dL in patients with ARF, although the ESR may be lower (>30 mm/hour) in high-risk populations.

A number of other clinical features are often observed in patients with ARF but are not included as manifestations in the Jones Criteria, including lethargy, abdominal pain, and epistaxis, as well as rapid sleeping pulse rate and tachycardia out of proportion to fever (Stollerman, Markowitz, Taranta, Wannamaker, & Whittemore, 1965; The Committee on Standards and Criteria for Programs of Care, 1956). Full blood examination frequently reveals a normochromic and normocytic anemia and leukocytosis.

Diagnosis of Acute Rheumatic Fever

There is no single confirmatory test for ARF. Instead, the diagnosis of initial or recurrent ARF relies on patients fulfilling a set of clinical criteria. The most famous of these, the Jones criteria, underwent its fifth revision in 2015 (Gewitz, et al., 2015; Table 1), which expands its applicability to both high and low-risk populations. The revisions also include the results of sub-clinical carditis as a major criterion, which is diagnosed through echocardiographic evaluation. Staying true to its original form, the diagnosis of initial ARF continues to require two major or one major and two minor Jones criteria, along with evidence of a preceding streptococcal infection (Table 1). Chorea and chronic indolent rheumatic carditis remain as exceptions to this requirement, and are

considered in isolation as sufficient evidence of ARF. The Jones criteria continues to emphasize that ARF must be a disease of exclusion, with an active search for other systemic diseases (Gewitz, et al., 2015).

In low-risk populations, defined as an "ARF incidence < 2 per 100,000 school-aged children per year or an all-age prevalence of RHD of ≤ 1 per 1000 population per year" (Gewitz, et al., 2015), the Jones criteria continue to emphasize high specificity to avoid false positive diagnoses. Major criteria include carditis (clinical or subclinical), polyarthritis, chorea, erythema marginatum, and subcutaneous nodules. Minor criteria include polyarthralgia, fever (≥ 38.5°C), ESR ≥ 60mm in the first hour and/or CRP ≥ 3.0 mg/dL, and a prolonged PR interval (unless carditis is already counted as a major criteria, as shown in Table 1).

In moderate- to high-risk populations, which are defined as any population with a prevalence or incidence of infection outside the definition of low risk (see above), the criteria emphasize high sensitivity to avoid false negative diagnoses. Since the last revision (1992), data emerged from endemic regions that restricting joint involvement to the classic migratory polyarthritis led to an under-diagnosis of ARF (Carapetis & Currie, 2001; Cann, Sive, Norton, McBride, & Ketheesan, 2010; Parks, Kado, Colquhoun, Carapetis, & Steer, 2009; Noonan, et al., 2013). Polyarthritis, monoarthritis, or polyarthralgia can now fulfill a major criterion. Slight changes to minor criteria in moderate- to high-risk populations further improve sensitivity and include monoarthralgia, a lowered fever requirement of ≥ 38.0°C, and a lowered ESR cutoff of ≥ 30mm in the first hour (Table 1).

The 2015 Jones revision also provides specific diagnostic criteria for ARF reoccurrences, which have been vague in previous iterations. In patients with a reliable history of ARF or RHD and documentation of a recent streptococcal infection, a reoccurrence can be diagnosed through fulfilling two major, one major and two minor, or three minor criteria. There is also provision for the diagnosis of "possible ARF" both for initial and recurrent ARF, for cases where there is a high clinical suspicion of ARF but incomplete fulfillment of the criteria. Lack of criteria could occur secondary to the unavailability of laboratory or echocardiographic testing, poor clinical history, and/or late presentation. In these circumstances, the Jones criteria find it reasonable to offer secondary prophylaxis with clinical and echocardiographic re-evaluation after one year (Gewitz, et al., 2015).

Table 1: Jones Criteria; (ARF: Acute Rheumatic Fever, RHD: Rheumatic Heart Disease, ESR: Erythrocyte Sedimentation Rate, CRP: C - reactive protein; * Subclinical carditis: Seen only on echocardiography without ausculatory findings, **Accounting for age variability & only if carditis NOT counted as a major criteria). Content reproduced and used with permission from (Gewitz, et al., 2015).

2015 Jones Criteria for the Diagnosis of ARF		
	Low-Risk Population ARF incidence ≤ 2 per 100,000 school-aged children or all-age RHD prevalence of ≤ 1 per 1000 population year	Moderate/High Risk Population Children not clearly from a low-risk population.
Major Criteria		
Carditis	Clinical and/or Subclinical*	Clinical and/or Subclinical*
Arthritis	Polyarthritis	Monoarthritis, Polyarthritis, and/or Polyarthralgia
	Chorea	Chorea
	Erythema Marginatum	Erythema Marginatum
	Subcutaneous Nodules	Subcutaneous Nodules
Minor Criteria		
Carditis	Prolonged PR interval**	Prolonged PR interval**
Arthralgia	Polyarthralgia	Monoarthralgia
Fever	≥ 38.5°C	≥ 38°C

Table 1 continued from previous page.

2015 Jones Criteria for the Diagnosis of ARF		
Markers of Inflammation	Peak ESR ≥ 60mm in 1 hour and/or CRP ≥ 3.0 mg/dL	Peak ESR ≥ 30mm in 1 hour and/or CRP ≥ 3.0 mg/dL
Evidence of Preceding Streptococcal Infection (any one of the following)		
Increased or rising anti-streptolysin O titer or other streptococcal antibodies (anti-DNASE B)		
A positive throat culture for group A B-hemolytic streptococci		
A positive rapid group A streptococcal carbohydrate antigen test in a child whose clinical presentation suggests a high pre-test probability of streptococcal pharyngitis		

Medical Management of ARF

The first aim of management of ARF is to confirm the diagnosis, for which a high index of suspicion is needed, especially in patients presenting with acute arthritis in geographic settings where ARF is endemic. All patients with suspected ARF should be hospitalized so that the clinical course can be closely observed and so that key investigations can be undertaken (Box 1). Additional investigations, such as joint ultrasound, joint aspiration, tests for other causes of arthritis, and tests for other causes of chorea are directed by the clinical presentation and course. Hospitalization also provides an opportunity for education about ARF, especially the need for secondary prophylaxis.

Beyond diagnosis, the priorities in management of ARF are: eradication of the group A streptococcus from the throat and commencement of secondary prophylaxis; symptomatic treatment of arthritis and/or arthralgia; management of carditis and/or heart failure; management of chorea; and patient and family education.

BOX 1: Key investigations for the diagnosis of acute rheumatic fever.

Full blood count

Acute phase reactants: C-reactive protein and erythrocyte sedimentation rate

Echocardiogram

Electrocardiogram

Chest radiograph

Throat swab for bacterial culture

Streptococcal serology (anti-streptolysin O titer and anti-deoxyribonuclease B titer)

Eradication of the group A streptococcus and starting secondary prophylaxis

Patients with ARF are treated with an antibiotic to eradicate *S. pyogenes* (Shulman, et al., 2012). A pragmatic approach is to administer long-acting intramuscular benzathine penicillin G, which serves two purposes: 1) to eradicate *S. pyogenes* carriage; and 2) as the first dose of four-weekly secondary prophylaxis. The first dose of intramuscular BPG given in a hospital setting can begin the process of education about the importance of secondary prophylaxis. Secondary prophylaxis with benzathine penicillin G is the cornerstone of the long-term management of patients with ARF (Shulman, et al., 2012); see Table 3).

Table 3: Recommendations for dosing and duration of secondary prophylaxis. Content reproduced and used with permission from (Gerber, et al., 2009). (Rating indicated classification of recommendation and Level of Evidence (LOE) eg, IA indicates class I, LOE A).

Secondary Prevention of Rheumatic Fever (Prevention of Recurrent Attacks)			
Agent	**Dosage**	**Route of Administration**	**Rating**
Benzathine Penicillin G	**Children (27kg / 60lb or less)** 600 000 U **Those (weighing more than 27kg / 60lb)** 1 200 000 U Every 4 weeks*	Intramuscular	IA
Penicillin V	250 mg twice daily	Oral	IB
Sulfadiazine	Patients (27kg / 60lb or less) 0.5 g once daily Patients (weighing more than 27kg / 60lb) 0.5 g once daily	Oral	IB
For patients allergic to penicillin and sulfadiazine			
Macrolide or Azalide	Variable	Oral	IC

* In high risk situations, administration every 3 weeks is justified and recommended.

Management of joint symptoms

Anti-inflammatory treatment is the mainstay of symptomatic management of the acute joint symptoms of ARF (Illingworth, Lorber, Holt, & Rendle-Short, 1957; Dorfman, Gross, & Lorincz, 1961). First-line therapy has traditionally been aspirin, which remains the most widely used anti-inflammatory medication for ARF. There is increasing experience in using newer NSAIDs, including naproxen for older patients and ibuprofen for younger children (Cilliers, 2003; Czoniczer, Amezcua, Pelargonio, & Massell, 1964; Hashkes, et al., 2003; Uziel, Hashkes, Kassem, Padeh, Goldman, & Wolach, 2000), which some prefer, because of their more convenient dosing frequency and the reduced risk of toxicity or Reye's syndrome, as compared to salicylates. A single small, randomized controlled trial supports the use of naproxen in ARF (Hashkes, et al., 2003). Ibuprofen has been used successfully in younger children with rheumatic fever. In patients with mild arthralgia, acetaminophen or paracetamol may provide adequate treatment.

Aspirin and NSAIDs quickly control joint symptoms, but should be continued until all symptoms have resolved (Dorfman, Gross, & Lorincz, 1961). Most patients require treatment for one to two weeks, although some patients require a longer course. Arthritis can recur when dosage of anti-inflammatory treatment is reduced—this phenomenon is known as "rebound" and means that a longer course of treatment is required (Holt, 1956). If corticosteroid treatment is used for management of carditis or for another reason, aspirin or NSAIDs can be ceased during the period of corticosteroid therapy, but should be re-started following the cessation of treatment.

Management of carditis

There is no evidence that anti-inflammatory therapy alters the long-term outcome of patients with ARF, although some experts recommend corticosteroids treatment in patients with severe carditis (ARF/RHD Writing Group, 2012; Bywaters & Thomas, 1961; Rheumatic Fever Working Party of the Medical Research Council of Great Britain and the Sub-Committee of the Principal Investigators of the American Council on Rheumatic Fever and Congenital Heart Disease, American Heart Association, 1965). Therefore, the management of carditis consists of treatment of heart failure in those with severe carditis. Valve surgery is rarely necessary in patients with rheumatic carditis, but can be life-saving in patients with acute rupture of a valve leaflet or with chordae tendinae (al Kasab, al Fagih, Shahid, Habbab, & al Zaibag, 1988).

All patients with severe carditis, including those with significant cardiomegaly, congestive heart failure, or a third-degree heart block, require an urgent echocardiogram and assessment by a cardiologist. Diuretics, fluid restriction, and bed rest are mainstays in the management of heart failure. Angiotensin-converting enzyme inhibitors are recommended for some patients with symptomatic aortic regurgitation and/or left ventricular dysfunction (Thatai & Turi, 1999). Many experts recommend the use of corticosteroids in severe acute carditis with heart failure, despite an absence of high-quality evidence. The side effects of corticosteroids, including gastrointestinal bleeding and fluid retention, can worsen cardiac failure.

Cardiac surgery is generally avoided until the acute inflammation has subsided, so that the repair is technically easier and so that a better long-term outcome can be achieved (Skoularigis, Sinovich, Joubert, & Sareli, 1994). The exception to this is torrential mitral or aortic regurgitation, which is often due to chordae tendinae rupture with a flail leaflet and requires urgent life-saving surgical repair (al Kasab, al Fagih, Shahid, Habbab, & al Zaibag, 1988). Where possible, valve repair rather than replacement is always the preferred surgery, in order to avoid the long-term anticoagulation treatment that is required for mechanical valves (Remenyi, et al., 2013b).

There are no reliable data to support the use of anti-inflammatory drugs (aspirin, NSAIDs, corticosteroids, intravenous immune globulin) in acute carditis, with regard to subsequent development of heart valve lesions and cardiac disease (Cilliers, Manyemba, & Saloojee, 2003; Voss, et al., 2001; Rheumatic Fever Working Party of the Medical Research Council of Great Britain and the Sub-Committee of the Principal Investigators of the American Council on Rheumatic Fever and Congenital Heart Disease, American Heart Association, 1965).

Management of chorea

Rheumatic chorea is usually benign and self-limiting with resolution within weeks to months, and as a result, treatment other than rest and a calm environment is often not necessary (Lessof & Bywaters, 1956), especially as medications used for chorea have potential serious adverse effects. However, if choreiform movements substantially interfere with normal activities of daily living, place the person at risk of injury, or are distressing to the patient and their family, then treatment should be considered (ARF/RHD Writing Group, 2012).

Valproic acid and carbamazepine are considered first-line treatments for chorea (Daoud, Zaki, Shakir, & al-Saleh, 1990; Genel, Arslanoglu, Uran, & Saylan, 2002; Peña, Mora, Cardozo, Molina, & Montiel, 2002). A small, prospective comparative study found that valproic acid was the more effective agent (Daoud, Zaki, Shakir, & al-Saleh, 1990). However, some experts recommend carbamazepine as initial therapy for severe chorea, because of the potential risk of liver toxicity with valproic acid (Heart Foundation, 2014). Valproic acid is contra-indicated in females of child-bearing age, due to the risk of teratogenicity. Short-course corticosteroid treatment can be also considered for severe or refractory chorea (Barash, Margalith, & Matitiau, 2005; Cardoso, Maia, Cunningham, & Valença, 2003; Paz, Silva, & Marques-Dias, 2006). Aspirin and NSAIDs have no significant effect on rheumatic chorea.

Education

Patients and their caregivers require adequate information and education about rheumatic fever and rheumatic heart disease, as well as the importance of adhering to secondary prophylaxis (Shulman, et al., 2012). In addition, it is important to educate patients about seeking treatment for sore throats, as well as emphasizing the role of dental care.

The transition to chronic rheumatic heart disease

Approximately 35–72% of patients that experience ARF will develop clinical carditis (Bland & Duckett Jones, 1951; Wilson & Lim, 1956; Ash, 1948; Lawrence, Carapetis, Griffiths, Edwards, & Condon, 2013; Meira, Goulart, Colosimo, & Mota, 2005), with an additional 18% showing evidence of sub-clinical cardiac involvement by echocardiography (Tubridy-Clark & Carapetis, 2007). However, resolution of acute carditis can occur,

particularly with excellent compliance with prophylaxis, and not all of these patients will transition to chronic RHD. Resolution is seen most often in the first year following ARF, but has been documented as far out as 10 years following the initial cardiac insult (Bland & Duckett Jones, 1951). Acute carditis limited to pure mitral regurgitation is the most likely lesion to regress, with regression almost never seen with aortic valve involvement (Bland & Duckett Jones, 1951). Factors that consistently predispose patients to development of chronic RHD include a younger age at first episode of ARF, more severe carditis at first episode of ARF, and frequency and number of ARF reoccurrences (Bland & Duckett Jones, 1951; Wilson & Lim, 1956; Ash, 1948).

It is also possible for children who do not have clinical carditis during initial ARF presentation to develop chronic RHD. This may be due to subclinical recurrences, poor compliance with prophylaxis, or delayed development of clinically-apparent disease, and highlights the need for serial echo data.

In the pre-penicillin, pre-echocardiography era, 24% of patients at 10 years and 44% of patients at 20 years who had no clinical carditis at ARF presentation had ausculatory findings of chronic RHD, despite only one-third having documented ARF reoccurrences (Bland & Duckett Jones, 1951). Contemporary data from Australia supports this finding, with 35% of children demonstrating RHD at 1 year following ARF, a number that increased to 61% 10 years after the initial ARF presentation (Lawrence, Carapetis, Griffiths, Edwards, & Condon, 2013).

Rheumatic Heart Disease

Clinical Manifestations, Features and Diagnosis of RHD

Echocardiography is the primary evaluation tool for patients with suspected and confirmed RHD, as it delineates the distribution and severity of valvular involvement and excludes alternate pathology (Saxena, 2013). While the carditis associated with ARF is a pancarditis, valvular pathology almost exclusively dominates chronic RHD. Left-sided cardiac involvement is most commonly seen; it involves the mitral valve almost 100% of the time and involves the aortic valve in 20–30% of cases. The tricuspid valve is affected histologically in 15–40% of patients with RHD (Kitchin & Turner, 1964; Roguin, Rinkevich, Milo, Markiewicz, & Reisner, 1998; Chopra & Bhatia, 1992), but this finding is rarely of clinical importance except in the most severe cases (Kitchin & Turner, 1964; Carpentier, et al., 1974). The pulmonary valve is almost never affected, though there are reports of pulmonary autografts, which have been used in the aortic position during a Ross procedure, subsequently developing graft failure and showing signs of rheumatic carditis (Choudhary, et al., 1999).

Mitral Regurgitation

Mitral regurgitation is the most commonly seen valvular pathology in RHD (Tissier, et al., 2005; Chockalingam, Gnanavelu, Elangovan, & Chockalingam, 2003), particularly in children and young adults, where pure mitral regurgitation is the most common RHD presentation (Rheumatic Fever Working Party of the Medical Research Council of Great Britain and the Sub-Committee of the Principal Investigators of the American Council on Rheumatic Fever and Congenital Heart Disease, American Heart Association, 1965; The Rheumatic Fever Working Party of the Medical Research Council of Great Britain and the Subcommittee of Principal Investigators of the American Council on Rheumatic Fever and Congenital Heart Disease, American Heart Association, 1960). Rheumatic mitral regurgitation primarily results from morphological changes that reflect chronic scarring of the mitral valve and mitral valve apparatus. Morphological mitral valve features are well-visualized on standard 2D echocardiography, though emerging data suggests that 3D echocardiography may improve the sensitivity and specificity of RHD detection in cases of mild valvular involvement and facilitate improved surgical planning for those with advanced disease (Beniwal, Bhaya, Panwar, Panwar, & Singh, 2015).

Valvular thickening, which is seen in 56–100% of patients with rheumatic carditis, is the most common morphological feature on both echocardiography (Yuko-Jowi & Bakari, 2005; Vasan, et al., 1996; Atalay, Uçar,

Ozçelik, Ekici, & Tutar, 2007; Câmara, Neubauer, Câmara, & Lopes, 2004) and direct visualization (Skoularigis, Sinovich, Joubert, & Sareli, 1994; Ungar & Ben-Ishay, 1965; van der Bel-Kahn & Becker, 1986). Such thickening is most commonly seen at the free edge of the leaflet, where the chordal structures can fuse with the leaflet tips (Ungar & Ben-Ishay, 1965); though nodularity, or beading, along the length of the leaflet can also be seen (Atalay, Uçar, Ozçelik, Ekici, & Tutar, 2007). The 2012 WHF criteria advise objective measurement of the mitral valve without harmonic imaging and set criteria for an abnormally thickened mitral valve based on age (Reményi, et al., 2012); see Table 2). Chordal thickening, a purely subjective measure on echocardiography (Reményi, et al., 2012), is also a common feature and results in the restricted movement of the anterior and/or posterior mitral leaflets, and the incomplete valvular coaptation characteristic of rheumatic mitral regurgitation (Vasan, et al., 1996; Atalay, Uçar, Ozçelik, Ekici, & Tutar, 2007; Câmara, Neubauer, Câmara, & Lopes, 2004). Another less common mechanism for chronic rheumatic mitral regurgitation and poor mitral coaptation involves excessive motion of the anterior mitral leaflet tip, which is sometimes referred to imprecisely as mitral prolapse. Though it is more commonly seen in acute rheumatic carditis, excessive anterior leaflet motion is defined as the displacement of the tip of the leaflet (rather than the leaflet body in classic mitral valve prolapse) towards the left atrium, and results from elongation of the primary mitral chords (Reményi, et al., 2012; Kalangos, et al., 2000; Chauvaud, et al., 2001).

Pure mitral regurgitation is generally well tolerated, and particularly that of mild to moderate severity. However, mitral regurgitation can progress over time (Enriquez-Sarano, et al., 1999) through continued scarring of the valve and valve apparatus and/or through compensatory left ventricular dilation, which further prevents proper coaptation by altering the size and position of the mitral annulus (Otsuji, et al., 1997). Patients with significant mitral regurgitation will show increased precordial activity with displacement of the ventricular impulse that is consistent with the degree of ventricular dilation. The classic murmur of mitral regurgitation is a holosystolic apical murmur, which can be best heard with the patient in the left lateral decubitus position. The intensity of the murmur generally correlates with the severity of regurgitation. A normal EKG and chest X-ray are most common with mild mitral regurgitation, but both will show left atrial and left ventricular dilation as the severity of regurgitation increases. Atrial fibrillation is uncommon with pure mitral regurgitation, particularly in the pediatric population, but occurs more commonly as left atrial size increases (Okello, et al., 2013). Except for those with severe acute mitral regurgitation, most patients will remain asymptomatic for years. Over time, mitral regurgitation can result in left ventricular dysfunction and symptomatic patients usually present with signs of left heart failure, decreased exercise tolerance, and shortness of breath with exertion (Nishimura, et al., 2014; Otto, 2003).

Mitral Stenosis

RHD is the most common etiology of mitral stenosis worldwide (Ratnakar, Rajagopal, & Somaraju, 1989; Waller, Howard, & Fess, 1994; Iung, et al., 2007). Development of mitral stenosis is associated with the number, though not necessarily with the severity, of carditis during ARF reoccurrences (Bland & Duckett Jones, 1951; Walsh & Nestor, 1956), and is more common in women (Rheumatic Fever Working Party of the Medical Research Council of Great Britain and the Sub-Committee of the Principal Investigators of the American Council on Rheumatic Fever and Congenital Heart Disease, American Heart Association, 1965; Stollerman, 2001). Mitral stenosis was also commonly seen as a late complication of chorea in up to 25% of children in the pre-antibiotic era (Bland & Duckett Jones, 1951). Rheumatic mitral stenosis was classically thought to be a late presentation in RHD, occurring in the third to sixth decade of life (Horstkotte, Niehues, & Strauer, 1991). However, rheumatic mitral stenosis has a more aggressive course in sub-Saharan Africa and other low-resource settings. For example, in Ethiopia, cases have been documented in a child as young as five years, and mitral stenosis was found in one-third of children diagnosed with clinical RHD (Tadele, Mekonnen, & Tefera, 2013).

Rheumatic mitral stenosis occurs later on the continuum of valvular scarring, and shares many morphological features with rheumatic mitral regurgitation. Typically, the mitral valve leaflets are thick and relatively immobile.

Restricted leaflet mobility generally results from shortening and fusion of the mitral valve chords (seen in 100% of patients with severe mitral stenosis) (Anwar, et al., 2010), calcification, and/or commissural fusion (Reményi, et al., 2012; Ratnakar, Rajagopal, & Somaraju, 1989; Carpentier, 1983), which results in a funnel-shaped mitral valve orifice.

Mild mitral stenosis is well tolerated and usually asymptomatic. However, with increasing stenosis, the left atrial pressure rises, which leads to increased pulmonary venous pressure and eventually to pulmonary hypertension. Early symptoms reflect decreased cardiac output and include fatigue and decreased exercise tolerance. Later, symptoms of pulmonary edema appear with shortness of breath, cough, wheeze, orthopnea, and paroxysmal nocturnal dyspnea. With development of pulmonary hypertension, patients can present in extremis with syncope, hemoptysis, and right heart failure (Nishimura, et al., 2014). Findings on physical examination reflect the severity of disease. The classical mitral stenosis murmur consists of a low-pitched rumbling apical diastolic murmur, which is best heard in the left lateral decubitus position (Nishimura, et al., 2014). Murmurs of longer duration reflect more severe stenosis. If the patient has developed pulmonary hypertension, P2 may be accentuated and patients can show an increased right ventricular impulse. Chest x-ray shows an enlarged left atrium, with or without pulmonary edema, and/or enlarged pulmonary arteries depending on disease severity. ECG evaluation is of critical importance, as patients with significant mitral stenosis are at high risk of atrial fibrillation.

Aortic Regurgitation

Rheumatic aortic regurgitation is most commonly seen in combination with rheumatic mitral valve pathology (Zühlke, et al., 2014). Pure aortic valve disease is uncommon (Zühlke, et al., 2014), occurring in only 4.5% of individuals < 18 and 2.8% of those >18 in a large series of patients from India (Chockalingam, Gnanavelu, Elangovan, & Chockalingam, 2003). If valve disease is suspected, a comprehensive search for other aortic pathologies should be undertaken (bicuspid aortic valve, sub-aortic membrane, infective endocarditis, and connective tissue diseases). The mechanism for rheumatic aortic regurgitation is most commonly restricted aortic leaflet motion, which occurs as a result of leaflet retraction and thickening (Cohen, et al., 1996). Surgical studies have demonstrated that between 41–100% of patients with rheumatic aortic regurgitation show aortic valve thickening, which is often irregular and nodular in appearance and results in a central coaptation defect (Myers, et al., 2010; Talwar, Saikrishna, Saxena, & Kumar, 2005b; Bernal, et al., 1998; Bozbuga, et al., 2004; Tekumit, et al., 2010; Grinda, et al., 2002). Prolapse of the aortic valve is a second, less common mechanism for rheumatic aortic regurgitation (Cohen, et al., 1996), but other primary causes of aortic prolapse are more common and should be ruled out.

Similar to chronic mitral regurgitation, patients with chronic aortic regurgitation can remain asymptomatic for years. However, aortic regurgitation creates both a volume and pressure load on the left ventricle and results in compensatory ventricular dilation (Nishimura, et al., 2014). Over time, ventricular dysfunction can occur and patients may present with symptoms of left heart failure, such as decreased exercise tolerance, fatigue, and shortness of breath with exertion. On physical exam, patients with significant aortic regurgitation will show a widened pulse pressure, bounding pulses, and increased precordial activity with leftward deviation of the ventricular impulse secondary to left ventricular dilation. The classical aortic regurgitation murmur is a high-pitched diastolic murmur heard at the left lower sternal boarder most prominently when a patient is leaning forwards and at end-expiration. Severity of regurgitation correlates with duration of the murmur, with a shorter murmur associated with more severe disease. Chest x-ray and ECG will be normal with mild disease, but with more advanced disease, long-standing aortic regurgitation evidence of left ventricular dilation will present, along with ascending aortic enlargement by chest x-ray.

Aortic Stenosis

Aortic stenosis is a less common rheumatic valvular pathology that is found in only 9% of study subjects in a recent large prospective data collection across Africa (Zühlke, et al., 2014). Stenosis occurs secondary to progressive leaflet thickening, commissural fusion, fibrosis, and calcification. Rheumatic aortic stenosis almost never occurs in isolation, and most patients have mixed aortic valve disease and concurrent mitral valve pathology. The prevalence of aortic stenosis increases with age and most commonly comes to attention anywhere from 20 to 40 years after the initial appearance of ARF (Bland & Jones, 1936).

The development of rheumatic aortic stenosis is usually gradual, which allows time for cardiac compensation and an asymptomatic period. As stenosis worsens, symptoms of left heart failure, poor cardiac output, and poor coronary perfusion develop, including angina, syncope, and shortness of breath with exertion. Patients with aortic stenosis may demonstrate a palpable thrill at the right upper sternal border or suprasternal notch. The classic murmur is a systolic ejection murmur at the right upper sternal border, often with a diastolic decrescendo murmur if there is concurrent aortic regurgitation. In contrast to congenital aortic pathologies, there is rarely an associated opening click (Nishimura, et al., 2014).

Mixed Valve Disease

Except in the young, where pure mitral regurgitation dominates, mixed valvular pathology is the most common finding in chronic RHD (Zühlke, et al., 2014). Mitral regurgitation and mitral stenosis most often develop along a continuum of disease, and many patients have both important regurgitating and stenotic components (Bland & Duckett Jones, 1951). These changes reflect the ongoing valvular scarring and remodeling that occur in RHD, even when recurrent episodes of ARF are absent. Similarly, aortic valve pathology is rarely seen in isolation (Zühlke, et al., 2014), and most commonly has an associated mitral pathology. The right-sided heart valves are infrequently involved and are never involved in isolation. Rheumatic tricuspid involvement is more common than pulmonary involvement, with clinically apparent disease in 3–5% of patients (Kitchin & Turner, 1964; Carpentier, et al., 1974).

Screening for Rheumatic Heart Disease

Not all patients who develop chronic RHD have a clinical history of ARF (Sliwa, et al., 2010; Carapetis, Currie, & Mathews, 2000). Emerging data from low-resource nations suggest that, at least in some endemic regions, latent RHD, or RHD absent a history of ARF, may even be the most common presentation among those with advanced rheumatic cardiac disease (Zhang, et al., 2013). RHD also typically shows a relatively long period between the initial cardiac insult and presentation with symptomatic cardiac disease. While the highest risk of ARF is in childhood, symptomatic RHD most commonly presents in the third and fourth decade (Zühlke, et al., 2014). This clinically silent period presents an opportunity for early RHD detection, and active surveillance for RHD has been advocated for endemic regions since the 1970s (WHO Expert Committee on the Prevention of Rheumatic Fever World Health Organization, 1966).

RHD fulfills the four criteria outlined by the 1994 Council of Europe for determination of diseases suitable for screening (Roberts, Colquhoun, Steer, Reményi, & Carapetis, 2013b). Within endemic areas, RHD exerts an enormous human and financial toll on individuals and communities (Günther, Asmera, & Parry, 2006; Kumar, Raizada, Aggarwal, & Ganguly, 2002; Carapetis & Currie, 1999; Milne, Lennon, Stewart, Vander Hoorn, & Scuffham, 2012b; Parks, et al., 2015), and also substantially contributes to the overall global burden of disease (Global Burden of Disease Study 2013 Collaborators, 2015). The natural history of untreated RHD is well documented, with progression of rheumatic valvular disease dependent on both the severity of the initial carditis and the number of ARF reoccurrences (Bland & Duckett Jones, 1951; Tompkins, Boxerbaum, & Liebman, 1972). Echocardiography, when interpreted through the 2012 World Heart Federation Criteria (WHF; Table 2) (Reményi, et al., 2012), appears to be highly sensitive (Roberts, Brown, Maguire, Atkinson, & Carapetis, 2013a; Beaton, et al., 2014; Colquhoun, et al., 2014; Engel, et al., 2015) and specific (Roberts, et al., 2014; Clark,

Krishnan, McCarter, Scheel, Sable, & Beaton, 2016; McGlacken-Byrne, Parry, Currie, & Wilson, 2015) screening test, though the clinical significance of the category "borderline RHD" remains unclear (see more below). And finally, initiation and maintenance of regular intramuscular penicillin injections (secondary prophylaxis), at least in those with early RHD and a documented history of ARF, improves the long-term prognosis by preventing recurrent ARF episodes and halting the progression of valvular damage (Feinstein, et al., 1964b; Stollerman, Rusoff, & Hirschfeld, 1955; Feinstein, Stern, & Spagnuolo, 1964a; Lue, Tseng, Lin, Hsieh, & Chiou, 1983).

Echocardiographic screening for RHD has emerged over the last decade as the most sensitive test for early RHD detection. A systematic review of echocardiographic screening studies to date calculated a pooled prevalence of RHD of 2.9 per 1000 people by auscultation, as compared to 12.9 per 1000 people by echocardiography (Rothenbühler, et al., 2014). However, studies directly comparing auscultation to echocardiographic screening have demonstrated auscultation to be both poorly sensitive and poorly specific (Viali, Saena, & Futi, 2011; Mehta, et al., 2014), and auscultation is no longer recommended for RHD screening (Reményi, et al., 2012). In 2011, an expert working group was convened by the World Heart Federation to develop the first evidence-based set of criteria for the echocardiographic diagnosis of RHD (Reményi, et al., 2012). These criteria use morphological and functional features of the mitral and aortic valves to categorize individuals as having no RHD, borderline RHD, or definite RHD. These criteria are meant for use only in individuals who have no clinical history of ARF and who reside in RHD endemic regions. It was the hope that standardized diagnostic criteria would improve both the sensitivity and specificity of screening, (particularly in limiting false positive diagnoses) and would provide a platform for sharing and collation of data across studies and sites (Table 2; Reményi, et al., 2012).

Screening by echocardiography has revealed a large burden of "borderline RHD"; a diagnostic category in the 2012 WHF criteria, which includes isolated functional or isolated morphological changes to either the mitral or aortic valves (Reményi, et al., 2012). The clinical significance of borderline RHD is not yet known, and the need for and role of secondary prophylaxis in this population is uncertain. The majority of short-term natural history studies appear to show a relatively benign prognosis for borderline RHD, particularly when the initial lesion is isolated pathological mitral regurgitation (Beaton, et al., 2014; Paar, et al., 2010; Mirabel, et al., 2015c; Bhaya, Beniwal, Panwar, & Panwar, 2011; Saxena, et al., 2011). However, a single study from Australia (with a follow-up at 2.5–5 years) showed that children with borderline RHD were at a significantly greater risk of ARF and progression of valvular disease, as compared to their age- and gender-matched peers (Rémond, et al., 2015). Clearly, more research in this area is needed. Currently, the most common practice for children with borderline RHD is close clinical follow-up, with initiation of secondary prophylaxis only in cases of recurrent ARF or progression of valvular disease.

Echocardiographic screening also faces significant implementation barriers, and echocardiographic screening as a public health measure is not yet widely supported (Roberts, Colquhoun, Steer, Reményi, & Carapetis, 2013b; Zühlke & Mayosi, 2013). Financial resources and skilled healthcare providers are scarce in most regions where RHD is endemic. Task-shifting of RHD screening to non-experts and the use of less expensive handheld echocardiography for screening have shown promise (Beaton, et al., 2015; Lu, et al., 2015; Ploutz, et al., 2016; Mirabel, et al., 2015b; Mirabel, et al., 2012; Engelman, et al., 2015), but further research is needed. Implementation research that examines the best screening strategies and environments is sparse, and the integration of screening into existing healthcare structures is only beginning. Additionally, in most RHD-endemic regions, an investment in infrastructure development will be needed prior to wide-spread screening, to ensure a reliable supply of penicillin and skilled care across the continuum of RHD severity. Finally, while there are preliminary data supporting the overall cost-effectiveness of echocardiographic RHD screening (Manji, et al., 2013; Zachariah & Samnaliev, 2015), these data are based largely on modeling. Future studies will need to include the direct costs from ongoing screening programs in a variety of clinical environments in order to justify investment in echocardiographic screening as a sustainable public health policy.

Medical Treatment and Management of Chronic RHD

Medical management for chronic RHD is largely based on the presence or absence of cardiovascular symptoms. Most patients with mild to moderate valvular involvement will remain asymptomatic for years. Strict adherence to secondary prophylaxis (see above) should be emphasized, as poor adherence and reoccurrence of ARF have independently been associated with an increased risk of RHD complications and death. Heart failure should be considered a surgical disease, with no long-term role for medical management, except in cases where surgery is unavailable or contraindicated (Nishimura, et al., 2014; Borer & Bonow, 2003). Exercise restrictions are based both on the severity of valvular disease and the intensity of the desired activity, and should be guided by the 2005 Bethesda Guidelines (Maron & Zipes, 2005). Emerging data suggests that statins may slow the progression of rheumatic mitral and aortic stenosis, but prospective adult trials have shown mixed results (Antonini-Canterin, et al., 2009; Antonini-Canterin, et al., 2010; Cowell, et al., 2005; Rossebø, et al., 2008). Statins are not currently recommended in patients with chronic RHD.

Mitral Regurgitation

There is no role for medical management in patients with severe mitral regurgitation and preserved left ventricular function (Nishimura, et al., 2014; Borer & Bonow, 2003). Data on the impact of afterload reduction on hemodynamics is mixed, with some studies reporting improvement (Gupta, Kapoor, Garg, Tewari, & Sinha, 2001; Sampaio, et al., 2005) and others reporting hemodynamic worsening (Wisenbaugh, Essop, Rothlisberger, & Sareli, 1992; Röthlisberger, Sareli, & Wisenbaugh, 1994). Importantly, afterload reduction has not been shown to slow symptom development, preserve left ventricular function, or improve survival rates; and as a result, it is not a recommended course of treatment (Nishimura, et al., 2014). Patients who develop symptoms or have decreased left ventricular function should be referred for surgical intervention (Nishimura, et al., 2014). Surgical intervention should also be considered in patients with severe left ventricular enlargement but preserved left ventricular function, if the chances of repair vs. replacement are high, the mortality risk is <1%, and if the surgery can be performed at a Heart Valve Center of Excellence (Nishimura, et al., 2014). Additionally for patients with severe mitral regurgitation and preserved function who have developed atrial fibrillation or resting systolic pulmonary artery hypertension (>50mmHg), surgical interventions should be considered if the chances of repair vs. replacement are high (Nishimura, et al., 2014). If surgical intervention is unavailable or contraindicated, medical therapy for systolic dysfunction is considered a reasonable course of treatment to manage symptoms (Nishimura, et al., 2014).

Mitral Stenosis

Medical management of mitral stenosis centers on the prevention of thromboembolic events. Anticoagulation is indicated for patients with mitral stenosis and atrial fibrillation, and/or a prior embolic event, and/or a left atrial thrombus (Nishimura, et al., 2014). Additional heart rate control can be beneficial in patients with mitral stenosis and atrial fibrillation with rapid ventricular response, or in those whom have mitral stenosis and symptoms that are associated with exercise (Nishimura, et al., 2014). A pilot study examining the utility of Bosentan, an endothelin receptor antagonist, has shown early promise in improving the functional status of patients with pulmonary hypertension secondary to rheumatic mitral stenosis (Vlachogeorgos, et al., 2015), but larger studies are needed before a universal recommendation can be made. As with mitral regurgitation, the timing of intervention is based on development of cardiovascular symptoms, as well as the overall favorability of the valve for a percutaneous vs. a surgical intervention (Nishimura, et al., 2014).

Aortic Regurgitation

Medical therapy has a limited role in treating patients with rheumatic aortic regurgitation. Recent evidence on the role of afterload reduction in the asymptomatic patient with severe aortic insufficiency and preserved left ventricular function has been mixed (Evangelista, Tornos, Sambola, Permanyer-Miralda, & Soler-Soler, 2005;

Scognamiglio, Rahimtoola, Fasoli, Nistri, & Dalla Volta, 1994), and afterload reduction is not currently recommended for these patients (Nishimura, et al., 2014). The asymptomatic patient with significant aortic regurgitation should be monitored for systemic hypertension (systolic BP >140 in an adult), and if present, appropriately treated with afterload reduction, such as calcium-channel blockers, ACE inhibitors, or angiotensin-receptor blockers (Nishimura, et al., 2014). Surgical intervention is indicated for all symptomatic patients with severe aortic insufficiency, even if the left ventricular function is preserved. Surgery should also be considered in asymptomatic patients with severe aortic insufficiency if there is decreased left ventricular function or severe left ventricular dilation (Nishimura, et al., 2014). If surgery is unavailable or otherwise contraindicated, it is reasonable to attempt symptom management with B-blockade and afterload reduction, such as ACE inhibitors or an angiotensin receptor blockade (Nishimura, et al., 2014).

Aortic Stenosis

There is no effective therapy for symptomatic rheumatic aortic valve stenosis (Nishimura, et al., 2014). Timing of surgical intervention is based both on the severity of the disease and the presence of cardiovascular symptoms. Unlike some congenital pathology, catheter-based balloon angioplasty has low efficacy in patients with rheumatic aortic stenosis, and should be reserved for symptomatic patients who are not surgical candidates (Nishimura, et al., 2014; Otto, et al., 1994; Lieberman, et al., 1995).

Endocarditis Prophylaxis

Endocarditis is an important co-morbidity in patients with chronic RHD, and a large percentage of endocarditis in low-and-middle income nations occurs in patients with RHD (Mirabel, et al., 2015a; Moges, et al., 2015; Watt, et al., 2015). However, prophylaxis antibiotics prior to dental and other high-risk procedures are not universally recommended. Current American Heart Association guidelines recommend the use of antibiotic prophylaxis only in patients with prosthetic cardiac valves, for the first six months in patients after placement of intracardiac or intravascular prosthetic material, and in patients with a previous history of endocarditis (Wilson, et al., 2007a). If endocarditis prophylaxis is prescribed for patients with chronic RHD who already receive regular secondary prophylaxis, oral flora should be assumed to be relatively penicillin/amoxicillin resistant and alternative therapies such as clindamycin, azithromycin, or clarithromycin should be utilized. Good oral hygiene should be emphasized for all patients with chronic RHD to decrease the lifetime risk of infective endocarditis (Wilson, et al., 2007a).

Atrial Fibrillation

Patients with mitral stenosis are at the greatest risk for developing atrial fibrillation, though it is also seen in patients with severe mitral regurgitation and significant left atrial dilation. Among patients with mitral stenosis, risk factors for development of atrial fibrillation include older age, higher right atrial pressure, and lower left ventricular ejection fraction (Pourafkari, Ghaffari, Bancroft, Tajlil, & Nader, 2015). Atrial fibrillation can initially present as episodic, but often becomes persistent as valvular disease progresses. Patients with rheumatic mitral and aortic disease have limited cardiovascular reserves, and the combination of decreased atrial contribution to cardiac output (up to one-third) and decreased ventricular filling time secondary to rapid ventricular rate may be poorly tolerated. Additionally, atrial fibrillation, even the transient sub-clinical form (Karthikevan, et al., 2014), increases the risk of systemic embolism and requires anticoagulation to prevent thromboembolic events (Nishimura, et al., 2014).

Surgical and Catheter-Based Treatment of Chronic Rheumatic Heart Disease

As noted above, the primary indications for surgical or catheter-based intervention in chronic RHD are the development of cardiovascular symptoms and/or decreased left ventricular function. Additionally, if a patient with multivalvular disease meets the criteria for intervention on one valve, the team should consider concurrently addressing the other involved valves, even if the valves don't individually meet the criteria, in order

to limit future additional surgeries (Nishimura, et al., 2014). The timing of surgery in children, especially asymptomatic children, is particularly challenging, as chronic RHD often exhibits a lifetime of progression, and the risk of recurrent ARF is highest during youth. Absent specific guidelines for children, most clinicians extrapolate from the adult guidelines (Nishimura, et al., 2014). Decisions on the type of surgical intervention and on surgery vs. catheter intervention are complex and cannot be covered here in full. More complete recommendations were published by the American Heart Association and American College of Cardiology in 2014 and should be consulted prior to making these decisions (Nishimura, et al., 2014).

Mitral Valve Repair vs. Mitral Valve Replacement

When a good result can be achieved, mitral valve repair is preferable to mitral valve replacement, particularly in children (Wang, Zhou, Gu, Zheng, & Hu, 2013). Techniques to repair the mitral valve include classical approaches such as thinning of the anterior mitral leaflet, commisuroplasty, and mitral annuloplasty (Talwar, Rajesh, Subramanian, Saxena, & Kumar, 2005a; Kitamura, Uemura, Kunitomo, Utoh, & Noji, 2000; Chauvaud, et al., 1986; Rumel, Vaughn, & Guibone, 1969), as well as newer techniques such as tricuspid autografts, neo-chordae, and pericardial patches (El Oumeiri, et al., 2009; Pomerantzeff, et al., 2009). A 2013 meta-analysis that compared mitral valve repair to mitral valve replacement found lower early and late mortality and fewer major adverse events in patients undergoing mitral valve repair. As compared to replacement, repair avoids the need for anticoagulation, and a decrease in hemorrhage and thromboembolic complications contributed to the reduction in risk. However, patients undergoing repair showed a higher rate of re-operation (Wang, Zhou, Gu, Zheng, & Hu, 2013). In most populations, the authors concluded that repair should be favored over replacement, though they have still accepted the potential need for re-operation (Wang, Zhou, Gu, Zheng, & Hu, 2013). However, in resource-limited settings (particularly those without self-sustained cardiovascular surgery programs), operative decisions need to account both for the risks of anticoagulation (which favors repair or the use of bioprosthetic valves) and the feasibility of re-operation (which favors replacement).

Surgical Mitral Valvotomy vs. Catheter-based Valvotomy

In selected patients and in the hands of a skilled operator, percutaneous mitral balloon valvotomy shows comparable results to open mitral commissurotomy, with high success rates. Percutaneous intervention is also highly effective in juvenile rheumatic mitral stenosis, and when possible, should be attempted as the primary procedure (Karur, Veerappa, & Nanjappa, 2014). Selection of patients with favorable mitral valves predicts success. Patients that are most likely to have immediate and lasting improvement have valves that are non-calcified, relatively mobile, and that lack severe leaflet thickening or subvalvular pathology (Wilkins, Weyman, Abascal, Block, & Palacios, 1988). Pre-existing left atrial thrombus and/or significant mitral regurgitation are relative contraindications to a percutaneous approach (Nishimura, et al., 2014). Current practice favors earlier intervention in patients who meet criteria for percutaneous intervention, with practitioners waiting longer to intervene if a surgical approach is needed (Nishimura, et al., 2014; Carabello, 2005).

Management of RHD during Pregnancy

Another aspect of management for RHD occurs during pregnancy, due to its associated increase in cardiac output. By monitoring pregnant women with RHD, healthcare providers can ensure that the extra cardiac output during pregnancy does not lead to heart damage or failure in RHD patients—especially since in some cases, the presence or severity of RHD may only become apparent during pregnancy, and as a result, secondary prophylaxis would not have been previously administered (Sawhney, et al., 2003). This management involves careful family planning through use of contraceptives, education about the risks of pregnancy, and the use of an anticoagulant that ensures both maternal and fetal safety.

Prevention of ARF and RHD

Group A Streptococcus, Acute Rheumatic Fever, and Rheumatic Heart Disease

Streptococcus pyogenes, also known as group A Streptococcus, is a Gram-positive bacterial pathogen responsible for a range of human diseases (Walker, et al., 2014). Superficial *S. pyogenes* infections, such as pharyngitis and impetigo, do not require long-term care. Nevertheless, these diseases are significant due to their prevalence and the required use of antibiotics for treatment that may be scarce in low-resource settings. Furthermore, superficial infections can lead to serious invasive diseases, including toxic shock, necrotizing fasciitis, and cellulitis (Moreland, et al., 2014). *S. pyogenes* infections can also lead to post-infectious, immune-mediated sequelae, which require costly, long-term treatment.

The most significant sequelae to *S. pyogenes* infection are ARF and RHD, and there are many strategies available to prevent these diseases. The challenge is to ensure that they are available in all settings, including resource-constrained environments, and that novel strategies are explored to help eliminate or eradicate ARF and RHD.

Primordial Prevention

Primordial prevention for ARF refers to the reduction of risk factors for *S. pyogenes* exposure and infection in susceptible individuals, given that these factors and infections would normally precede ARF (ARF/RHD Writing Group, 2012). While often underemphasized, primordial prevention is the major explanation for the control of ARF and RHD that occurred in affluent settings during the 20th century (Gordis, 1985). Figure 3 demonstrates that the major reduction in RF deaths in the USA occurred prior to the availability of antibiotics—these reductions can be attributed to improved living conditions and reductions in poverty (Gordis, 1985).

One poverty-associated risk factor for *S. pyogenes* infection is household crowding (Maguire, Carapetis, Walsh, & Brown, 2012; Rheumatic Fever Working Party of the Medical Research Council of Great Britain and the Sub-Committee of the Principal Investigators of the American Council on Rheumatic Fever and Congenital Heart Disease, American Heart Association, 1965; Gordis, Lilienfeld, & Rodriguez, 1969). The effects of overcrowding were analysed in a study showing that when beds were moved closer to one another in military barracks, rates of *S. pyogenes* infection increased (Wannamaker, 1954). Another study in Australia found a strong association between overcrowded housing in Aboriginal communities and rates of streptococcal impetigo (McDonald, et al., 2006). The association is highly plausible because crowding allows for rapid *S. pyogenes* bacterial transmission. Thus, the improvement of housing is crucial to preventing the spread of *S. pyogenes* infections.

Another potential risk factor for *S. pyogenes* infection is poor nutrition in childhood (Steer, Carapetis, Nolan, & Shann, 2002). While this factor may merely be correlated with overcrowded housing, rather than being solely responsible for *S. pyogenes* infection susceptibility, insufficient nutrition can alter antibody responses, with evidence that poorly-nourished individuals may be more likely to develop ARF subsequent an *S. pyogenes* infection (Steer, Carapetis, Nolan, & Shann, 2002). Overall, risk factors resulting from poverty are associated with high rates of ARF and RHD, making it crucial to tackle these factors and avoid the requirement for expensive treatments and reductions in the quality of life once individuals have already contracted *S. pyogenes* infections, ARF, and RHD.

Primary Prevention

While primordial prevention addresses the socioeconomic risk factors for *S. pyogenes* infection, primary prevention serves to prevent *S. pyogenes* colonization, infection, and transmission, as well as preventing the spread of *S. pyogenes* infections (ARF/RHD Writing Group, 2012).

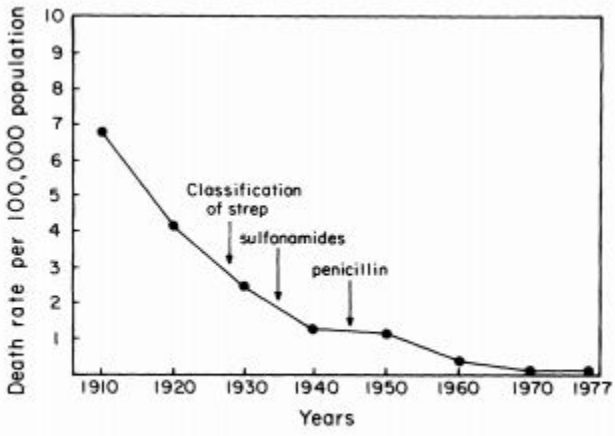

FIGURE 7. Crude death rates from rheumatic fever, United States, 1910–1977.

Figure 3. Crude death rates from rheumatic fever in the USA, 1910–1977. Figure reproduced from (Gordis, 1985) with permission.

The most desirable primary prevention method is vaccination against the *S. pyogenes* bacterium, which would prevent *S. pyogenes* colonization and infection, and thus prevent post-infectious sequelae, such as ARF and RHD (Sheel, Moreland, Fraser, & Carapetis, 2016). Based on the range of diseases caused by *S. pyogenes* that include superficial, invasive infections to post-infectious sequelae, a vaccine against *S. pyogenes* could effectively prevent all of these *S. pyogenes*-mediated diseases (Sheel, Moreland, Fraser, & Carapetis, 2016). However, few pharmaceutical companies have shown interest in developing an *S. pyogenes* vaccine until recently. This may be partially due to antibiotics being readily available to treat *S. pyogenes* pharyngitis in wealthy areas, which makes a vaccine potentially unnecessary. Until an effective, globally-relevant *S. pyogenes* vaccine reaches the market, other methods must be relied upon to prevent the development of invasive infection and post-infectious sequelae upon *S. pyogenes* infection.

The current mainstay of primary prevention of ARF and RHD is the administration of antibiotics for *S. pyogenes* pharyngitis (ARF/RHD Writing Group, 2012), which eliminates bacteria before they can trigger an autoimmune response. To treat pharyngitis and prevent ARF, healthcare providers may administer one dose of intramuscular BPG or a ten-day course of oral penicillin or amoxicillin, commencing within nine days of the onset of the *S. pyogenes* infection (Bass, Crast, Knowles, & Onufer, 1976; Lennon, Farrell, Martin, & Stewart, 2008; Clegg, et al., 2006; Robertson, Volmink, & Mayosi, 2005). This is known as primary prophylaxis.

There are two main strategies to the implementation of primary prophylaxis. The first involves the incorporation of primary prevention efforts, such as administering antibiotics following *S. pyogenes* infection, into primary health care delivery efforts, while another strategy could involve a systematic surveillance approach for identifying and treating *S. pyogenes* throat infections at the community level. In almost all settings, the more practical strategy would be the implementation of primary prophylaxis into primary care delivery efforts; however, in resource-poor settings, various challenges (such as poor health seeking behaviors in those who may have a sore throat), the logistics, cost, limited supply, availability and timely delivery of BPG may hinder this approach (Karthikeyan & Mayosi, 2009). For effective primary prophylaxis intervention, individuals with sore throats must present for health care; health staff must have awareness, the appropriate training and resources to diagnose and treat *S. pyogenes* pharyngitis; and finally, the treatment must be delivered and the treatment schedule followed. Very few resource-poor countries are capable of providing these key elements in their entirety, in addition to the added cost and limited feasibility of obtaining a bacteriological diagnosis, leaving the diagnosis of *S. pyogenes* pharyngitis using imperfect clinical algorithms as the only practical option.

However, even under ideal circumstances, primary prophylaxis may potentially prevent a minority of ARF cases, since many ARF episodes do not follow a significant sore throat, with outbreaks of ARF documented over the past decades in Utah (Veasy, et al., 1987), Columbus (Hosier, Craenen, Teske, & Wheller, 1987), Ohio (Congeni, Rizzo, Congeni, & Sreenivasan, 1987), and Pennsylvania (Wald, Dashefsky, Feidt, Chiponis, & Byers, 1987). Although the cause of these particular outbreaks remains unknown and they affected mainly mid-socioeconomic family groups, there is a possibility that they may have been related to particularly virulent bacterial strains or other factors.

Discouraging as this may be, primary prophylaxis remains an important step for preventing new episodes of ARF and the subsequent development of RHD. Encouraging sore throat presentations along with the correct diagnosis and appropriate treatment of *S. pyogenes* infections remains a significant means of preventing new episodes of ARF; however, this may have a minimal impact on the incidence of ARF at the population level (Carapetis, 2010).

An alternative strategy that could be undertaken is active surveillance of at-risk children in an effort to diagnose and appropriately treat *S. pyogenes* pharyngitis. This has been attempted in different ways, although the more common approach is through the utilization of a school-based sore throat surveillance system (Lennon, Stewart, Farrell, Palmer, & Mason, 2009). The only rigorous study of this approach was a large randomized trial conducted in New Zealand that found a 22–28% non-significant reduction in ARF incidence in schools with sore-throat clinics (Lennon, Stewart, Farrell, Palmer, & Mason, 2009). Despite the lack of statistical significance found in this study, New Zealand has since embarked on a large implementation of school-based sore throat surveillance and treatment, combined with a number of other strategies to try to reduce the incidence of ARF. An interim evaluation found evidence of a significant reduction in ARF incidence that was confined to the region of the country with the highest incidence rate, but was not directly attributable to the sore throat clinics in schools (Jack, et al., 2015). Moreover, the approach that was undertaken in New Zealand was costly and expensive, and unlikely to be affordable in the vast majority of countries with a high incidence of ARF. At this stage, active sore throat surveillance and treatment remains a costly exercise and may not be routinely recommended.

There is some evidence that skin infection may play a role in the pathogenesis of ARF (Williamson, et al., 2015), which raises the prospect that control of skin infections may lead to subsequent reductions in ARF incidence. There is also considerable momentum currently in Australia around community-based interventions to control skin infections and underlying scabies (Andrews, McCarthy, Carapetis, & Currie, 2009; Steer, et al., 2016) but the potential for this to be a form of primary prevention for RF and RHD remains unproven. While primary prevention measures are necessary to ensure that *S. pyogenes* throat or possibly skin infections do not lead to ARF, the challenge remains for the implementation of these available strategies in resource-constrained settings. The development of a *S. pyogenes* vaccine would greatly facilitate primary prevention in these areas.

Secondary Prevention

The goal of secondary prevention of ARF and RHD (otherwise known as secondary prophylaxis), is to prevent recurrence of *S. pyogenes* infection in those previously diagnosed with ARF (Wyber, et al., 2014). The most effective approach to secondary prophylaxis involves the use of benzathine penicillin G (BPG), the long-acting depot form of penicillin G (Medsafe, 2012; Stollerman & Rusoff, 1952; Stollerman, Markowitz, Taranta, Wannamaker, & Whittemore, 1965). Once BPG is intramuscularly injected, the drug is slowly released from the muscle into the systemic circulation, where it is activated via *in-vivo* hydrolysis, and produces prolonged serum concentrations of benzylpenicillin (Medsafe, 2012). This prolonged release BPG formulation is vital for the treatment and prevention of ARF and RHD, where serum concentrations of benzylpenicillin are expected to remain at or above 0.02 μg/ml, the accepted minimum inhibitory concentration (MIC) for preventing *S. pyogenes* colonization and preventing ARF recurrences (Gutmann & Tomasz, 1982; Currie B. , 2006; Medsafe, 2012).

The use of oral penicillin V is an alternative regimen, but even with 100% adherence, it is not as protective against recurrent *S. pyogenes* infection or ARF as BPG (Manyemba & Mayosi, 2003). Most patients who report an allergy to penicillin may not necessarily have experienced an allergic reaction to the drug, but since penicillin is the drug of choice for secondary prophylaxis, it is recommended that a thorough assessment of possible penicillin hypersensitivity be conducted before accepting that an ARF or RHD patient cannot receive penicillin prophylaxis. In these rare circumstances, the American Heart Association recommends sulfadiazine or sulfasoxazole, or an oral macrolide or azalide as a suitable alternative (Gerber, et al., 2009).

To maintain protective serum concentrations, the recommended regimen for secondary prophylaxis requires the injection of 1.2 million units of BPG once every four weeks for a minimum of 10 years, and in some cases, even for a lifetime (Wyber, et al., 2014). While current protocols for secondary prophylaxis suggest four-weekly BPG injections, pharmacokinetic studies show that serum penicillin G levels may be below the minimum inhibitory concentration (MIC) detection level of 0.02 μg/ml for *S. pyogenes* by 10–21 days after injection (Broderick, et al., 2011; Neely, Kaplan, Blumer, Faix, & Broderick, 2014). More frequent BPG dosing has been suggested (Broderick, et al., 2011; Neely, Kaplan, Blumer, Faix, & Broderick, 2014); however, adherence to such intensive injection schedules may be problematic. Despite the low serum penicillin G levels, current evidence suggests that dosing BPG every 28 days can prevent recurrences of ARF (ARF/RHD Writing Group, 2012). Therefore, the World Heart Federation recommends BPG injections every four weeks for those on secondary prophylaxis, unless the patient has a history of breakthrough ARF recurrence on prophylaxis or lives in a particularly high-risk setting. While more frequent administration may offer better protection, the recommendation for BPG injection every four weeks is also influenced by patient acceptability, adherence rates, logistical barriers, and costs involved with the delivery of secondary prophylaxis, as well as the supply of high quality penicillin. In New Zealand, where dosing once every four weeks has long been the standard of care, the recurrence rates of ARF remain low (Pennock, et al., 2014; Robin, Mills, Tuck, & Lennon, 2013; Siriett, Crengle, Lennon, Stonehouse, & Cramp, 2012; Spinetto, Lennon, & Horsburgh, 2011).

Even with the use of injections every four weeks, rather than more frequent BPG administration schedules, patient adherence to secondary prophylaxis is often low (Gasse, et al., 2013). While adherence to as few as 80% of BPG doses can significantly lower a patient's chances of contracting an *S. pyogenes* infection, in some regions with high rates of RHD, nearly half of all patients fail to meet this threshold (Spinetto, Lennon, & Horsburgh, 2011). Moreover, it is suspected that in low socioeconomic populations of many countries, access to and availability of BPG is so problematic that most ARF and RHD patients receive no secondary prophylaxis at all.

There are many community-specific barriers for the adherence of BPG secondary prophylaxis schedules, which can include poor healthcare delivery, a lack of relationships with local healthcare providers, limited availability of transportation, and fear of anaphylaxis (Gasse, et al., 2013; Huck, et al., 2015; Musoke, et al., 2013; Tullu, Gandhi, & Ghildiyal, 2010; Stewart, McDonald, & Currie, 2007). These barriers must be addressed by tailoring implementation of secondary prophylaxis to the needs of those receiving treatment. There are also secondary prophylaxis adherence barriers that are shared across ARF and RHD patients, which could potentially be addressed through BPG reformulation. One significant example that is responsible for lack of patient adherence to secondary prophylaxis is the frequency of injections. This is particularly problematic in remote and low-resource areas, where patients may not be able to access clinics every four weeks (Mincham, Toussaint, Mak, & Plant, 2003; Huck, et al., 2015). The pain associated with the monthly BPG intramuscular injections is also another significant factor, as children in particular often comment on the pain they experience when receiving their injections (Gasse, et al., 2013; Stewart, McDonald, & Currie, 2007; Huck, et al., 2015).

Difficulties with the administration of BPG injections are exacerbated by the use of a powdered BPG formulation, which often crystallizes and causes needle blockage after resuspension in diluent (Public Health Agency of Canada, 2013). The alternate, premixed formulation of BPG requires refrigeration and is very expensive, making it impractical for use in remote locations (Currie B. , 2006). Therefore, the pain and

inconvenience of secondary prophylaxis remains inevitable whenever the premixed formulation of BPG cannot be used.

There are also concerns that even those who adhere to treatment schedules may not be receiving high-quality BPG (Wyber, Taubert, Marko, & Kaplan, 2013). The issue of needle-blockage has raised concerns about the quality of BPG, since it is unclear whether this problem is caused by the active ingredient or by contaminants and by-products, which may also cause allergic reactions and anaphylaxis. Furthermore, concerns have also been raised as to whether available formulations of BPG contain enough of the active ingredient to protect patients for the expected drug-release period (Currie, 1996; Currie, Burt, & Kaplan, 1994; Lue, Wu, Hsieh, Lin, Hsieh, & Chiou, 1986; Lue, Wu, Wang, Wu, & Wu, 1994; Lue, Wu, Wang, Wu, & Wu, 1996; Broderick, Hansen, Russell, Kaplan, Blumer, & Faix, 2011). Limited global data are available on the quality of BPG, and there are few quality control guidelines in place for BPG manufacturing (Wyber, Taubert, Marko, & Kaplan, 2013). Thus, secondary prophylaxis patients may not be receiving high-quality BPG, which increases their risk of contracting *S. pyogenes* infections. In the long term, concerns regarding BPG quality and low secondary prophylaxis adherence must be addressed through the development of a safe, effective and less painful BPG reformulation that supports adherence. The preferred characteristics of a long-acting BPG reformulation that could improve adherence to secondary prophylaxis schedules were recently published (Wyber, et al., 2016). These characteristics included a BPG reformulation that was less painful and featured an extended dose interval, therefore reducing dose frequency, was heat stable, and is cheaper than currently available products (Wyber, et al., 2016). Secondary prophylaxis for ARF and RHD is crucial in preventing cardiac damage in patients, often children, with a history of ARF. Thus, it is important to improve the delivery and effectiveness of secondary prophylaxis.

Tertiary Prevention

Tertiary prevention refers to the provision of medical and surgical interventions to prevent morbidity and mortality from cardiac damage that has resulted from ARF (ARF/RHD Writing Group, 2012; World Health Organization). These interventions include management of heart failure, anticoagulation, arrhythmia, prevention of endocarditis and pregnancy-related complications, along with surgical interventions (Remenyi, Carapetis, Wyber, Taubert, & Mayosi, 2013a)—these are covered in previous sections.

Tools for the Prevention of ARF and RHD: Register-Based Control of RHD

The purpose of a register-based approach to RHD control is to improve the delivery of secondary prophylaxis by keeping track of those who are receiving treatment (Carapetis & Zühlke, Global research priorities in rheumatic fever and rheumatic heart disease, 2011). The use of registers as a tool to improve delivery of secondary prevention for ARF and RHD was first established across the USA in the 1950s, and while the significant decreased prevalence of ARF and RHD in the USA cannot be solely attributed to these programs, the register-based approach is still considered to be an effective tool (McDonald, Brown, Noonan, & Carapetis, 2005). Due to the success of registers in the USA, the WHO launched a register for ARF and RHD control in 1972 (McDonald, Brown, Noonan, & Carapetis, 2005). These WHO-coordinated registers were established in various locations, and while the use of these registers differed based on location, the proportion of individuals receiving ten or more doses of BPG per year increased for patients on the register (Strasser, et al., 1981). This study revealed that a register-based approach could significantly improve the delivery of secondary prophylaxis, though the registers in this study did have lower coverage rates (McDonald, Brown, Noonan, & Carapetis, 2005).

The WHO-coordinated registers were replaced in 1990 with a global program that helped establish registers in 16 countries, but funding for this program ended in 2001 (Thornley, McNicholas, Baker, & Lennon, 2001). Since then, few countries have been able to establish significant regional or national registers.

It is important to distinguish between a register, which is a database used to monitor and improve patient care, and a control program, which may use a register as its core data collection tool, but which also incorporates broader strategies around patient care, disease control, and education. The most successful RHD control programs currently in use are based in New Zealand and Australia (Thornley, McNicholas, Baker, & Lennon, 2001). The New Zealand approach includes management and surveillance programs, where management programs coordinate delivery of secondary prophylaxis through nursing services, while surveillance programs keep track of those receiving prophylaxis without providing the treatment themselves (Thornley, McNicholas, Baker, & Lennon, 2001). Overall, these two types of registers are effective because they both help track RHD prevalence and centralize treatment delivery (Thornley, McNicholas, Baker, & Lennon, 2001). Overall, a register-based approach may help ensure consistent delivery of secondary prophylaxis as well as medical and surgical care to ARF and RHD patients, which will help lower the recurrence of ARF and morbidity and mortality from RHD. This approach can help prevent cardiac damage, which is costly and detracts from patients' quality of life.

Conclusion

The progression of RHD, beginning with *S. pyogenes* infection and continuing through ARF and subsequent cardiac valve damage, offers many opportunities for the prevention of "the next step" in disease prognosis. Thus, it is important to ensure that areas with high rates of ARF and RHD have adequate resources for all of these stages of prevention. These include programs to improve housing and sanitation, antibiotics to treat *S. pyogenes* infection and an *S. pyogenes* vaccine (once developed), a stable supply of high-quality BPG for those diagnosed with ARF, and access to appropriate medical and surgical treatments to treat cardiac damage.

These strategies are not equally effective in preventing ARF and RHD, nor have they been assigned equal focus. While efforts have been directed towards improving access to secondary prophylaxis, fewer resources have been dedicated to preventing the initial attack of ARF through vaccine development, and even more fundamentally, through the improvement of housing and sanitation in resource-poor areas. While it is crucial to improve the delivery of secondary prophylaxis by ensuring that BPG is available and that dosing strategies are effective in those receiving this treatment, and while secondary prophylaxis has been determined to be cost-effective, this does not mean efforts should only be directed at this prevention effort (Steer & Carapetis, 2009). While cardiac surgery would ideally be made accessible to those who have been impacted by RHD, this strategy is costly and may not necessarily ensure a high quality of life for those affected. It is crucial that investment also be directed at primordial and primary prevention of *S. pyogenes* infection; for, as observed through the rarity of ARF and RHD in wealthy communities, the prevention and treatment of initial infection through less crowded housing, vaccination, and the treatment of *S. pyogenes* pharyngitis (and possibly skin infection), can prevent ARF and RHD (Steer & Carapetis, Prevention and treatment of rheumatic heart disease in the developing world, 2009). Secondary and tertiary prevention strategies certainly prevent cardiac damage and death from ARF and RHD, but primordial and primary prevention strategies can prevent the onset of these conditions in the first place, which will ultimately help eliminate (or ideally, eradicate) ARF and RHD.

Vaccine Development

The Development of a Vaccine for *Streptococcus pyogenes*

Please refer to the following chapter article in this series:

Ferretti, J. J., Stevens, D. L., & Fischetti, V. A. (2016). Current Approaches to Group A Streptococcal Vaccine Development--Streptococcus pyogenes: Basic Biology to Clinical Manifestations.

The CANVAS Initiative to Facilitate *S. pyogenes* Vaccine Development

To make a safe and effective *S. pyogenes* vaccine available for use, vaccine development efforts must be complemented by projects that facilitate the development and distribution of this vaccine. One such project is the trans-Tasman CANVAS (Coalition to Advance New Vaccines for Group A *Streptococcus*) initiative, which offers three deliverables to accelerate *S. pyogenes* vaccine development (Steer, et al., 2016). One deliverable is an economic evaluation, which will help determine whether investment in a *S. pyogenes* vaccine is economically viable (Steer, et al., 2016). Based on the high costs of treatment for *S. pyogenes*-mediated diseases such as ARF and RHD, it is likely that vaccination against *S. pyogenes* will be cost-effective, particularly in developing nations with high rates of ARF and RHD (Steer & Carapetis, 2009; Steer, et al., 2016). Furthermore, pharmaceutical companies, including Merck and GSK, as well as governmental organizations have supported *S. pyogenes* vaccine development in the past (Steer, et al., 2016). Overall, while a rigorous economic evaluation of a potential *S. pyogenes* vaccine has yet to be published, it is likely that the development of a *S. pyogenes* vaccine will be economically efficient, due to the high duration of treatment for *S. pyogenes*-mediated diseases, such as ARF and RHD, and the loss of productivity that is due to the severity of these diseases (Sheel, Moreland, Fraser, & Carapetis, 2016).

The CANVAS initiative seeks to create a panel composed of a selection of the most common strains of *S. pyogenes*, which would help determine which strains should be covered by a vaccine. Current vaccine candidates will be tested against this panel (Sheel, Moreland, Fraser, & Carapetis, 2016). These CANVAS initiative projects aim to facilitate the rapid development and availability of a *S. pyogenes* vaccine.

Conclusions

The need for a vaccine against *S. pyogenes* is evident, given the high rates of *S. pyogenes* infection globally and the severity of some *S. pyogenes*-mediated diseases, particularly in low-resource areas. Unfortunately, efforts to develop a *S. pyogenes* vaccine have been hindered by safety concerns and by lingering perceptions that *S. pyogenes* infections are harmless or easily treatable, since invasive infections and post-infectious sequelae are rare in wealthy regions, which are where vaccine development will likely take place. The development of a safe and effective vaccine against *S. pyogenes* will help prevent severe diseases that often affect children, and will improve the quality of life for individuals susceptible to *S. pyogenes* infections, particularly in developing countries and in indigenous populations in wealthy nations.

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Pediatric Autoimmune Neuropsychiatric Disorders Associated with Streptococcal Infections (PANDAS)

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Foreword

The inclusion of a chapter on pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections (or PANDAS) is essential to provide a history of the disease and provide current information about its association with *Streptococcus pyogenes* (group A streptococci), tics, obsessive compulsive disorder (OCD) and its relationship to Sydenham chorea (SC), which is the neurologic manifestation of acute rheumatic fever. PANDAS has been misunderstood and confusing to doctors since its discovery, but the original group of the first 50 cases as described by Dr Susan Swedo (Swedo, et al., 1998) has a similarity to Sydenham chorea that distinguishes this initial group from tic and OCD cases. As this chapter will examine, the acute onset is an important feature of these disorders, as are their piano-playing choreiform movements, enuresis, night-time fears, separation anxiety, learning regression, and handwriting disabilities.

The most current literature, which has been recently published in the *Journal of Child and Adolescent Psychopharmacology* (Murphy, et al., 2015b; Murphy, Parker-Athill, Lewin, Storch, & Mutch, 2015a; Toufexis, et al., 2015; Gerardi, Casadonte, Patel, & Murphy, 2015; Chang, et al., 2015), provides new insight into the clinical phenotype of PANDAS; namely, a subgroup of pediatric acute-onset neuropsychiatric syndrome (PANS), which has been proposed to have multiple etiologies, including those that are genetic and immunologic, and that present either with or without preceding infections, such as with *Streptococcus pyogenes* (Toufexis, et al., 2015). PANS is a subtype of obsessive compulsive disorder (OCD) that presents with an abrupt onset or exacerbation of neuropsychiatric symptoms (Murphy, et al., 2015b), including moderate or severe OCD. Elevated anti-streptococcal antibody titers tended to have higher OCD severity and the symptoms tended to lead to sudden and severe impairment, due to comorbidities, such as anxiety, behavioral regression, depression, and suicidality. Comorbid tics in PANS were associated with decline in school performance, visuomotor impairment, eating disorders, deterioration of handwriting skills, and lower quality of life, as compared to children without tics (Murphy, et al., 2015b). In addition, clinical evaluation of youth with PANS and PANDAS and recommendations for diagnosis were reported from the 2013 PANS conference held at Stanford University where a group of clinicians and researchers who were academicians with clinical and research interest in PANDAS and PANS (Chang, et al., 2015). PANDAS is clearly a subtype of PANS (Murphy, et al., 2015b; Murphy, Parker-Athill, Lewin, Storch, & Mutch, 2015a; Chang, et al., 2015) and not all PANS cases have an underlying streptococcal infection—but all PANDAS cases are associated with streptococcal infections, at least temporally.

When these diseases appear, treatment with antibiotics can be successful, and a treatment trial of cefdinir by Murphy and colleagues indicated that therapy with cefdinir, a β lactam antibiotic, provided notable improvements in tic symptoms rated by the Yale Global Tic Severity Scale (YGTSS) and OCD symptoms rated

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by the Children's Yale-Brown Obsessive Compulsive Scale (CY-BOCS). However, the differences within the groups as a whole were not significant. β-lactam antibiotics have been proposed to be neuroprotective above and beyond their antibiotic efficacy (Murphy, Parker-Athill, Lewin, Storch, & Mutch, 2015a). Anti-neuronal autoantibodies against the brain in SC and PANDAS react with brain antigens including dopamine receptors (Cox, et al., 2013; Brimberg, et al., 2012), lysoganglioside (Kirvan, Swedo, Heuser, & Cunningham, 2003; Kirvan, Swedo, Snider, & Cunningham, 2006a), and tubulin (Kirvan, Cox, Swedo, & Cunningham, 2007), as well as the activation of the calcium calmodulin-dependent protein kinase II (CaM KII) in human neuronal cells (Kirvan, Swedo, Heuser, & Cunningham, 2003). Human anti-brain antibodies expressed in Tg mice targeted dopaminergic neurons and signaled the dopamine D2 receptor (D2R) (Cox, et al., 2013). Evidence strongly suggests that human anti-brain autoantibodies induced by *Streptococcus pyogenes* infections target the dopamine receptors (Cox, et al., 2013; Brimberg, et al., 2012) and that animal models immunized with the *S. pyogenes* antigen develop obsessive behaviors and movement problems, along with antibodies that react with the dopamine receptors and signal the CaMKII, similar to antibodies found in humans with SC and PANDAS (Brimberg, et al., 2012; Lotan, et al., 2014a).

Introduction and Background

In the last half of the 1990s, a group of clinical researchers, including Swedo et al. (Swedo, et al., 1998; Snider, et al., 2002) at the National Institutes of Mental Health (NIMH) described a subgroup of children who presented with obsessive-compulsive disorder (OCD) and/or tic disorders following an infectious illness, in particular after streptococcal infections, and proposed the term pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections (PANDAS) for this subgroup (Swedo, et al., 1998).

The background information for this proposal stems from different sources: some anecdotal reports on the relationship between OCD or tic symptoms and infectious illness (Selling, 1929; von Economo, 1931; Kondo & Kabasawa, 1978; Kiessling, Marcotte, & Culpepper, 1993); the observation of OCD, as well as tic symptoms, in patients with Sydenham chorea (SC) (Langlois & Force, 1965; Kerbeshian, Burd, & Pettit, 1990; Swedo, et al., 1989); and the observation of a fluctuating, infectious-related course of OCD in some patients without choreoathetoid movements of SC (Allen, Leonard, & Swedo, 1995; Swedo, 1994).

The original working criteria established by the NIMH group for the diagnosis of PANDAS included: 1) the presence of OCD and/or a tic disorder; 2) a pediatric onset; 3) an episodic course of symptom severity; 4) an association with streptococcal infections; 5) an association with neurological abnormalities, including piano-playing choreiform movements of the fingers and toes, which suggests that PANDAS may be similar to SC. Moreover, besides these core features, the first 50 cases described in the original series showed emotional lability (66%), deteriorated school performance (60%), personality changes (54%), separation anxiety (46%), nightmares (18%), bedtime rituals (50%), deterioration in handwriting (36%), oppositional behaviors (32%), and motoric hyperactivity (50%), as seen in Table 1.

During the following years, the concept of PANDAS has become very popular and at the same time has sparked a heated debate among researchers and clinicians. To date, a large number of studies on different aspects of PANDAS have been published, as well as some comprehensive and recent reviews (Murphy, 2013; Macerollo & Martino, 2013).

Several researchers have examined two main critical aspects of PANDAS: the difficulty in establishing a tight link between the inciting streptococcal infection/exposure and the onset/recrudescence of OCD or tic symptoms, and the lack of reliable biological markers. These difficulties have led to a recent revision of the diagnostic criteria and to the proposal of a new clinical entity, the pediatric acute-onset neuropsychiatric syndromes (PANS), in which the key clinical feature is "acute and dramatic symptom onset." There are some changes in the presenting symptoms (with special relevance to OCD and anorexia, and loss of prominence of tics) without any

reference to their relationship with streptococcal infections (Swedo, Leckman, & Rose, 2012), although PANDAS would be included under a broader PANS group (Figure 2).

In this chapter, we will review some clinical, microbiological, and immunological aspects of PANDAS. Because tics and OCD symptoms are the main clinical features of PANDAS, a short review on their prevalence, appearance, and natural history will be provided first.

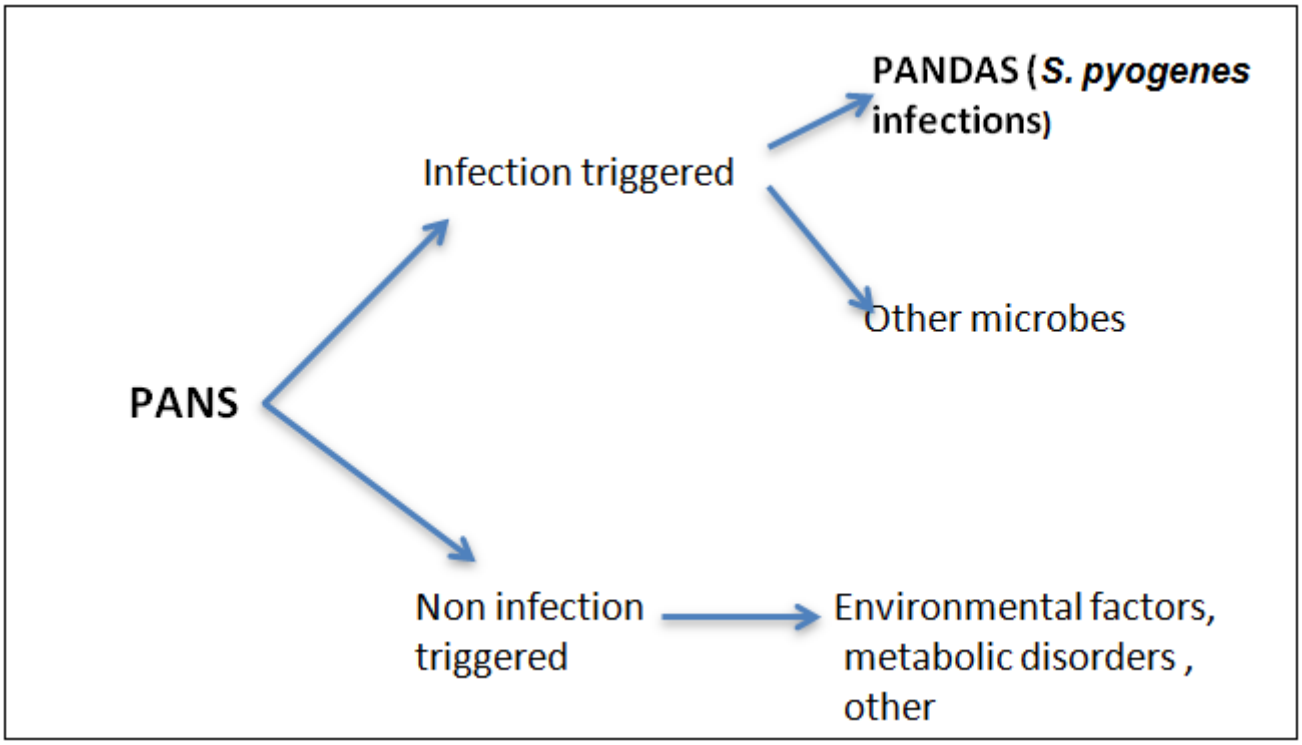


Figure 2. Evolution criteria of PANDAS and PANS (modified from (Swedo, Leckman, & Rose, 2012)

Table 1: Historical PANDAS diagnostic criteria (from (Swedo, et al., 1998))

All five criteria must be met	
	Presence of obsessive-compulsive disorder (OCD) and/or a tic disorder
	Prepubertal symptom onset
	Acute symptom onset and episodic (relapsing remitting) course
	Temporal association between S. pyogenes infection and symptom onset /exacerbation
	Associated with neurologic abnormalities (choreiform movements, motoric hyperactivity)

A brief review of tic disorders

Tics are rapid, recurrent, non-rhythmic, and stereotyped movements or vocalizations that can be simple or complex; they are usually suggestible, may be preceded by premonitory urges, and may be suppressed voluntarily. The last edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) categorizes tic disorders in three main groups, based on the length of the disorder (a duration of more or less than 1 year) and on the signs of symptoms (motor or phonic): "provisional tic disorder," with motor or phonic tics that last less than 1 year); "persistent tic disorder," with motor or phonic tics that last more than 1 year; and "Tourette's disorder" (or TS), with motor and phonic tics that last more than 1 year.

Tics are considered the most prevalent movement disorder in childhood, even if their exact prevalence in the general population is unknown. In fact, many cases of tics don't come to clinical attention, probably because of mild symptoms, they cause little functional or psychosocial impairment, or that the tics don't cause parental concern in parents. This is true for all the clinical forms of tics, including the chronic ones. Moreover, the epidemiological studies on the incidence and the prevalence of tic disorders are biased by different factors, such as sampling methods, sample sizes, rate of subject participation, assessment methods, and diagnostic thresholds

used to define cases. With these cautions, the prevalence of transient tics in school-age children is estimated to be from 11 to 20% (Snider, et al., 2002; Cubo, et al., 2011; Kurlan, et al., 2001; Linazasoro, Van Blercom, & de Zárate, 2006), while the prevalence of TS in school-age children is likely to fall somewhere between 5 and 7 cases per 1,000. For all tic disorders, there is a clear male prevalence, with the male-to-female ratio ranging from 2 to 1 to as high as 3.5 to 1.

Tic disorders are mainly a childhood and adolescent disturbance. In persistent or chronic forms, the onset of tics occurs between 2 and 7 years; the worst period of tic expression usually peaks around pre-adolescence (9–12 years); then there is a phase of stabilization and attenuation of symptoms during adolescence and early adulthood. Some studies report that more than around 40% of the TS children have no more tics during adult life; another 40% have minimal or mild tics that cause no interference in their lives; and only 20% continue to show moderate or even severe symptoms.

From a symptomatic point of view, in the majority of cases, the first tics are motor tics, eye tics (Martino, Cavanna, Robertson, & Orth, 2012) or facial movements. However, in some cases, vocal tics (shouts or vocalizations) and other motor tics (arm jerking, trunk spasms or other more complex movements) can be the first sign of the disorder, and can often pose problems of differential diagnosis with other movement disorders, particularly if their appearance is abrupt.

Typically, the onset of symptoms is sub-acute: the tics tend to slowly increase in frequency and intensity during months or years, and parents often have difficulties in recalling a precise date when tics began. The tics rarely appear acutely (seemingly overnight), though when this occurs, their intensity and frequency are very high from the beginning. This can often cause a great deal of anxiety in parents, who turn to emergency departments for consultations.

The severity of tic symptoms generally varies over time, and the waxing and waning course of chronic tic disorders is an universally recognized feature. Beside the natural course of chronic tic disorders, as described before, this variation of severity occurs over a period of weeks to months, and can even occur over the same day. A relationship between tics and environmental contingencies or emotional factors has been proposed: in particular, psycho-social stress (Lin, et al., 2010) or abnormalities in the cortisol circadian rhythm (Corbett, Mendoza, Baym, Bunge, & Levine, 2008) have been reported to affect the modulation of tic severity, but for many tics, it is difficult to establish a link between these fluctuations and a specific situation or environmental cause.

However, in the absence of a general agreement on the cut-off that defines a true exacerbation from the "normal" fluctuation of symptoms, the notion of tic exacerbation is quite vague. In a few studies, including those on some PANDAS patients (Lin, et al., 2002; Luo, et al., 2004; Kurlan, Johnson, Kaplan, & Tourette Syndrome Study Group, 2008; Leckman, et al., 2011; Martino, et al., 2011), tic exacerbation thresholds that incorporated the change score from the previous month and the current symptom score were estimated by using state-of-the-art bootstrap methods. Such methods agreed with the judgement of clinical experts. A seven-points increase of the global score (without impairment score) at the Yale Global Tic Severity Scale (YGTSS) has been considered in some studies to be a reliable cut-off that defines an exacerbation. Unfortunately, in an attempt to provide such a crucial definition, little help has come from the psychopharmacological studies on anti-tic medications, in which the definition of responsiveness or refractoriness to a single drug is quite vague.

Brief Review of OCD

OCD is a disorder with a lifetime prevalence of 1–3% in the general population. The disorder is characterized by the presence of obsession (i.e., recurrent and persistent thoughts, urges, or images that are experienced as intrusive and unwanted) and/or compulsions (i.e., repetitive behaviors or mental acts that an individual feels driven to perform in response to an obsession or according to rules that must be rigidly applied).

In children and adolescents, OCD shows a bimodal age of onset: the first one peaks around 8-12 years, which is the so-called "early onset OCD" that is characterized by a frequent comorbidity with tics/TS, a male prevalence (nearly 25% of males with the disorder have an onset before 10 years), a different content of obsessions/compulsions or the presence of compulsions without obsessions, a different and reduced response to the pharmacological treatment, and a poorer prognosis. The second one peaks after puberty and is characterized by a slight female prevalence, a content of obsessions/compulsions similar to those seen in adulthood, a good response to treatment, and a better prognosis.

The bimodal age of the onset of OCD suggests some different etiological factors. In particular, patients with an early onset are likely to have a stronger genetic or biological component than patients with a late onset. In particular, family studies revealed higher familial aggregation among relatives of early-onset subjects (Geller, 2006).

The abrupt overnight onset of initial OCD symptoms reported in cases of PANDAS or PANS is characteristic of these disorders. However, in typical OCD, the onset of symptoms is more gradual. OCD is described as a chronic disorder with a fluctuation of symptoms (waxing and waning), even if an episodic course is described in some cases. Notably, in some longitudinal studies when both tic and OCD symptoms were present, there was a significant degree of covariation (Lin, et al., 2010; Luo, et al., 2004; Leckman, et al., 2011).

Pathophysiology of tics, TS, and OCD

In the last twenty years, a growing number of studies have investigated the neural and pathophysiological underpinnings of tics, TS, and OCD. Given the known role of the basal ganglia in motor control and in other movement disorders, these structures have been the primary focus of many studies that have investigated the neurobiology of these disorders.

The basal ganglia comprise a set of subcortical nuclei that include the caudate nucleus, putamen, globus pallidus, subthalamic nucleus, and substantia nigra. Their functional connections to several cortical regions have led to the conceptualization of the corticostriatal-thalamo-cortical (CSTC) circuits; namely, multiple parallel, segregated feedback circuits with outputs from striatum that target primary motor areas, and specific pre-motor and prefrontal cortical areas. The primary function of the CSTC circuits is to control and select goal-directed motor, cognitive and motivational behavior. Further, CSTC circuits are involved in inhibitory control (Aron, Behrens, Smith, Frank, & Poldrack, 2007) and habit formation (Graybiel, 2008).

Even if a clear explanation for the occurrence of tics hasn't yet emerged, the most compelling finding so far is increased supplementary motor area (SMA) activity just prior to tic onset (Hampson, Tokoglu, King, Constable, & Leckman, 2009), which suggests that the SMA plays a role in the sensory phenomena that precede the execution of tics (premonitory urges).

Structural MRI studies have revealed reduced caudate volumes in children and adults with TS (Peterson, et al., 1993), with a negative correlation between caudate volume in childhood and the severity of symptoms later in life (Bloch, Leckman, Zhu, & Peterson, 2005).

The sensorimotor cortices are intuitive candidate cortical areas for investigation in TS due to the motor nature of tics and the sensory disturbances that frequently accompany them. MRI studies that have measured cortical thickness and grey matter volume in sensorimotor cortices in TS are limited in number, but they have provided consistent results. In particular, Sowell et al. (2008) found cortical thinning in sensorimotor cortex, along with other regions (ventral frontal cortex, dorsal parietal cortex), in children with pure TS (Sowell, et al., 2008).

To date, neuroimaging studies of TS (and especially functional MRI studies) are limited and many study results are inconsistent. These inconsistencies could be due to the large heterogeneities in the samples that have been studied.

With regard to OCD, in recent years, a growing number of studies have identified the CSTC circuits as centrally implicated in the pathophysiology of the disorder (Saxena & Rauch, 2000). Most especially, the limbic or orbitofrontal circuit (orbitofrontal cortex, anterior cingulate cortex, and caudate nucleus) has consistently been shown to be involved in OCD symptoms. Imaging research (which concerns structural, functional, and connectivity investigations) have shown a particularly high degree of concordance across the studies and have led to the conceptualization of the CSTC model of OCD. This model has received further support through neuropsychological and treatment studies (Menzies, et al., 2008).

PANDAS phenotype

Most of the studies published on PANDAS investigated a possible relationship between the onset or recrudescence of symptoms—mainly tics—and clinical or biological signs of *S. pyogenes* infections in different populations (such as tics or TS patients) observed in cross-sectional or longitudinal ways; furthermore, other research was conducted to search for possible markers of the proposed autoimmune process. In most of these studies, the definition of PANDAS cases was made after a retrospective review of clinical records. As a matter of fact, up until now, little attention has been paid to the clinical signs that could differentiate PANDAS from tics, TS, or OCD patients, besides the inclusion criteria. To date, four studies reported data that was useful for a comparison.

In 2008, Kurlan et al. compared 40 PANDAS patients with 40 OCD or chronic tic disorder matched subjects, followed for a period of 24 months. From a clinical point of view, the groups were comparable, with the exceptions that the PANDAS case subjects seemed to more often have a psychiatric diagnosis other than tic disorder or OCD (Leckman, et al., 2011).

In 2010, Bernstein et al. compared 21 PANDAS children with a control group of 19 children with non-PANDAS OCD, with respect to ancillary symptoms, types of obsessions and compulsions, symptom severity, and co-morbid DSM-IV diagnoses. Both groups were retrospectively defined by reviewing their medical records. PANDAS children were significantly more likely to present with separation anxiety, urinary urgency, hyperactivity, impulsivity, deterioration in handwriting, and decline in school performance during their initial episode of neuropsychiatric illness, as compared with children with non-PANDAS OCD. The total number of tics was higher and the vocal tics were more severe in PANDAS children. Separation anxiety disorder and social phobia were more prevalent in non-PANDAS OCD children, and children with non-PANDAS OCD were significantly more likely to include others in their rituals (Bernstein, Victor, Pipal, & Williams, 2010).

In 2011, Leckman et al. conducted a multi-centric longitudinal study that compared 31 children who met the criteria for PANDAS with 53 TS or OCD non-PANDAS subjects. Both groups showed a similar severity of symptoms and a similar rate of tic or OCD symptom exacerbations. Only a quarter of exacerbations identified in the PANDAS group were associated with a simultaneous sudden acute onset with increase in anxiety, depression, and/or attention deficit and hyperactivity disorder (ADHD) symptoms (Martino, et al., 2011).

Finally, in 2012, Murphy et al. examined 109 children showing tics, TS, or OCD; the assignment to the PANDAS (41 subjects) or the non-PANDAS (68 subjects) group was based on the presence of PANDAS operational criteria, as developed by Swedo et al. in 1998. The clinical assessment didn't show any clinical difference between groups. Children classified as PANDAS had a high rate of dramatic symptoms onset and clumsiness; however, it should be noted that these are two of five criteria for PANDAS (Murphy, Storch, Lewin, Edge, & Goodman, 2012). (A subset of the non-PANDAS group would have met the criteria for PANS, but did not have the temporal association normally found with streptococcal infections).

Although these studies did not provide overwhelming evidence for the existence of "PANDAS-specific" phenomenological features, PANDAS is similar to SC and shows the choreiform movements of fingers and toes that may not have been observed in earlier comparative studies. The presence of these movements may

constitute a red flag that signals a PANDAS diagnosis. However, a lack of choreiform movements does not exclude PANDAS, and these signs have to be regarded with caution, as they are also present in typically developing children and in children with other childhood disorders, such as ADHD and developmental coordination disorders.

PANDAS vs. Sydenham chorea

Sydenham chorea (SC) has provided a model for the conceptualization of PANDAS, and Swedo and the NIH group have shown that PANDAS is similar to SC and is characterized by choreiform piano-playing movements of the fingers and toes (Snider & Swedo, 2004). Both PANDAS and SC have immunological similarities, as a later section will show. Human sera studies in immunoassays suggest that human dopamine D2 receptor (D2R) is the target of autoantibodies that are produced in both SC and PANDAS (Cox, et al., 2013). Tics or OCD symptoms are often present in the early prodromal phases of SC, with choreoathetoid movements following. Deterioration in handwriting and irritability, often seen in early phases of SC, are also accompanying symptoms of PANDAS. However, the clinical course of PANDAS vs. SC is different: SC often is a monophasic illness, even if recurrent or persistent cases have been described (Cardoso, Vargas, Oliveira, Guerra, & Amaral, 1999). In contrast, the recurrence of tics or OCD symptoms after streptococcal (or other) infections has been one of the basic criteria for a PANDAS diagnosis. Finally, echocardiographic abnormalities (valvular incompetencies) are present in nearly 80% of SC patients, and they constitute a feature that often leads to the right diagnosis in the anamnestic or clinical doubtful cases. Conversely, PANDAS patients don't generally show signs of cardiac involvement (Snider, Sachdev, MacKaronis, St Peter, & Swedo, 2004); however, minimal echocardiographic abnormalities have been described in some patients with *S. pyogenes* related tics disorders (Cardona, et al., 2007) and in some children with PANDAS (Segarra & Murphy, 2008).

Association of *S. pyogenes* infections with tics/OCD

To fully understand the different studies published on the relationship between *S. pyogenes* and some neuropsychiatric disorders (such as tics, TS, or OCD) is not an easy task and has often raised more questions than answers. Some of these studies strongly support this association (Lin, et al., 2010; Murphy, Storch, Lewin, Edge, & Goodman, 2012; Cardona & Orefici, 2001; Leslie, et al., 2008; Mell, Davis, & Owens, 2005; Murphy & Pichichero, 2002) while others firmly deny it (Macerollo & Martino, 2013; Luo, et al., 2004; Kurlan, Johnson, Kaplan, & Tourette Syndrome Study Group, 2008; Leckman, et al., 2011), but all agree on the necessity of more research for a definitive demonstration of the existence/absence of this relationship and of the basic cellular and immune mechanisms involved. Table 2 provides a list of these studies.

The difficulty of having definitive results partially reflects the complexity and the possibly multifactorial nature of neuropsychiatric disorders. Animal models used (generally mice and rats) are not completely satisfactory and should be interpreted with caution; since humans are the only natural reservoir for *S. pyogenes*, these animal models may not exactly reproduce a human disorder. However, animal models in mice and rats have been very instructive in PANDAS and SC, with several studies indicating that immunization of rats and mice leads to behavioral alterations similar to SC and PANDAS (Brimberg, et al., 2012; Hoffman, Hornig, Yaddanapudi, Jabado, & Lipkin, 2004). In addition, passive transfer of anti-streptococcal antibodies to naïve rats or mice led to behavioral changes (Lotan, et al., 2014a; Hoffman, Hornig, Yaddanapudi, Jabado, & Lipkin, 2004).

After reviewing human studies, it is clear that many are looking at heterogeneous human populations; as a result, it may not be appropriate to analyze these data, which can be misleading when considered together. In some cases, the population studied is not in prepubertal age (Bencivenga, Johnson, & Kaplan, 2009; Schrag, et al., 2009; Morshed, et al., 2001); in others, there is a larger than usual presence of females; other studies are enhanced with more tic spectrum patients than acute onset OCD patients (Kurlan, Johnson, Kaplan, & Tourette Syndrome Study Group, 2008; Leckman, et al., 2011), which indicates some bias in the selection of patients; in others the original population with tics/OCD described by Swedo for PANDAS (Garvey, Giedd, & Swedo, 1998)

is enlarged by the inclusion of ADHD cases (Swedo, et al., 1998). In some studies, the involvement of *S. pyogenes* is investigated only through the detection of antibodies against one or two *S. pyogenes* antigens, without looking for the presence of the bacterium. It is important to note these differences when considering the results.

Streptococcus pyogenes is known to be a complex organism with a vast repertoire of virulence factors produced for bacterial adhesion and invasion, for evasion of phagocytosis, or for modulating host defenses (Sjöholm, Karlsson, Linder, & Malmström, 2014). It is able to change over time by the acquisition of new mechanisms and structures to avoid host defenses (Bryant, et al., 2014; Hertzén E. , et al., 2012) or to have long intracellular persistence (Wang, Li, Southern, & Cleary, 2006; Hertzén E. , et al., 2010; Kaplan, Gastanaduy, & Huwe, 1981; Kaplan, Chhatwal, & Rohde, 2006). The same strain may cause suppurative diseases, non-suppurative sequelae, toxic shock, or may colonize carriers without provoking an infection.

Many human studies have focused more on clinical findings or on host immune responses to specific antigens than on the bacterium itself, and as a result, only the presence or absence of *S. pyogenes* is reported. Microbiologists often act as simple blinded operators, and no information is reported on the methods used for taking specimens or isolating the strain. This result is evident from differences in the percentage of *S. pyogenes* positive samples found in different studies, or in multicenter studies, by the different microbiology results between participating centers.

Unfortunately, while a *S. pyogenes*-positive sample demonstrates the real presence of the bacterium in the infected or carrier host throat, a negative swab is not really informative; in particular, a swab that contains just a few colonies may be not detected as positive by routine laboratory methods. Since a true infection may be accompanied by very few colonies in the throat swab (Johnson, Kurlan, Leckman, & Kaplan, 2010), the percentage of positive samples found largely depends on the methods used to detect them. In our experience, at the time of their first visit for tics, children rarely present clinical signs of pharyngitis, and a percentage of them often carry even fewer than 10 colonies/plate in their throat swabs. That requires great care in taking the swab, to avoid *S. pyogenes* being covered by a too large amount of saprophytic flora present in the sample, and the use of selective media and careful methods in processing the swab, even different from those routinely in use for pharyngitis. If samples of children with tics but without clinical signs of pharyngitis are processed in the same way as those of children with sore throats, there are strong possibilities that the result will be below the threshold of detection and that the culture will be considered negative. *S. pyogenes* colonizing tonsillar criptae have been found in greater than 30% of children undergoing tonsillectomy for recurrent *S. pyogenes* tonsillopharyngitis and in children with no history of previous frequent infections who underwent surgery for different reasons, who were selected as healthy controls (Roberts, et al., 2012). These percentages are higher than all those found in PANDAS studies or in carriers, which indicates that even with accurate methods, many positive subjects are not detected; but, in the studies on the association between *S. pyogenes* infection/exposure and neuropsychiatric symptoms, false negative results can result in the true differences in positivity between cases and controls being entirely undetectable.

A second important point to consider is that *S. pyogenes* is not only an extracellular pathogen, but can survive to phagocytosis inside the cells. M-protein–expressing *S. pyogenes* strains can survive after phagocytosis by human neutrophils (Staali, Mörgelin, Björck, & Tapper, 2003) and the surface M-anchored protein has been identified as the pivotal factor that affects the phagosomal maturation in macrophages (Hertzén, et al., 2012; Hertzén, et al., 2010). During the intracellular phase, the expression of many genes—namely, the majority of those involved in cell wall synthesis and energy production—is significantly altered; after a replicative phase, *S. pyogenes* egress after having destroyed the host cells, and are fit to infect new cells and may persist in the throat for a long time, releasing any type of streptococcal antigens and causing the permanence of high-antibody titers even in the absence of overt disease. This may also account for the intermittent presence of the same serotype in the throat of tic patients and for the high percentage of carriers seen in some studies after treatment (Pichichero, et al., 1999). Host cells are a useful niche to escape many antibiotic drugs used against *S. pyogenes* (such as penicillin,

for instance), and microorganisms during carriage or infection are selected on the basis of their capacity to enter and survive the treatment (Kaplan, Gastanaduy, & Huwe, 1981; Sela, Neeman, Keller, & Barzilai, 2000; Park, Francis, Yu, & Cleary, 2003).

The last point to consider concerns the level of antibodies (Anti Streptolysin O, or ASLO, and Anti DnaseB, or ADB) used to indicate the infection. In several cases (Johnson, Kurlan, Leckman, & Kaplan, 2010), a true infection causes a moderate increase in this level, though it remains below the threshold considered the upper limit of normal titers (ULN) and, in the absence of an accurate monitoring of the subject, it is disregarded. On the other hand, choosing a too low level of antibodies as the ULN may result in undetectable differences between cases and controls.

All these considerations show the difficulties in studying the association between *S. pyogenes* infection/exposure and neuropsychiatric symptoms and in explaining why, despite the large number of studies published, the demonstration of PANDAS following the original definition given by Swedo et al. (Garvey, Giedd, & Swedo, 1998) is debated. Initially, studies of the association of SC with streptococcal infection determined that chorea can occur anywhere from several weeks to nine months following a streptococcal infection (Cardoso, Vargas, Oliveira, Guerra, & Amaral, 1999).

Epidemiologic evidence for some *S. pyogenes* involvement in tic disorders comes from administrative data from a health maintenance organization in the Seattle area where 144 new cases of TS and OCD/tics were matched with 609 controls (Mell, Davis, & Owens, 2005). A significant association was found (13-fold more for TS) with prior *S. pyogenes* infections diagnosed either 3 months or 1 year before the onset of disturbance. The presence of multiple *S. pyogenes* infections in the previous 12 months significantly increased the risk of TS, which indicates a sort of threshold of anti-streptococcal antibodies to be reached before the onset of the manifestation.

A strong association with a prior *S. pyogenes* infection was also found in an USA national health insurance study where 479 cases of OCD, tics, and TS were matched with 3647 controls, but the results of the study did not include a rigorous ascertainment of tic or OCD from consistent diagnostic criteria (Leslie, et al., 2008).

In a retrospective study, 80 consecutive children (15–17 years) were investigated through a structured clinical interview to establish if infection and an abrupt onset of symptoms could be identified; 53% of the patients reported such an abrupt onset and 21% of this subset had it within 6 weeks of infection (Singer, Giuliano, Zimmerman, & Walkup, 2000). It was suggested that in some cases, the abrupt onset might have been exaggerated by a biased memory of parents.

In the UK, Schrag et al. (Schrag, et al., 2009), on the contrary, were unable to support an association with *S. pyogenes* infection through a database analysis. However, in this case, both the mean age of their patients (16 years instead of prepubertal age) and the lack of established analysis parameters to determine the time between streptococcal infection and onset of tics/OCD (up to 3 years) could have accounted for the temporal association and thus the different results.

The age of the population studied is very important: in a large study that included 3006 school children, the percentage with motor or vocal tics was 22.3% for preschool children, 7.8% for elementary school, and 3.4% for secondary school, with the male/female ratio of 3.8/1 in the elementary school group and 6.1/1 for the secondary school group (Gadow, Nolan, Sprafkin, & Schwartz, 2002). Therefore, results using patients not in prepubertal age or with a too low rate of male/female subjects need to be carefully evaluated, since the population may be different from that of other studies.

Many of the studies performed on tic disorders are cross-sectional: clinical, serological, and microbiological data are collected at the time of the neuropsychiatric manifestation, onset, or increase of tics /OCD, but are not monitored over the time. This type of study may give useful insights (and they often do) to demonstrate that patients with tics differ from healthy normal people for a higher exposure to *S. pyogenes* antigens, but these

studies are inadequate to demonstrate the overall PANDAS concept (*S. pyogenes* clinical infection with the subsequent or antecedent rise of antibodies associated with onset /recrudescence of symptoms in a pediatric population), which can be only assessed through sequential observations.

In a case-control study performed between March 1996 and November 1998, 150 children were examined for sudden onset, recrudescence, or protracted duration of their tic disorders (Cardona & Orefici, 2001). The controls were 150 healthy children without tics. In this study, 38% of the cases (in comparison with 2% of the controls had ASLO titers higher than 500 IU with a mean ASLO titer of 434 IU, in comparison with 155 IU in controls ($p < 0.01$). Moreover, 58% had a family history of tics and frequent upper respiratory tract infections and 17% had a throat swab positive for *S. pyogenes*. At the time of the visit, none of the patients had clinical evidence of pharyngitis and, if analyzed by standard methods, the rate of *S. pyogenes* positive specimens was very low, with only a few colonies per plate; as a result, an old pour plate method was used (Taranta & Moody, 1971) that gave better results than the one routinely used for pharyngitis (Johnson, et al., 1997), which made it easier to detect and isolate every single colony (see Figures 1A and B).

In several studies, the increase of the immune response to those streptococcal antigens (ASLO and ADB) generally used as indicators of *S. pyogenes* infection is considered to be evidence of infection. It is interesting that when studying more or less the same matter (i.e., the possible involvement of *S. pyogenes* in tic disorders), the results and the conclusions produced by different groups were different. Loiselle et al. (Loiselle, Wendlandt, Rohde, & Singer, 2003) were unable to confirm differences in ASLO, anti-DNase B, and anti-basal ganglia (ABGA) titers in 41 children with TS and ADHD and 38 controls, even if ASLO titers were significantly higher in children with ADHD, as compared to the non-ADHD group.

In another study, sera from 30 children with PANDAS, 30 with TS, and 30 controls were examined for ABGA. Though more antibody positivity and a higher immunofluorescence against human striatum samples were found in samples from children with PANDAS and TS in comparison with controls, no statistically significant association was found between immunofluorescent reactivity and the diagnosis (Morris, Pardo-Villamizar, Gause, & Singer, 2009).

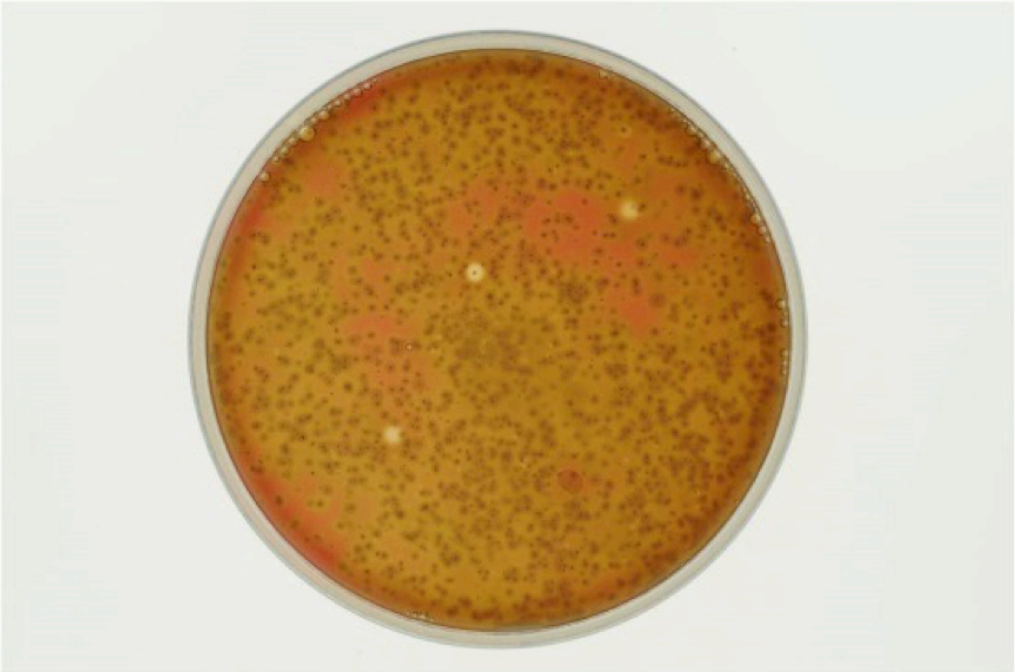
In a cross-sectional study on 100 British patients with TS (50% children), Church et al. (Church, Dale, Lees, Giovannoni, & Robertson, 2003) found that 64% of children and 68% of adults had a significantly higher ASLO titer, as compared to 15% of children with recent uncomplicated streptococcal pharyngitis, but no attempts to study the presence of *S. pyogenes* was reported.

A higher ASLO titer in comparison with controls was also found in an American study on 81 patients with TS (Morshed, et al., 2001); antistreptococcal higher titers (ASLO, ADB, anti-M12 and anti-M19) were also found in a sample of German patients (Corbett, Mendoza, Baym, Bunge, & Levine, 2008).

In a cross-sectional case-control study on 69 Italian TS children, Martino and Rizzo et al. found that 59% of the patients had significantly high ASLO titers (> 400 IU), in comparison with 19% of the controls (Martino, et al., 2011); a high percentage of children with high ABGA was also found. Finally in another cross-sectional study (Geller, 2006), the mean ASLO titers (246 IU vs 125 IU, $p>0.01$), the number of positive *S. pyogenes* throat cultures (8%, compared with 2%; $p=0.009$) and the positivity of anti-basal ganglia antibodies (ABGA) (23% in comparison with 8%; $p >0.001$) were significantly higher in TS patients than in controls, but no difference in ASLO titers was detected between ABGA-positive and ABGA-negative children

These are just some examples that show the elusiveness of the matter and the difficulty in reaching firm conclusions from published studies. Retrospective studies from health maintenance data may be a good source of information, even if they are not wholly collected with an ad hoc questionnaire.

A.



B.

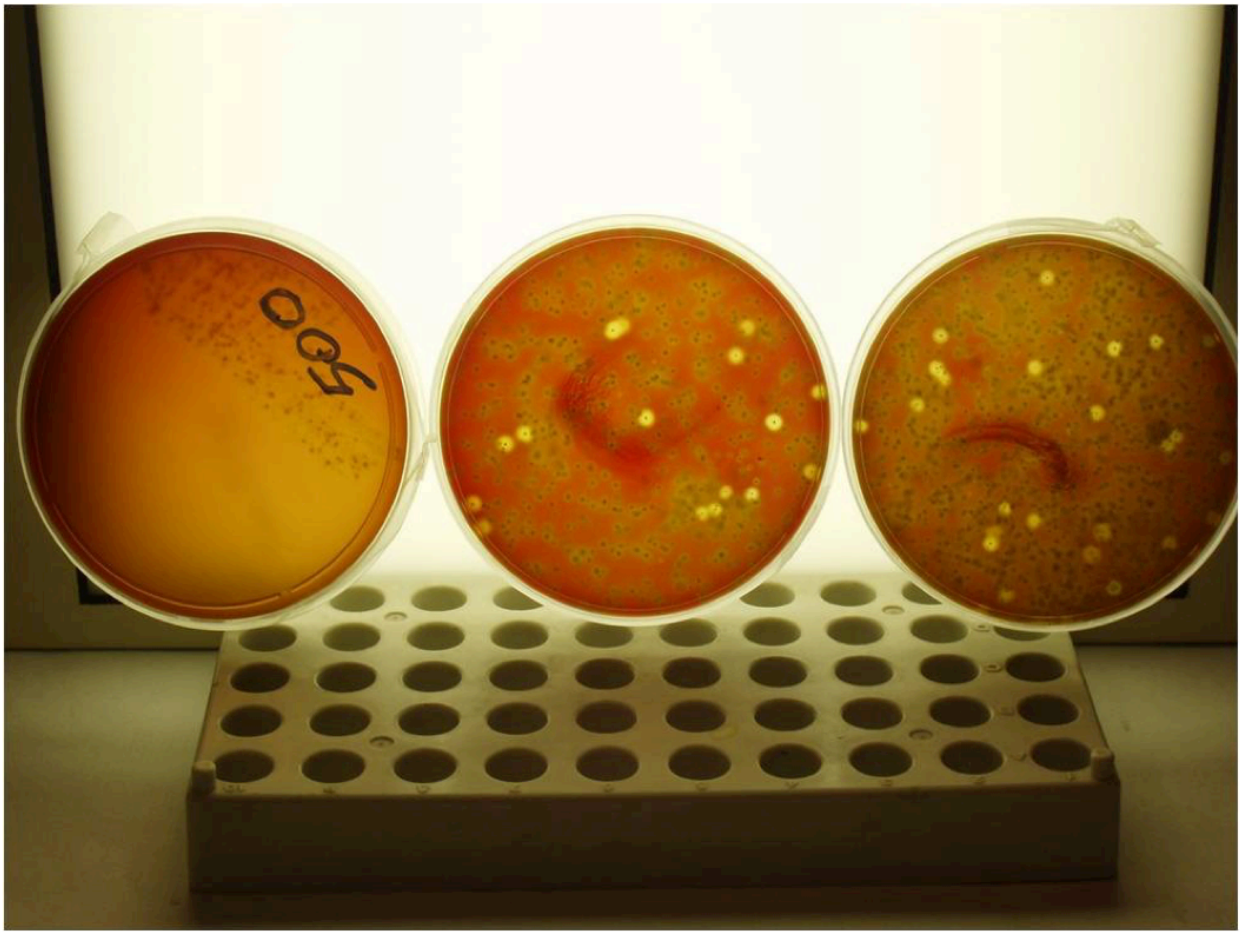


Figure 1. **A.** 300 *S. pyogenes* colony-forming units (CFUs) were suspended in 5 mL Todd Hewitt broth in an 0.1mL plated (pour plate) in blood Columbia agar. The recovery was about 50%. **B.** 500 *S. pyogenes* CFUs put in 5 mL Todd Hewitt Broth. 0.1mL and 0.2 ml were pour plated in blood Columbia agar and compared to 0.1 mL directly streaked on the surface.

Table 2: Studies to establish the association of *S. pyogenes* and other infections or stress situation with some neuropsychiatric disorder (tics/OCD, TS)

	Pros/Conclusive	Cons/Inconclusive
Anti-*S. pyogenes* antibodies and neuropsychiatric behaviors in animal models of group A streptococcal immunization and passive antibody transfer	(Hoffman, Hornig, Yaddanapudi, Jabado, & Lipkin, 2004) (Yaddanapudi, et al., 2010) (Brimberg, et al., 2012) (Lotan, et al., 2014a) (Lotan, Cunningham, & Joel, 2014b)	
Retrospective studies associate *S. pyogenes* with tics and OCD	(Mell, Davis, & Owens, 2005) (Singer, Giuliano, Zimmerman, & Walkup, 2000) (Cox, et al., 2015)	(Schrag, et al., 2009)
Cross sectional studies associate *S. pyogenes* with tics and OCD	(Swedo, et al., 1998) (Cardona & Orefici, Group A streptococcal infections and tic disorders in an Italian pediatric population, 2001) (Cardona, et al., 2007) (Garvey, Giedd, & Swedo, 1998) (Macerollo & Martino, 2013)	
Longitudinal studies associate *S. pyogenes* with tics and OCD	(Murphy & Pichichero, 2002) (Murphy, et al., 2004) (Martino, et al., 2011) (Murphy, et al., 2007) (Singer, et al., 2015)	(Luo, et al., 2004) (Kurlan, Johnson, Kaplan, & Tourette Syndrome Study Group, 2008) (Leckman, et al., 2011) (Morris-Berry, et al., 2013)
Antibodies against streptococcal antigens in tics and OCD	(Morshed, et al., 2001) (Müller, et al., 2001) (Church, Dale, Lees, Giovannoni, & Robertson, 2003) (Lin, et al., 2010) (Martino, et al., 2011) (Bombaci, et al., 2009)	(Loiselle, Wendlandt, Rohde, & Singer, 2003)
Antibodies against anti-basal ganglia in *S. pyogenes* sequelae, tics and OCD	(Dale, et al., 2001) (Kirvan, Swedo, Heuser, & Cunningham, 2003) (Kirvan, Swedo, Snider, & Cunningham, 2006a) (Martino, et al., 2011) (Dale, et al., 2012) (Cox, et al., 2013)	(Loiselle, Wendlandt, Rohde, & Singer, 2003) (Morris, Pardo-Villamizar, Gause, & Singer, 2009)
Other pathogens associated with tics and OCD	(Riedel, Straube, Schwatz, Wilske, & Müller, 1998) (Müller, et al., 2004) (Hoekstra, Manson, Steenhuis, Kallenberg, & Minderaa, 2005) (Krause, et al., 2010)	
Involvement of psychosocial stress in tics and OCD	(Chappell, et al., 1994) (Lin, et al., 2007) (Corbett, Mendoza, Baym, Bunge, & Levine, 2008) (Buse, Kirschbaum, Leckman, Münchau, & Roessner, 2014)	

Longitudinal studies to demonstrate the relationship between new infections and recrudescences of symptoms: the problem of carriers

The main difficulty in demonstrating the concept of PANDAS is showing the association of a new *S. pyogenes* infection with a sudden exacerbation of tics or OCD. In fact, this association can't be demonstrated by cross-sectional studies and requires monitoring the microbiological and serological parameters of patients and

controls for a long time, both to detect infections and to establish that these infections are new and are caused by *S. pyogenes* types that were not previously present. Longitudinal studies (in particular, those that follow patients from the initial tic manifestation) are informative, but are much more difficult to perform, and only a limited number of patients can be followed.

A prospective microbiology study analyzed the data of 160 children with tics enrolled in two clinical studies with identical protocols (Johnson, Kurlan, Leckman, & Kaplan, 2010). The goal of the study was to provide a description of the long-term kinetics of the immune response in patients with tics, either with *S. pyogenes* infections or who were carriers. The presence of *S. pyogenes* in the throat samples, M types, and the variation of antibody responses to ASLO and ADB were reported without specific attempts to correlate *S. pyogenes* infection with the concurrent recrudescences of tics. Pharyngeal swabs were taken every month for 120 weeks, and blood was examined approximately every three months. The study was very accurate, and shows that a true rise in anti-streptococcal antibodies may occur at levels below the ULN used in many studies. It stresses the need to use at least two antibodies (ASLO and ADB) for the diagnosis of new infection (since sometimes only a single antibody titer increases) and clarifies that a true infection with a significant antibody response may be associated with cultures with <10 colonies per plate, which demonstrates that many infections would be unidentified without a longitudinal observation. Obviously, only a limited number of example cases were reported; therefore, not much detailed information is available on the total population studied (number of *S. pyogenes* positive children, total number of infections seen during the entire study, characteristics of all *S. pyogenes* isolated) to enable comparisons to other large studies.

The conclusions of this research are convincing. Unfortunately, the same accuracy is difficult to reach in clinical practice, where the clinician is not necessarily informed on the basal level of anti-streptococcal antibody titers before the onset of the clinical manifestation or on the previous microbiology of *S. pyogenes* in the throat.

S. pyogenes throat infections and carriage are rather common in school-age children: several previous studies (Kaplan E. L., 1980) and in a recent meta-analysis (Shaikh, Leonard, & Martin, 2010) found 37% *S. pyogenes* positivity in children with sore throat and a 12% positivity in healthy children. In the Johnson study (Johnson, Kurlan, Leckman, & Kaplan, 2010) and in other studies, patients bearing the same *emm* type for a long time without changes in antibody levels were considered "carriers," since the presence of *S. pyogenes* in these cases could not be considered a new infection. Nevertheless, the status of a "carrier" is difficult to define: historically, *carriage* was defined as the prolonged permanence of *S. pyogenes* in the pharynx without evidence of immune or inflammatory response (Kaplan, 1980; Tanz & Shulman, 2007). Carriage is a very complex phenomenon, in which the *S. pyogenes* strain, the host immune response, and environmental factors all play roles, as demonstrated by the fact that the same strain can provoke pharyngitis, invasive disease, or simply be carried by a healthy population. We agree with Kaplan (Kaplan, 1980) in that "antibody titers remain elevated as long as the organism is present in the upper respiratory tract," and that "in many so-called prolonged carriers, there was a continual anti-streptococcal immune response."

In Johnson's study and other examples, there are some cases where the same strain was repeatedly isolated and the antibody titers remained high for a long time (therefore, correctly defined as "no new infection"), which made it difficult to differentiate a true carriage from a persistent infection. In these cases, it would be interesting to see if these "carriers" are more frequent in the tic population, and to characterize these strains to evaluate if this long presence of *S. pyogenes* (either simply as carriage or as a persistent infection), together with other environmental factors, may be associated with a higher frequency of tic recrudescences. The model of colonization followed by a long "carriage" with a rise in antibody titers without evidence of clinical infection has been described by Ashbaugh in baboons (Ashbaugh, et al., 2000), and had already been described by Kaplan in humans (Kaplan, 1980). In any case, patients with tics and long-lasting antibody titers should be carefully studied, even if the patients have negative swabs. If that does not change anything for the clinical treatment of a single patient, it may nevertheless give new insights into the role played by *S. pyogenes* in these movement, tic,

and neuropsychiatric disorders. In 2007, Murphy et al., a school study showed that those with repeated *S. pyogenes* infections had higher rates of behavioral and movement findings. Although the strains were not characterized, the findings suggest that a carrier state could contribute to neuropsychiatric symptoms (Murphy, et al., 2007).

In other longitudinal studies, patients were followed for a long time for clinical and immunological findings to verify if recrudescences may be temporally associated with a *S. pyogenes* infection, as described in the PANDAS definition. In a 3-year prospective study, 12 children with new-onset PANDAS were followed (Murphy & Pichichero, 2002). All tested throat-positive for *S. pyogenes*, and had neuropsychiatric symptoms along with signs of tonsillo-pharyngitis with rises in antibody titers. Antibiotics were effective in both eradicating *S. pyogenes* and suppressing tics at the first episode and at recurrences.

In another prospective longitudinal study on 47 patients and 19 controls, *S. pyogenes* infection rate in children with TS and/or OCD was 0.42 per year, as compared with 0.28 per year in non-tic patients**,** but the association of symptom exacerbations and new *S. pyogenes* infections was not higher than those on the basis of chance. Therefore, the study suggested no clear relationship between exacerbations and new *S. pyogenes* infections (Luo, et al., 2004). Since school-aged children may have a higher rate of *S. pyogenes* positivity or carriage due to classroom exposures, it may be difficult to demonstrate new infection and its association with exacerbation in some studies.

Murphy et al. (Murphy, et al., 2004) investigated the relationship between *S. pyogenes* infections and symptom fluctuations in 25 children followed for 9–22 months with visits every 6.2 weeks on average for each subject. Authors reported that a part of their patients named ESC (episodic/sawtooth course) closely approximated the criteria described for PANDAS. In this study, beside ASLO and ADB titers, antibodies against the streptococcal capsular polysaccharide (ACHO) were measured. The typical pattern reported for one of these patients showed that, unlike ASLO and ADB, ACHO antibodies that target the group A streptococcus carbohydrate after a rapid increase remain elevated for a long time; moreover, a positive correlation between YGTSS and ACHO ($p=0.063$) or CYBOCS and ACHO ($p=0.013$) was found. This observation is interesting since in tic, TS, OCD or PANDAS studies, only the variation of antibody titers against protein antigens (ASLO and ADB) is usually considered, while ACHO is known from studies in SC to be a key antigen in rheumatic disease (Martins, et al., 2008; Cunningham, 2012). Moreover, it has been observed that for many patients with PANDAS, symptoms appeared only after repeated *S. pyogenes* infections.

Mell also reported this higher risk of tic development in children with frequent infections (Mell, Davis, & Owens, 2005), who noticed that for some PANDAS patients, symptoms emerged only after repeated *S. pyogenes* infections and that increases in behavioral and motor symptoms were found in children with repeated *S. pyogenes* infections (Murphy, et al., 2007); these findings suggest that a threshold of antibodies is needed to trigger symptoms. It is still unclear and unproven whether a true *S. pyogenes* clinical infection is really needed to develop symptoms, or whether repeated exposure to *S. pyogenes* antigens (maybe together with other external agents such as stress or a concomitant viral infection) may stimulate recrudescences on its own.

An accurate analysis was used to validate the PANDAS entity in a longitudinal study (Kurlan, Johnson, Kaplan, & Tourette Syndrome Study Group, 2008) which followed a group of 40 PANDAS cases, matched to 40 controls with TS without any documented association of recrudescences with *S. pyogenes* infection, for two years. Results showed that even if not statistically significant, the group of PANDAS had more exacerbations than controls (65 clinical exacerbations in total: 40 in PANDAS, 25 in the control group). Moreover, among the 43 definite or probable *S. pyogenes* infections, 31 were in 22 PANDAS cases and 12 were in 9 subjects of the control group. The number of exacerbations associated with *S. pyogenes* infections (defined as hits) was outside the 95% confidence limit for the mean number of hits, which suggests that PANDAS exacerbations are significantly associated with an antecedent *S. pyogenes* infection. On the other hand, 75% of exacerbations had no observed temporal relationship with *S. pyogenes* infection. After noting that the number of recrudescences was lower and milder

than expected, the authors concluded that the vast majority of PANDAS clinical recrudescences could not be linked to *S. pyogenes* infections, and that children with PANDAS represented a subgroup of patients with TS or OCD who are susceptible to *S. pyogenes* infections as part of their initial symptoms. An interesting note is that 22.5% of the PANDAS cases (compared with 5.3% of the controls) have a family history of rheumatic fever, which might indicate a special genetic predisposition in PANDAS cases that perhaps makes them more prone to develop *S. pyogenes* infections. Similar data were also reported by other authors (Cardona & Orefici, 2001).

Following the same study design and the same protocol, Leckman et al. (Leckman, et al., 2011) did not find an increase in exacerbations in the group of PANDAS, but on the contrary, a higher number was detected in the non-PANDAS group. As in the Kurlan study (Kurlan, Johnson, Kaplan, & Tourette Syndrome Study Group, 2008), the total number of recrudescences and *S. pyogenes* infections reported was lower than previously estimated, which raises suspicions that the study was underpowered; in contrast to what had been described in the definition of PANDAS, only a small number of recrudescences was associated with a sudden increase of tic /OCD severity. Again, 20 out of 31 children in the PANDAS group (in comparison to 8 of 53 in the non-PANDAS group) had a family history positive for rheumatic fever, which suggests some genetic predisposition and susceptibility to *S. pyogenes* sequelae. It should be noted that in both these latter studies, patients could continue to have their usual tic medications and that based on laboratory results, physicians were free to prescribe antibiotics. In particular, 28% of the controls (in comparison with 60% of the PANDAS group) were treated with antibiotics. This might partially account for the results achieved, and could be an unintentional indirect support for antibiotic treatment to suppress recrudescences.

In another multicenter longitudinal study on TS patients (Martino, et al., 2011), *S. pyogenes* infections ASLO titers, ADB, and anti-basal ganglia antibodies (ABGA) antibodies were compared among 168 children with TS, and 177 matched controls with epileptic or sleep disorders without tics. Seven definite (2%) and 32 possible (10%) infections were reported with a rise in ASLO titers in 26 (18%) of the subjects and in 11 (8%) of the ADB titers; 14% of patients had ABGA test persistently positive over at least 2 consecutive visits, and 20% became ABGA positive throughout the study. Nevertheless, it was not possible to correlate *S. pyogenes* infections with exacerbations, and the occurrence of a new identification of ABGA did not predict the occurrence of an exacerbation. Authors concluded that children and adolescents with TS show an increased exposure to and immune response against *S. pyogenes* and increased expression of antineuronal antibodies. This supports the view that patients with TS, independently of the PANDAS definition, may be more prone to *S. pyogenes* infections and may develop stronger immune responses against streptococcus, possibly as a result of immune dysregulation.

Other infections

S. pyogenes is not the only pathogen thought to be associated with the onset or recrudescence of tics. Other pathogens, such as intracellular microorganisms with the capacity of living and replicating inside host cells (Riedel, Straube, Schwatz, Wilske, & Müller, 1998; Müller, et al., 2004; Krause, et al., 2010), have been described as possibly being involved in these movement disorders, and particularly associated with recrudescences, but no strict observance of common parameters have been reported. Hoekstra (Hoekstra, Manson, Steenhuis, Kallenberg, & Minderaa, 2005) found a recrudescence association with the common cold, but it is difficult to evaluate this report because of the vague assessment of "common cold" and of the low isolation of *S. pyogenes* from patients.

Since the involvement of *S. pyogenes* in PANDAS has never been completely demonstrated, and there is no complete evidence of which *S. pyogenes* antigen(s) (if any) could be associated with tics, it is also impossible to define the way in which these other microorganisms are related to tics: could they be the causes, or could they collaborate with *S. pyogenes* in triggering symptoms? Are some of their antigenic determinants common with *S. pyogenes*? Or are none of them really associated with tics? In any case, even if some reports have been published, they could support the hypothesis that the genetic background of these patients (including any

immunodeficiencies) may generally make them more susceptible to certain infectious organisms and more prone to develop antibodies against microbial and brain antigens.

Plurality of antigens involved

This last hypothesis stated above seems to be supported by the results of a study (Bombaci, et al., 2009) that used a protein array to test the antibody responses of children with tics to a panel of more than 100 recombinant *S. pyogenes* antigens. These patients had chronic tic disorders, but no overt pharyngitis and no previous rheumatic diseases; their results were compared with those of healthy control children without tics and with children with microbiologically demonstrated *S. pyogenes* pharyngitis. The results showed that a group of 25 antigens were recognized by sera of all three groups; 21 antigens reacted with sera of tic and pharyngitis patients, but poorly with control sera; and 5 antigens were preferentially recognized by sera from children with chronic tics. Moreover, the overall response to the tested antigens appeared to be stronger in tic patients than in pharyngitis cases. What is most interesting is that this strong response to streptococcal antigens in the absence of clinical evidence of pharyngitis was independent of ASLO titers or an *S. pyogenes* positive throat culture. The results of the study indicated that a subgroup of tic patients show a typical profile of subjects who mount a broad, specific immune response to *S. pyogenes* antigens in the absence of clinical pharyngitis, and suggests that in genetically predisposed patients, a strong anti- *S. pyogenes* response due to a lengthy exposure to streptococcal antigens (like in long-term carriers or children with frequent pharyngitis) may produce a cumulative threshold of antibodies that are needed to produce recrudescences. The effect of other environmental factors (stress or other concomitant infections) may help in triggering the disorder in the absence of overt infection. The results show how ASLO, ADB, and the positive *S. pyogenes* throat swab may be sufficient to predict children who are at risk of developing neuropsychiatric movement or tic-like symptoms.

The high number of proteins tested in the study, and the strong preferential response by the tic sera to five of them, does not necessarily mean that these antigens are involved in tics, since only protein antigens have been examined, and because of the broad plasticity demonstrated by *S. pyogenes* in switching on or off the genes (Hertzén, et al., 2012) on the basis of the intracellular or extracellular environment. However, the stronger response in the children with tics suggests that they responded more strongly to streptococcal antigens than did children with pharyngitis or children in the control group. This could be due to repeated streptococcal infections, as is believed to occur with rheumatic fever.

Characterization of the strains

No attempts to characterize the strains from tic patients for their possible specific virulence factors or antibiotic resistance have been made, nor have studies been attempted of M proteins (the fundamental antigens of *S. pyogenes*), which are involved in pathogenicity and used in characterization of the strains. Could M proteins play any role in the development of tics, or are specific M types more frequently found in these movement disorders? In one study (Müller, et al., 2001), Mueller detected antibodies against M1 and M 23, and in the Johnson study (Johnson, Kurlan, Leckman, & Kaplan, 2010) some M types of the strains isolated from swabs are reported, but not many studies that specifically examine the M types of the *S. pyogenes* isolated in these patients have been published.

Creti et al. (Creti, et al., 2004) examined 100 strains collected from 368 children with tics during years 1996-2001. Strains were typed by M protein agglutination and *emm* molecular typing. Sixty-seven children (18%), 53 males and 14 females had one or more throat swab test positive for *S. pyogenes*. Notably, while no problems were found with the molecular typing, 35% of the isolates resulted in being non-typable by anti M-protein sera even after repeating the typing in 3 centers (Rome, Prague, and Minneapolis), which indicates a very scarce presence of M protein on the surface.

No specific *emm* types associated with these patients were found, but 5 types, namely M12 (11.40%), M22 (11.40%), M5 (8.86%), M3 (6.32%), and M89 (6.32%) accounted for 44.3% of the strains, while M4, M2, and M1 accounted for 5.06% each. A large number of different types was found, and in some cases, a type was represented by only one or two isolates. M3 and M5 were generally associated with ASLO titers higher than 407 IU, but the numbers were too small to make a comparison to other M types. It is interesting that, even if the rank order appeared different from that of strains from pharyngitis (Dicuonzo, et al., 2001) or invasive disease (Creti, et al., 2007) isolated from the same area in the same period, the same M types presented the same "virulence and antibiotic string" (*spe* A, *spe* C, *mef*A, *erm*A, *erm*B), independent from the source of isolation (tic, pharyngitis or invasive disease). In Italy, M12 is the M type most frequently isolated from carriers, and M4 is frequently associated with scarlet fever epidemics; the M types typical for rheumatic disease, like M1, M3, M5, and M18 (Shulman, Stollerman, Beall, Dale, & Tanz, 2006), when found, were never related to previous histories of rheumatic fever.

Even when taken with caution (due to the small amount of data available), the M types found represent the serotypes present in a "normal" population with no specific M types or particular virulence factors. In this sense, the observation of the scarce typability by anti-M protein sera, the fact that different *emm* types were isolated in strains in the following years (2002–2007)—as happens in the natural selection of the strains—and that strongly mucoid strains, which are traditionally "rheumatogenic," were never found, all support the hypothesis that the strains found were those that circulate in the normal population. On the other hand, changes in the rank of frequency can be expected when a limited number of strains are studied; this possibly reflects the normal epidemiological relationship between circulating types and the population immunological condition and dynamics (when antibodies have been raised against one serotype among the population, this type decreases its frequency). Nevertheless, this finding could be important, along with immunologic and genetic study of these patients, to determine why individuals are more prone to streptococcal infections, to improve the knowledge about these isolated strains, and to detect if there is any specific reason that facilitates their colonization or their long-term residence in the throat.

The effect of a genetic predisposition for rheumatic disease has already been described by Bryant et al. (Bryant, et al., 2014), who investigated whether any difference in immune response detectable by gene expression can be found between individuals susceptible to ARF and those who are not. The authors found that 34 genes were significantly and differentially expressed between ARF-susceptible and ARF-resistant subjects, 7 of which were involved in immune response genes, chemotaxis, and apoptosis.

Kotb et al. (Kotb, et al., 2008), used *S. pyogenes* as a model to demonstrate in cell cultures and in animal models (including transgenic mice) how the host's genetically determined response may be modulated by environmental factors and how the response to some streptococcal superantigens may account for the different severity of the invasive disease determined. The same type of study might be useful for PANDAS and for tics in general.

The role of stress

Studies on TS/OCD report the relevance of psychosocial stress, which suggests that these disorders are sensitive to stress and show a high stress response (Corbett, Mendoza, Baym, Bunge, & Levine, 2008; Chappell, et al., 1994; Buse, Kirschbaum, Leckman, Münchau, & Roessner, 2014); these findings offer further evidence that many different factors contribute to these movement disorders. A cohort of 86 children diagnosed with TS/OCD and 41 matched controls were followed in a longitudinal study to verify if TS/OCD patients showed higher levels of psychosocial stress, as compared to the healthy population (Lin, et al., 2007). Notably, while levels of psychosocial stress were modest but were significant predictors of future tic symptom severity, current tic severity was not a significant predictor of psychosocial stress.

The same group in a longitudinal study monitored 45 cases (with 11 defined as PANDAS) and 41 healthy controls for 2 years with thrice-yearly visits and monthly telephone conversations to examine the impact of new

S. pyogenes infections and psychosocial stress on future fluctuations of tic/OCD and the severity of depressive symptoms. PANDAS cases had higher (even if not significant) number of *S. pyogenes* infections compared to normal controls or non-PANDAS cases. Psychosocial stress and newly defined (or possible diagnosed) *S. pyogenes* infections were significant predictors of future symptom severity: newly diagnosed *S. pyogenes* infections increased by a factor of more than three, which indicates the power of psychosocial stress to predict future symptom severity. The study suggests that a minority of children with tics or OCD are sensitive to antecedent *S. pyogenes* infections, and that psychosocial stress is a potent factor associated with future worsening of tics (Lin, et al., 2010).

Clinical trial in PANDAS

Another source of information on the relationship between *S. pyogenes* and neuropsychiatric symptoms comes from clinical trials. Based on experience with acute rheumatic fever (ARF), in which secondary prophylaxis with penicillin reduced recurrences of ARF or SC by preventing *S. pyogenes* infections, some studies were conducted on patients with PANDAS.

The first one was an 8 month, double-blind, balanced cross-over study (Garvey, et al., 1999). Thirty-seven children who met the five classical criteria for PANDAS, were randomized to receive either 4 months of the active compound (twice daily oral 250 mg penicillin V) followed by 4 months of a placebo, or a placebo followed by penicillin V. Subjects were evaluated monthly for eight consecutive visits in order to assess the clinical features (ratings of tics, obsessive compulsive symptomatology, anxiety, and depression) as well as undergo laboratory evaluation (including serum titers of antistreptolysin-O (ASLO), anti-deoxyribonuclease B (anti-DNaseB) and throat cultures). These results showed no significant difference between the two phases in the number of both streptococcal infections and symptom exacerbations. The authors reported a lack of compliance by 26 of the children, which they attributed to an overall failure to achieve the aims of the study.

Some years later, the same group conducted another double-blind, randomized controlled trial (Snider, Lougee, Slattery, Grant, & Swedo, 2005); in this study, 23 subjects with PANDAS received an antibiotic prophylaxis with penicillin or azithromycin for 12 months. In particular, subjects were randomized in a double-blind fashion to receive either penicillin V-K 250 mg two times a day, or azithromycin 250 mg capsules two times a day, on one day of the week and placebo capsules taken two times a day on the other six days. The rate of streptococcal infections and symptom exacerbations were assessed during the study year by monthly visits and laboratory evaluation (ASLO and Anti-DNase B titers) and were then compared with those of a baseline year (for which subjects/parents were asked to retroactively recall the number of clinical exacerbations and streptococcal infections; medical records were also reviewed). Results showed a significant reduction (96%) of the rate of streptococcal infections, as well as of neuropsychiatric symptoms (64%) in both groups.

The authors concluded that both penicillin and azithromycin are effective in preventing *S. pyogenes* infections, and that both penicillin and azithromycin may be effective in preventing *S. pyogenes*-triggered neuropsychiatric exacerbations in children in the PANDAS subgroup. However, they also suggest great caution when interpreting the data, due to the small number of patients and the lack of a placebo. It should be mentioned that this study received several criticisms, including from the Tourette's Syndrome Study group (Budman, et al., 2005), about many of the study's methodological aspects. Most recently, a report published by Murphy et al. suggested that a reduction of symptoms using the β-lactam antibiotic cefdinir was observed in PANS (Murphy, Parker-Athill, Lewin, Storch, & Mutch, 2015a), which supports the potential usefulness of antibiotics in these diseases.

To our knowledge, apart from the above-mentioned studies and a case series report (Murphy & Pichichero, 2002), no other data have been published to establish a treatment protocol for antibiotic therapy or prophylaxis in PANDAS subjects. Caution is advised against the over-use of antibiotic treatments of PANDAS or PANS patients, as well as of subjects that show only a single exacerbation of neuropsychiatric (tics or OCD) symptoms, which has been reported in the US (Gabbay, et al., 2008) and in other countries.

Anti-Neuronal Autoantibodies in Sydenham Chorea and Pediatric Autoimmune Neuropsychiatric Disorder Associated with Streptococci (PANDAS)

Sydenham chorea (SC) is well established as the neurologic manifestation of acute rheumatic fever (Taranta & Stollerman, 1956), and is characterized by antibodies found in the cytoplasm of neurons in the caudate and putamen regions of the human brain (Husby, van de Rijn, Zabriskie, Abdin, & Williams, Jr., 1976). Little was known about the antibodies and how they affected the brain until human mAbs were derived from SC (Kirvan, Swedo, Heuser, & Cunningham, 2003) and were found to react with the group A streptococcal carbohydrate epitope N-acetyl-beta-D-glucosamine and brain antigens lysoganglioside (Kirvan, Swedo, Heuser, & Cunningham, 2003) and tubulin (Kirvan, Cox, Swedo, & Cunningham, 2007). Evidence from studies of human chorea-derived mAbs strongly suggests that autoantibody crossreactivity between streptococci and brain is an important feature in Sydenham chorea (Kirvan, Swedo, Heuser, & Cunningham, 2003; Kirvan, Cox, Swedo, & Cunningham, 2007; Kirvan, Swedo, Kurahara, & Cunningham, 2006b). Human mAbs and antibodies in sera or cerebrospinal fluid from the SC-activated calcium calmodulin dependent protein kinase II (CaMKII) in human neuronal cells (Kirvan, Swedo, Heuser, & Cunningham, 2003) and led to an increase in dopamine release from the human neuronal cell line using tritiated dopamine assays (Kirvan, Swedo, Kurahara, & Cunningham, 2006b). Further study indicated that chorea-derived mAb (24.3.1) induced tyrosine hydroxylase activity in dopaminergic neurons after the intrathecal transfer of purified human mAb 24.3.1 (Kirvan, Swedo, Heuser, & Cunningham, 2003) into a Lewis rat brain (Kirvan, Swedo, Kurahara, & Cunningham, 2006b). The removal of IgG from serum caused a loss of neuronal cell-signaling activity (Brimberg, et al., 2012; Kirvan, Swedo, Heuser, & Cunningham, 2003), and plasmaphoresis was found to improve chorea symptoms (Perlmutter, et al., 1999; Garvey, Snider, Leitman, Werden, & Swedo, 2005). Therefore, antibody-mediated neuronal cell signaling was induced by IgG antibodies in serum or cerebrospinal fluid from SC, and the presence of these signaling autoantibodies were associated with symptoms (Kirvan, Swedo, Heuser, & Cunningham, 2003; Ben-Pazi, Stoner, & Cunningham, 2013). Antibody-mediated neuronal cell signaling in SC is a novel pathogenic mechanism which is important in the movement and neuropsychiatric disorder of acute rheumatic fever (Kirvan, Swedo, Heuser, & Cunningham, 2003). SC may be a model for other movement and neuropsychiatric disorders associated with infections, such as PANDAS (Swedo, et al., 1998).

To further the studies of the antibodies in SC and related diseases, a novel transgenic mouse model expressing an SC-derived brain autoantibody was developed to gain insight into *in vivo* functional antibody targets that may be involved in the mechanisms of SC, and to test the hypothesis that autoantibodies from movement and behavioral disorders target neurons and possibly the dopamine D2 receptor (D2R) in the brain (Cox, et al., 2013). Transgenic mice expressed chorea-derived, human mAb 24.3.1, heavy and light chain variable region (V_H and V_L) genes as part of a chimeric (human V gene/mouse constant region) IgG1^a antibody construct (Figure 3). Mice transgenic for mAb 24.3.1 V genes were validated by characteristic cross-reactive anti-neuronal antibody specificities in serum, and of mAbs produced from lymphocytes from spleens of transgenic mice. In our SC transgenic mouse model, chimeric 24.3.1 antibody expressed in mouse B cells and serum penetrated the brain and dopaminergic neurons in the basal ganglia of transgenic mice. Expression of the V genes of SC mAb 24.3.1 (Cox, et al., 2013) in transgenic mice demonstrated that the SC antibody V gene expression in the serum of transgenic mice targeted dopaminergic tyrosine hydroxylase positive neurons in the basal ganglia of the transgenic mice (Cox, et al., 2013), as shown in Figure 2. These results were consistent with evidence seen in human SC (Husby, van de Rijn, Zabriskie, Abdin, & Williams, Jr., 1976). In addition, human mAb 24.3.1 from SC was shown to react with and signal the human dopamine D2 receptor expressed in transfected cell lines (Cox, et al., 2013). Evidence using a flag-tagged D2 receptor, as well as signaling of the human D2 receptor in transfected cell lines, demonstrated that human mAb, as well as human SC sera IgG, targeted the dopamine D2 receptor (Cox, et al., 2013). In addition, antibodies (IgG) were also present in serum against the human D1 receptor, and further studies suggested that the ratio of the anti-D1R/D2R antibodies correlated with symptoms

(Ben-Pazi, Stoner, & Cunningham, 2013). The studies also showed that anti-D1 receptor and anti-D2 receptor antibodies (IgG) were significantly elevated in serum from SC, as well as PANDAS, as described by Cox et al. (Cox, et al., 2013).

PANDAS shares similar antibodies against the dopamine receptors, as does SC (Cox, et al., 2013). The symptoms of PANDAS, as originally reported, appear as small choreiform piano-playing movements of the fingers and toes which were reported in the first 50 cases by Swedo et al. (Swedo, et al., 1998). PANDAS is characterized by tics and OCD; which, in addition to the fine choreiform movements, are not as obvious as those movements seen in SC (Garvey, Snider, Leitman, Werden, & Swedo, 2005; Garvey & Swedo, 1997). The fine choreiform movements of lower amplitude than chorea may go unnoticed in PANDAS and can lead to poor handwriting associated with learning and behavioral regression, enuresis, separation anxiety and night-time fears, and anorexia in approximately 17 percent of cases (Swedo, et al., 1998). The appearance of PANDAS is very striking because the onset is very sudden, such as overnight behavioral changes.

For years the focus of research on SC was primarily on the chorea and involuntary movements, with little attention given to the neuropsychiatric obsessive-compulsive symptoms which predate the chorea and characterize the neurological manifestations of acute rheumatic fever (Ben-Pazi, Stoner, & Cunningham, 2013). These manifestations may be seen in other types of infections, and in these cases, is termed pediatric acute onset neuropsychiatric syndrome or PANS (Swedo, Leckman, & Rose, 2012). There have been many questions about PANDAS/PANS, which current research is attempting to answer. Clearly, the original PANDAS group has many similarities to SC, including a previous *S. pyogenes* infection; however, unlike SC, PANDAS has a male predominance (Swedo, et al., 1998; Murphy, Parker-Athill, Lewin, Storch, & Mutch, 2015a; Swedo, et al., 1989; Swedo, 1994; Swedo, Leckman, & Rose, 2012; Snider & Swedo, 2004). PANS and more chronic types of tics and OCD are not always associated with *S. pyogenes* infections. More chronic tics and OCD may not display the small choreiform piano-playing movements of the fingers and toes, and are not similar to SC in their anti-neuronal antibody patterns of antibodies against the dopamine D2 receptor (Cox, et al., 2015; Singer, et al., 2015). More chronic forms of tics and OCD do not have the IgG antibodies against the D2 receptor (Cox, et al., 2015; Singer, et al., 2015; Morris-Berry, et al., 2013). PANDAS with small choreiform piano-playing movements of the fingers and toes (Swedo, et al., 1998) share the antibodies against both D1 and D2 receptors with SC (Cox, et al., 2013; Brimberg, et al., 2012; Ben-Pazi, Stoner, & Cunningham, 2013) and also have elevated antibodies against tubulin and lysoganglioside (Cox, et al., 2013; Brimberg, et al., 2012; Kirvan, Cox, Swedo, & Cunningham, 2007; Ben-Pazi, Stoner, & Cunningham, 2013). Both tics and OCD, including the original PANDAS (Swedo, et al., 1998) and the more chronic tics and OCD are both temporally associated with *S. pyogenes* infection and have a significantly elevated abnormal CaMKII (Kirvan, Swedo, Snider, & Cunningham, 2006a; Cox, et al., 2015; Singer, et al., 2015). More studies of PANS are required to study children who have OCD and tics that are not associated with *S. pyogenes* infection.

Animal models of movement and obsessive compulsive symptoms have been studied in a mouse model and Lewis rat model, where both models show positive evidence that symptoms are associated with anti-streptococcal antibodies. Immunization of a mouse model (Hoffman, Hornig, Yaddanapudi, Jabado, & Lipkin, 2004) with streptococcal components in Freund's complete adjuvant led to behavioral alterations and compulsions, and a subset of mice with antibody deposits in several brain regions, including deep cerebellar nuclei (DCN), globus pallidus, and the thalamus (Hoffman, Hornig, Yaddanapudi, Jabado, & Lipkin, 2004). Group A streptococcal immunized mice with increased deposits of IgG in the deep cerebellar nuclei exhibited increased rearing behavior, as compared to controls. These data suggested that immune responses against *S. pyogenes* were associated with motoric and behavioral disturbances, and suggested anti- *S. pyogenes* antibodies that cross-react with brain components may lead to symptomatology (Hoffman, Hornig, Yaddanapudi, Jabado, & Lipkin, 2004). Passive transfer of anti-streptococcal antibodies from the immunized mice into naïve mice led to autoantibody deposits in the brain, as well as behavior changes (Yaddanapudi, et al., 2010).

Another animal model of SC, and potentially PANDAS, was created in the Lewis rat (Brimberg, et al., 2012), which demonstrated that exposure to group A streptococcal antigens during immunization led to behaviors characteristic of SC and PANDAS. After at least two immunizations, rats were not able to hold a food pellet as well as control rats, and also could not traverse a narrow beam as well as control rats (Brimberg, et al., 2012). In addition, the rats demonstrated a compulsive grooming behavior. Antibody IgG deposits were observed in the Lewis rat striatum, thalamus, and frontal cortex, and concomitant alterations in dopamine and glutamate levels in the cortex and basal ganglia were observed, which were consistent with SC and its related neuropsychiatric disorder. In the rat model, serum from group A streptococcal immunized rats activated CaMKII in SKNSH neuronal cells (Brimberg, et al., 2012) like that observed for sera from acute SC (Kirvan, Swedo, Heuser, & Cunningham, 2003). The expression of SC mAb V genes in transgenic mice demonstrated that antibody in SC most likely targets the dopamine receptors on dopaminergic neurons, since the antibody was observed in the cytoplasm of dopaminergic neurons in the basal ganglia (Cox, et al., 2013) and was found to signal the dopamine D2 receptor, as well as associate with the flag-tagged D2 receptor on transfected cells (Cox, et al., 2013). The reactivity of chorea-derived mAb 24.3.1 or SC IgG with D2R was also confirmed by the blocking of Ab reactivity by an extracellular D2R peptide (Cox, et al., 2013).

To summarize, the anti-neuronal antibodies present in SC and PANDAS with fine choreiform piano-playing movements include anti-lysoganglioside (Kirvan, Swedo, Snider, & Cunningham, 2006a), anti-tubulin (Kirvan, Cox, Swedo, & Cunningham, 2007), anti-dopamine D2 receptor (D2R) (Cox, et al., 2013; Brimberg, et al., 2012; Ben-Pazi, Stoner, & Cunningham, 2013), and anti-dopamine D1 receptor (D1R) (Ben-Pazi, Stoner, & Cunningham, 2013) antibodies. In SC, the ratio of the anti-dopamine D2 receptor / anti-dopamine D1 receptor antibodies correlated with the UFMG-Sydenham's-Chorea-Rating-Scale (USCRS) clinical rating scale of neuropsychiatric symptoms (Ben-Pazi, Stoner, & Cunningham, 2013). Most importantly, these antibodies in both SC and PANDAS signaled the SKNSH human neuronal cell line and activated calcium calmodulin-dependent protein kinase II (CaMKII) (Kirvan, Swedo, Heuser, & Cunningham, 2003; Kirvan, Swedo, Snider, & Cunningham, 2006a), which may have led to excess dopamine release (Kirvan, Swedo, Kurahara, & Cunningham, 2006b). Figure 4 shows a model diagram from a recent review (Cunningham, 2012).

In our most recent studies of tics and OCD, anti-neuronal autoantibodies were investigated as well as antibody-mediated neuronal cell signaling activity as previously reported for SC and PANDAS to determine immunological profiles for a large cohort (n=742) of children with tics and/or OCD (Cox, et al., 2015). The goal of this study was to expand upon these earlier observations and to investigate whether sera from patients with OCD, tics, or both resulted in higher CaMKII induction, as compared to healthy controls, and also to see if sera from patients with OCD, tics, or both showed elevated reactivity to previously tested neuronal antigens, tubulin and lysoganglioside, and to dopamine D1 and D2 receptors, which appeared to be targets of autoantibodies in an animal model as well as human sera IgG from PANDAS sera (Brimberg, et al., 2012). In addition, the link between streptococcal infection and OCD, tics, or both was investigated. The study focused on 311 of the 742 participants who had a history of neuropsychiatric illness with streptococcal infections or not, and tics, OCD, or both. Not all 311 subjects fell into every category studied, which resulted in only 261 of the 311 participants with confirmed tics, OCD, or both. Of the 311 individuals, 222 (71%) had evidence of a confirmed group A streptococcal infection, which was associated with tics and/or OCD status (p=0.0087) (Cox, et al., 2015). In our study, the presence of OCD and/or tics was associated with positive streptococcal infection status ($p = 0.0087$). It was also found that subjects who were positive for streptococcal infection were more likely to have both OCD and tics (51%), as opposed to those who were negative for streptococcal infection (30%), while there was no significant association when tics or OCD were considered alone, relative to streptococcal infection.

Individuals with tics and/or OCD (n=261) had evidence of elevated serum IgG antibodies against human D1R ($p<0.0001$) and lysoganglioside ($p=0.0001$), and higher activation of CaMKII activity ($p<0.0001$) in a human neuronal cell line, as compared to healthy controls (n=16). Furthermore, children with tics and/or OCD had

significantly increased activation of CaMKII activity, as compared to children with only tics or only OCD ($p<0.033$ for each) (Cox, et al., 2015).

Our new study also revealed two important correlations that involved CaM kinase II activation: one, the presence of OCD and/or tics was positively associated with CaM kinase II activation ($n = 261$, $p = 0.0008$); and two, CaM kinase II activation was elevated for children with OCD and/or tics ($n=261$), with the median percentile of CaMKII increased values ranging from 149 to 162 percentile units above the baseline enzyme activity, while CaMKII activation remained unaffected in healthy controls, with a median of 94 (equivalent to baseline CaMKII activity at approximately 100) ($n = 16$, $p < 0.0001$) (Cox, et al., 2015). The difference in the median value for CaMK II activation between patient samples and healthy controls is similar to what was found for PANDAS sera and non-PANDAS sera in previous studies (Kirvan, Swedo, Snider, & Cunningham, 2006a).

Our study showed that sera IgG from cases with OCD, tics, or both reacted more significantly with human D1 receptor antigen, as compared to healthy controls in direct ELISA ($p \leq 0.0001$) (Cox, et al., 2015). Clearly, serum IgG from our tics and OCD cohort did not react significantly above normal values with the human D2 receptor and were determined to be more chronic, since the symptoms were present for >1 year or longer in our cohort. Reactivity of the original acute onset PANDAS and SC sera IgG as tested in direct ELISA reacted more significantly with the dopamine D2 receptor antigen, as compared to healthy controls, while and PANDAS sera reacted more significantly with both the D1 and D2 receptor antigens when compared with healthy controls. Additionally, when the sera of 261 patients diagnosed with OCD, tics, or both was found to react in a direct ELISA with lysoganglioside as the antigen, sera IgG had statistically significant higher titers than healthy controls ($p = 0.0001$) (Cox, et al., 2015). The direct ELISA with tubulin as the antigen did not show a statistically significant difference between sera from tics, OCD, or both, versus healthy controls.

To summarize this study, the presence of OCD and/or tics was associated with positive streptococcal infection status ($p = 0.0087$). It was also found that subjects who tested positive for streptococcal infection were more likely to have both OCD and tics (51%) versus those who tested negative for streptococcal infection (30%), while there was no significant association when tics or OCD were considered alone, relative to streptococcal infection. As a result, it is possible that subjects that present with both OCD and tics are more likely to have had streptococcal infections. Presentations of OCD and tics alone are potentially manifestations of disorders not associated with *S. pyogenes*. The study also suggested a significant correlation of streptococcal associated tics and OCD with elevated anti-D1R and anti-lysoganglioside anti-neuronal antibodies concomitant with the higher activation of CaMKII in human neuronal cells. The statistically significant correlation between a history of chronic tics/OCD with anti-neuronal antibodies against the D1R and lysoganglioside and functional activation of CaMKII suggests that at least some pediatric neuropsychiatric disorders may be associated with autoimmunity against the brain. The functional activity of the autoantibodies which signal CaMKII in human neuronal cells suggests that antibodies could target receptors in the brain and alter dopamine neurotransmission, which could lead to neuropsychiatric symptoms.

The mechanisms and effects of anti-neuronal antibodies on the brain include alterations in dopamine transmission, including the release of excess dopamine from neuronal cells. Excess dopamine was released from the SKNSH cell line when treated with a human mAb from SC (Kirvan, Swedo, Kurahara, & Cunningham, 2006b) and human mAb from PANDAS was found to cause alterations in the sensitivity of the receptors to dopamine (Zuccolo, 2015). Evidence in animal models and humans strongly suggest that antibodies mediate inflammatory consequences in SC, PANDAS, and PANS (Brimberg, et al., 2012; Lotan, et al., 2014a; Perlmutter, et al., 1999; Lotan, Cunningham, & Joel, 2014b). There may be other brain antigens targeted by autoantibodies in PANDAS/PANS and related autoimmune diseases that may affect memory and behavior (Hoffman, Hornig, Yaddanapudi, Jabado, & Lipkin, 2004; Yaddanapudi, et al., 2010; Huerta, Kowal, DeGiorgio, Volpe, & Diamond, 2006; Kowal, et al., 2004; DeGiorgio, et al., 2001).

Finally, molecular mimicry between *S. pyogenes* and the brain is supported by evidence from studies of human mAbs and serum IgG antibodies from rheumatic fever (Kirvan, Swedo, Heuser, & Cunningham, 2003; Galvin, Hemric, Ward, & Cunningham, 2000). The investigation of human mAbs from SC has supported the hypothesis that antibodies against the *S. pyogenes* carbohydrate epitope GlcNAc (Kirvan, Swedo, Heuser, & Cunningham, 2003) recognize crossreactive structures on neuronal cells in the brain, which may lead to the onset of SC. In the brain, antibody-mediated neuronal cell signaling may be a mechanism of antibody pathogenesis in SC. The emerging theme in mimicry suggests that crossreactive autoantibodies target intracellular antigens—but for disease pathogenesis, the antibodies must target the surface of neuronal cells by affecting the signaling pathways in neurons. These mechanisms of molecular mimicry lead to the effects seen in acute rheumatic fever and related autoimmune sequelae associated with *S. pyogenes* infections.

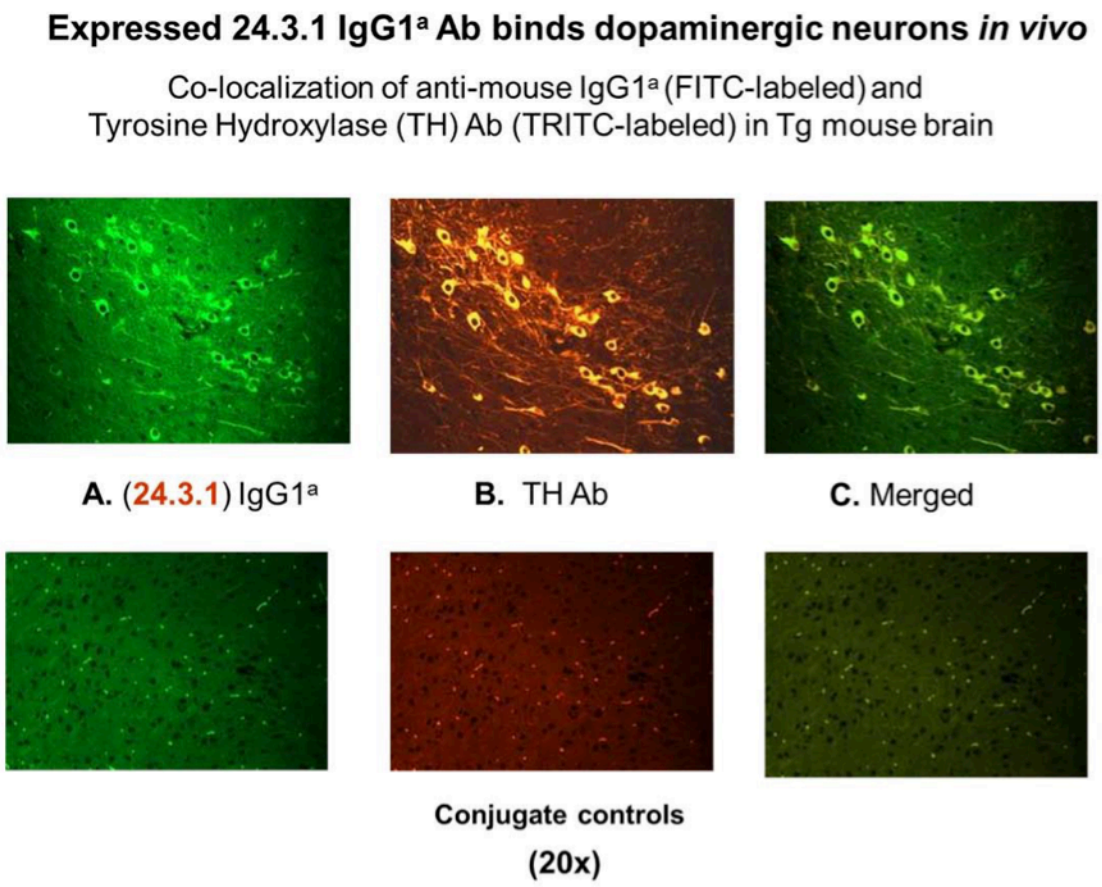


Figure 3. Human Sydenham chorea 24.3.1 V gene expressed as a human V gene-mouse IgG1a constant region in Transgenic(Tg) mice targets dopaminergic neurons in the basal ganglia (most likely substantia nigra, based on location). Chimeric Tg24.3.1 VH IgG1a Ab expressed in Tg mouse sera penetrated dopaminergic neurons in Tg mouse brain in vivo. Colocalization of Tg 24.3.1 IgG1a (anti-IgG1a Ab, green Left Panel) and Tyrosine Hydroxylase Antibody (anti-TH Ab, yellow Middle Panel). TH is a marker for dopaminergic neurons. Left panel shows IgG1a (FITC labeled), center panel shows TH Ab (TRITC labeled), and right panel is merged image (FITC-TRITC). Brain sections (basal ganglia) of VH24.3.1 Tg mouse (original magnification 320), showing FITC labeled anti-mouse IgG1a (A), TRITC-labeled anti-TH Ab(B), and merged image (C). Controls treated with secondary antibody are negative. Figure 3 is similar to the figure shown in Cox et al. (Cox, et al., 2013).

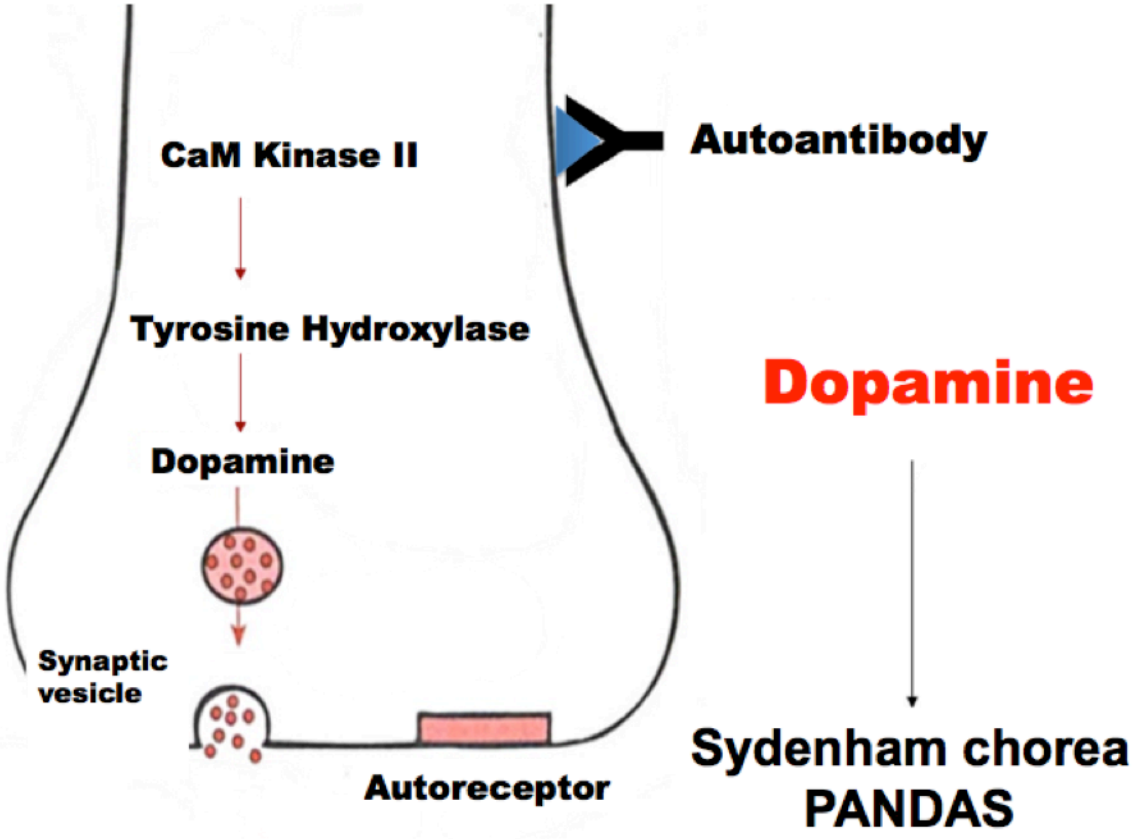


Figure 4. Simplified illustration of a potential pathogenic mechanism of antibody mediated neuronal cell signaling in Sydenham chorea and PANDAS. Antineuronal antibody (IgG) may bind to receptors (blue triangle) on the surface of neuronal cells and trigger the signaling cascade of CaMKII, tyrosine hydroxylase, and dopamine release, which may potentially lead to excess dopamine and the manifestations of Sydenham chorea. Similar to figure shown in Cunningham 2012 review (Cunningham, 2012).

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Post-Streptococcal Glomerulonephritis

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Abstract

Acute glomerulonephritis that results from streptococcal infections is the best-studied immune complex-mediated glomerulonephritis. Initially described in the convalescence of scarlet fever, the incidence of acute post streptococcal glomerulonephritis (APSGN) has decreased worldwide, particularly in developed countries where it is now rare and is limited to adult patients who have debilitating conditions. In developing countries, the annual burden of APSGN remains at a level of least 9 cases per 100,000 inhabitants. Two antigenic fractions of the streptococcus (streptococcal GAPDH/nephritis-associated plasmin receptor, and streptococcal pyrogenic exotoxin B and its zymogen precursor) are currently under scrutiny as putative nephritogens. Glomerulonephritis results from the glomerular deposition of circulating immune complexes and by the *in situ* formation of immune complexes. In-situ formation of immune complexes is a characteristic associated with cationic antigens that have a charge-facilitated penetration through the polyanionic glomerular basement membrane. The plasmin-binding capacity of streptococcal antigens favors immune complex deposition and inflammation. The typical pathological changes are endocapillary proliferation with varying degrees of leukocyte infiltration, and C3, IgG, and IgM immune deposits. Electron microscopy shows the hallmark lesion of subepithelial electron dense deposits ("humps"). The immediate prognosis is excellent in children, but adults have a significant early mortality, which partially results from cardiovascular disease. The long-term development of end-stage renal disease is exceptional in children. However, studies in aboriginal communities indicate that patients with a history of APSGN have a higher incidence of albuminuria, and that APSGN represents a risk factor for the subsequent development of chronic renal failure, if associated with diabetes and obesity.

Introduction

Acute poststreptococcal glomerulonephritis (APSGN) is the prototype of post-infectious glomerulonephritis and is associated with a previous skin or throat infection by group A streptococcus (*Streptococcus pyogenes),* or occasionally groups C or G streptococcus. According to Becker and Murphy (Becker & Murphy, 1968), the development of dark and scanty urine was a feared complication of the epidemics of scarlet fever in the fourteenth century, and clinical descriptions of the "dropsy that follows scarlet fever" have appeared in medical literature since at least 1812 (Wells, 1812). APSGN was probably the cause of death of Wolfgang Amadeus Mozart in 1791 (Zegers, Weigl, & Steptoe, 2009).

The observation that the disease appeared in the convalescent period of scarlet fever led Clemens von Pirquet (von Pirquet, 1911) to postulate that post-scarlatinal nephritis was caused by the development of harmful antibodies (as opposed to the beneficial antibodies in vaccination) and coined the term "*allergy*" (altered reactivity) to define this pathogenic modality. This landmark paper opened the field of immune complex-mediated diseases. The demonstration of the streptococcal etiology of scarlet fever (Dochez & Sherman, 1924) and the recognition that acute rheumatic fever and glomerulonephritis, both complications of streptococcal

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infections, have epidemiological and biological differences and rarely, if ever, occur in the same patient; this suggested the existence of rheumatogenic and nephritogenic strains of the bacterium to Seegal and Earle (Seegal & Earle, 1941), and prompted the search for nephritogenic antigens (reviewed in (Rodríguez-Iturbe & Batsford, 2007)).

Epidemiology

APSGN may occur in epidemic outbreaks or in clusters of cases, and it may occur in isolated patients. Epidemic outbreaks reported in the past as a consequence of upper respiratory or skin streptococcal infections have periodically appeared in specific regions of the world, such as the Red Lake Indian Reservation in Minnesota (Anthony, Kaplan, Wannamaker, Briese, & Chapman, 1969); in Port of Spain, Trinidad (Poon-King, et al., 1967); in Maracaibo, Venezuela (Rodríguez-Iturbe, 1984); and in the Northern Territory of Australia (Marshall, et al., 2011). The most recent epidemics have occurred in the indigenous communities of the Northern Territory of Australia, resulting from pyoderma after infection with *emm55* group A streptococcus (Marshall, et al., 2011) and in the rural region of Nova Serrana, Brazil, caused by the ingestion of unpasteurized milk obtained from cows with mastitis caused by *Streptococcus zooepidemicus* (Balter, et al., 2000). *Streptococcus zooepidemicus* has also caused clusters of cases (5–15 patients) reported in the last two decades in poor communities in industrialized countries (Nicholson, et al., 2000).

In recent decades, the incidence of APSGN has declined significantly worldwide. The reduction of the incidence of APSGN is probably the result of easier and earlier access to appropriate medical care for streptococcal infections. Reports from France (Simon, et al., 1994), Italy (Coppo, Gianoglio, Porcellini, & Maringhini, 1998), China (Zhang, Shen, Feld, & Stapleton, 1994), Chile (Berríos, et al., 2004), Singapore (Yap, et al., 1990), the United States (Roy & Stapleton, 1990), and Venezuela (Rodríguez-Iturbe & Musser, 2008) all indicate that APSGN is now an infrequent disease, and its rarity in affluent societies has been considered a factor for delayed diagnosis in patients who do not have gross hematuria (Pais, Kump, & Greenbaum, 2008).

In industrialized countries, APSGN is now a disease of elderly patients that tend to have debilitating conditions, malignancy, alcoholism, or diabetes (Montseny, Meyrier, Kleinknecht, & Callard, 1995). Nevertheless, APSGN remains a significant health problem in underdeveloped societies. Endocapillary glomerulonephritis, assumed to be of poststreptococcal etiology, is the most common glomerulonephritis found in children in developing countries (Rodríguez-Iturbe & Mezzano, 2005) and in aboriginal populations (Currie & Brewster, 2001). Two independent studies have estimated the incidence of APSGN in developing countries. Carapetis et al. (Carapetis, Steer, Mulholland, & Weber, 2005) analyzed 11 population studies and found that the annual burden of APSGN in developing countries was 9.3 cases per 100,000 people. We evaluated the incidence of APSGN in developing countries, using the reports of pediatric acute renal failure due to glomerulonephritis (Rodríguez-Iturbe & Musser, 2008). We assumed that the cases of acute glomerulonephritis were in fact APSGN, which was explicitly stated in most series, but not in all. These patients had severe renal failure because they were seen at a referral hospital and admitted to the intensive care unit, if one was available, and then dialyzed. The total number of cases in the general population was estimated, considering that uncomplicated cases of APSGN are 100 to 300 times more common than those of life-threatening disease. Using this approach, the annual incidence of APSGN in developing countries was estimated to be 9.5 (low estimate) to 28.5 (high estimate) cases per 100,000 people. This low value is remarkably close to the estimate of Carapetis et al. (Carapetis, Steer, Mulholland, & Weber, 2005) and the higher value exceeds it by three-fold—yet, these authors acknowledged that theirs was likely an underestimation and that the actual incidence was probably a great deal higher.

Etiology and pathogenesis

APSGN is an immune complex-mediated disease. Several mechanisms may participate in the pathogenesis of renal damage (Table 1). Nephritogenic immune complexes are formed in circulation and deposited in the

glomeruli; alternately, the antigen and antibody arrive separately and meet in or outside the glomerular basement membrane, causing *in situ* immune complex disease. Immune cell recruitment, production of chemical mediators and cytokines, and local activation of the complement and coagulation cascades drive an inflammatory response that is localized in the glomeruli. Glomerular deposition of circulating immune complexes depends on the antigen load, the antigen:antibody ratio, and the size of the immune complexes (Dixon, Feldman, & Vazquez, 1961; Germuth, Senterfit, & Dreesman, 1972). *In situ* formation of immune complexes is favored by cationic antigens that have a charge-dependent facilitated penetration into the polyanionic glomerular basement membrane, and tend to occur in conditions of antigen excess (Vogt, et al., 1990).

Table 1. Pathogenetic mechanisms participating in acute poststreptococcal glomerulonephritis

Mechanism	Evidence	Reference
Nephritogenic antigens (NAPlr, SPEB, streptokinase, others)	NAPlr and SEPB demonstrated in renal biopsies	(Oda, et al., 2010; Poon-King, Bannan, Viteri, Cu, & Zabriskie, 1993; Nordstrand, McShan, Ferretti, Holm, & Norgren, 2000)
Circulating immune complexes	Circulating anti-SPEB and anti-NAPlr antibodies in APSGN patients	(Yoshizawa, et al., 2004; Parra, et al., 1998)
In situ Immune complexes (cationic antigens)	SPEB co-localized with complement in glomeruli and demonstrated in the subepithelial electron-dense deposits ("humps") in APSGN	(Batsford, Mezzano, Mihatsch, Schlitz, & Rodríguez-Iturbe, 2005)
Autoimmunity (*anti-IgG, other*)	Neuraminidase is produced by some nephritogenic streptococci. Serum neuraminidase activity in APSGN patients	(Mosquera & Rodríguez-Iturbe, 1984; Rodríguez-Iturbe, Katiyar, & Coello, 1981; Asami, Tanaka, Gunji, & Sakai, 1985)
Anti-Ig (induced by the loss of sialic acid of the IgG or binding of the Fc fragment of IgG to type II receptors on the surface of group A streptococcus)	Serum anti-IgG titers Renal anti-IgG deposits	(Rodríguez-Iturbe B. , 1984; Parra, et al., 1998; Rodríguez-Iturbe, Rabideau, García, & McIntosh, 1980; Burova, et al., 2012)
Other autoimmune reactivity	Anti-DNA, anti.C1q, ANCA demonstrated in serum	(Kozyro, et al., 2006; Ardiles, Valderrama, Moya, & Mezzano, 1997)
Other		
Increased plasmin activity in glomeruli (facilitating immune complex deposition	Co-localization of plasmin and NAPlr in glomeruli. Increased urinary plasmin activity	(Yoshizawa, et al., 2004; Oda, et al., 2010; Oda, et al., 2008)
Neuraminidase-induced glomerular infiltration of desialised leukocytes	Desialised leukocytes accumulate in the glomeruli of patients with APSGN	(Mosquera & Rodríguez-Iturbe, 1986; Marín, Mosquera, & Rodríguez-Iturbe, 1995)

Nephritogenic antigens

Traditionally, APSGN was considered to be caused by an antigen present in group A streptococci. *Streptococcus pyogenes* of M types 1, 2, 4, and 12 were associated with epidemic nephritis resulting from upper respiratory infections and M types 47, 49 and 55 were associated with epidemic nephritis following pyoderma. However, nephritis may also follow infections with group C streptococci since *Str. zooepidemicus* has been identified as the cause of an epidemic in Brazil (Balter, et al., 2000) and several clusters of cases (Nicholson, et al., 2000; Francis, Nimmo, Efstratiou, Galanis, & Nuttall, 1993); therefore nephritogenic antigen(s) are present in streptococci from several groups.

Throughout the years, many putative streptococcal nephritogenic antigens have been studied without definite confirmation of their causal relationship with glomerulonephritis (Rodríguez-Iturbe & Batsford, 2007).

The nephritogenic potential of streptokinase was investigated by Nordstrand et al. (Nordstrand, Norgren, Ferretti, & Holm, 1998) in mice with the experimental tissue cage model, and demonstrated glomerular streptokinase deposits in association with hypercellularity, complement deposition, and proteinuria. Subsequent studies by this group evaluated allelic variants of streptokinase using site-specific integration plasmids, and demonstrated the nephritogenic properties of the nephritis-associated streptokinase gene *ska I*(Nordstrand, McShan, Ferretti, Holm, & Norgren, 2000).

Presently, two streptococcal antigenic fractions with substantial claims to nephritogenicity are being actively investigated. These are the nephritis associated plasmin receptor (NAPlr), identified as glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Oda, et al., 2010; Yoshizawa, et al., 2004), and the streptococcal pyrogenic exotoxin (erythrotoxin) B (SPEB) and its zymogen precursor (zSPEB) (Poon-King, Bannan, Viteri, Cu, & Zabriskie, 1993; Vogt, Batsford, Rodríguez-Iturbe, & García, 1983).

Work by Fujino et al. (Fujino, et al., 2007) has shown that the expression and gene sequence of NAPlr is present in streptococci from several groups. Anti- NAPlr antibody levels are found in 92% of the sera from convalescent APSGN patients and in 60% of the uncomplicated streptococcal infections in Japan (Yoshizawa, et al., 2004). NAPlr is present in early biopsies of APSGN and, since it is not co-localized with complement or IgG, its role as a nephritogen is thought to be related to its plasmin-binding capacity, which facilitates immune complex deposition and inflammation (Oda, et al., 2010). The nephritogenic potential of plasmin-binding activity has been noted, not only in NALPr but also in SPEB, streptokinase, and enolase. Oda et al. (Personal communication, discussed in (Rodríguez-Iturbe & Musser, 2008)) remarked that this property may represent a common nephritogenic property of both NALPr and SPEB, since both of these antigens were identified by their group in the mesangium of biopsies of APSGN. Along with this possibility, patients with APSGN exhibit an increase in urinary plasmin-like activity (Oda, et al., 2010; Oda, et al., 2008).

SPEB, an extracellular cysteine proteinase, is a cationic (pK>8.0) antigen that is co-localized in the glomeruli with complement and Ig deposits, and is the only streptococcal antigen that has been demonstrated within the electron dense subepithelial deposits (humps) that are the hallmark of APSGN (Batsford, Mezzano, Mihatsch, Schlitz, & Rodríguez-Iturbe, 2005). It is likely that this localization is facilitated by the cationic charge of this antigen, as earlier studies have postulated (Vogt, et al., 1990; Vogt, Batsford, Rodríguez-Iturbe, & García, 1983). High anti-SPEB/zSPEB antibody titers have been found in the vast majority of convalescent sera sampled from patients in Latin America (Parra, et al., 1998). SPEB/zSPEB induces chemotaxis and increases angiotensin II production by mesangial cells (Romero, Mosquera, Novo, Fernandez, & Parra, 1999; Viera, Pedreanez, Rincon, & Mosquera, 2009). Recent studies have demonstrated that the Fc portion of antibodies directed to SPEB bind to the C-terminal domain (rSPEB 345-398), and that immunization with this domain prevents group A streptococcal infection in mice (Tsao, et al., 2013). The attractiveness of a charge-related GBM penetration in the pathogenesis of APSGN (Oite, Batsford, Mihatsch, Takamiya, & Vogt, 1982) has led Zhang et al. (Zhang, Ignatowski, Spengler, Noble, & Stinson, 1999) to examine if histones that are cationic elements may be part of the pathogenesis of APSGN. Their studies showed that histones enter the circulation after streptococcal lysis and are capable of inducing *in situ* immune-complex formation.

Finally, recent evidence suggests that there is not a single nephritogenic antigen, since studies by Beres et al. (Beres, et al., 2008) have shown that the gene that encodes for SPEB was absent in the *Str. zooepidemicus* isolated from patients in the Brazil epidemic, and therefore that this antigenic fraction was not involved in this epidemic. Notably, in studies of the *Str. zooepidemicus* of the Brazil epidemic, Nicholson et al. (Nicholson, et al., 2000) found that 33 of 44 patients were seropositive for the Szp5058 fusion protein that was isolated from the causative streptococcal strain, and noted that the antiphagocytic properties of Szp proteins may play a role in the pathogenesis of glomerulonephritis.

Both NALPr and SPEB are capable of inducing monocyte chemoattractant protein 1 (MCP1) and IL-6 in mesangial cells (Khan, et al., 2007; Pedreanez, Viera, Rincon, & Mosquera, 2006; Rincon, Viera, Romero, & Mosquera, 2003) and SPEB elicits the release of a variety of cytokines and interleukins from peripheral blood leucocytes (Viera, Pedreanez, Rincon, & Mosquera, 2007).

Humoral and cellular immunity

Activation of the complement system is a consequence of the antigen/antibody reactivity found in glomeruli. The alternate pathway of complement activation is usually activated in APSGN and is manifested by a depression of C3 levels. However, some patients may also have a reduction in their levels of C1 and C4. In these patients, there may be a role for Protein H, a surface streptococcal protein that may activate the classic pathway of complement activation, in combination with IgG (Berge, Kihlberg, Sjöholm, & Björck, 1997). In addition, in some patients, there may also be complement activation by the lectin pathway (Ohsawa, et al., 1999); but APSGN may develop in individuals who are genetically unable to activate this pathway (Skattum, Akesson, Truedsson, & Sjöholm, 2006).

In addition to humoral immunity, cellular immune mechanisms are also activated in APSGN. It has long been known that there is an overexpression of cellular adhesion molecules (ICAM-1, LFA-1) and infiltration of lymphocyte and macrophages in the glomeruli of these patients. (Parra, Platt, Falk, Rodríguez-Iturbe, & Michael, 1984; Parra, Romero, Henriquez-La Roche, Pineda, & Rodríguez-Iturbe, 1994). Higher numbers of CD4-positive lymphocytes are present in renal biopsies obtained in the first 3–4 weeks of the disease and decrease afterwards (Parra, Platt, Falk, Rodríguez-Iturbe, & Michael, 1984). Increased glomerular expression of IL-8 correlates with neutrophil infiltration and transforming growth factor–β with mesangial expansion (Mezzano, Burgos, Olavarría, & Caorsi, 1997).

Autoimmune reactivity

High titers of anti-IgG rheumatoid factors have been reported in as many as two-thirds of the patients with APSGN in the first week of the disease (Rodríguez-Iturbe, 1984), and anti-human IgG deposits within the glomeruli are present in 29% of the biopsies (Rodríguez-Iturbe, 2008) and were eluted from the kidney of a fatal case of the disease (Rodríguez-Iturbe, Rabideau, García, & McIntosh, 1980). This anti-IgG reactivity may be due to autoantigenic changes to IgG modified by neuraminidase (sialidase). Using methods that measure the release of acetyl neuraminic acid from substrates by a thiobarbituric acid assay, neuraminidase activity was found in group A and B streptococci and, more specifically, in streptococcal strains isolated from patients with nephritis (Mosquera & Rodríguez-Iturbe, 1984). However, a more sensitive methylumbelliferyl acetyl neuraminidase fluorescence test failed to demonstrate neuraminidase activity in culture supernatants of a large number of *Streptococcus pyogenes* strains (Savic & Ferretti, 1994). Neuraminidase activity and free sialic acid have been found in the plasma of patients with acute APSGN (Asami, Tanaka, Gunji, & Sakai, 1985; Rodríguez-Iturbe, Katiyar, & Coello, 1981; Rodríguez-Iturbe, Rubio, & García, 1981). The existence of sialic acid depleted glomerular structures was investigated through the glomerular binding capacity of the lectin *Arachis Hypogaea* (peanut agglutinin), a lectin with a highly specific affinity for galactopyranosyl galactosamine radicals that are exposed after sialic acid removal. Positive intraglomerular staining with this FITC-lectin was detected in the majority of early APSGN biopsies. (Mosquera & Rodríguez-Iturbe, 1986). Neuraminidase may have the additional effect of facilitating the infiltration of desialized leucocytes in the glomeruli (Marín, Mosquera, & Rodríguez-Iturbe, 1995). The simultaneous presentation of APSGN and thrombotic microangiopathy was considered to be the result of streptococcal neuraminidase (Duvic, Desramé, Hérody, & Nédélec, 2000).

Another possible mechanism for the production of anti-Ig is the binding of the Fc fragment of IgG to type II receptors on the surface of group A streptococcus. This binding induces intense anti-IgG reactivity and

glomerulonephritis with anti-IgG deposits, which may have nephritogenic potential (Burova, et al., 2012; Burova, et al., 1998).

Other autoimmune phenomena that have been reported in patients with APSGN include anti-DNA antibodies, anti-C1q antibodies (Kozyro, et al., 2006), and antineutrophil-cytoplasmic autoantibodies (ANCAs). ANCAs have been found in two-thirds of the patients with azotemia, and in 70% of the patients with crescentic APSGN and a rapidly progressive course (Ardiles, Valderrama, Moya, & Mezzano, 1997). Despite the variety of findings of autoimmune reactivity, the clinical relevance of these phenomena remains undefined in APSGN.

Genetic aspects

The familial incidence of APSGN was noted in one of the earliest descriptions of the disease. In 1812, Wells (Wells, 1812) noted that "when one child of a family has been attacked with this disease, the other children of the same family who passed through scarlet fever are more liable to become dropsical, than the children of another family, who had also labored under that fever but among whom no instance of dropsy has occurred," and attributed this predisposition to "similarity of constitution derived from common parents." Subsequent studies have shown that the attack rate of APSGN in the general population in epidemic situations ranges between 5% and 28%, while the development of APSGN (clinical and subclinical) in siblings of index cases in non-epidemic conditions is 38% (Rodríguez-Iturbe, Katiyar, & Coello, 1981). Studies by Layrisse et al. (Layrisse, Rodríguez-Iturbe, García-Ramírez, Rodríguez, & Tiwari, 1983) suggested that DR4 HLA antigens were more common in unrelated patients with APSGN, and investigations in Japan (Naito, Kohara, & Arakawa, 1987) reported association between DR1 antigens and APSGN. Nevertheless, the genetic characteristics that are responsible for predisposition (or resistance) to the disease have not been identified.

Clinical and serological characteristics

As previously indicated, APSGN in developed countries is now a disease of patients with chronic debilitating diseases. The clinical characteristics and the prognosis in these patients are different from the milder clinical course in children. In children with APSGN massive proteinuria and cardiovascular complications are rare and early mortality exceptional. In elderly individuals with APSGN, proteinuria in the nephrotic range occurs in 20% of the patients, congestive heart failure in 43%, and azotemia in 83% (Melby, Musick, Luger, & Khanna, 1987).

The classic patient with APSGN is a child (the male:female ratio is 2:1) between the ages of 2 and 18. The latent period between upper respiratory infection and nephritis is 7–10 days and 2–4 weeks in cases that follow skin infection. The typical clinical presentation is of acute nephritic syndrome (hematuria, edema, hypertension, and oliguria); in a minority of cases, APSGN may be manifested by nephrotic syndrome; and in rare cases, by a rapidly progressive (crescentic glomerulonephritis) clinical course. In a typical case of post-streptococcal nephritis, improvement is observed after 2–7 days when the urine volume increases, followed rapidly by resolution of edema and return of the blood pressure to normal levels. Asymptomatic disease may be manifested by microscopic hematuria and a fall in serum complement levels, and is 4–5 times more common than clinical disease in non-epidemic conditions (Rodríguez-Iturbe, Katiyar, & Coello, 1981; Dodge, Spargo, & Travis, 1967).

The most consistent serological finding in the acute period is a reduction in serum complement levels, which return to normal levels in less than a month. In the first week of the disease, high rheumatoid factor titers and cryoglobulins may be found in as many as two-thirds of the patients (Rodríguez-Iturbe, 1984). The best markers for nephritogenic streptococcal infection are serum antibody levels to NALPr (Yamakami, et al., 2000) or SPEB/zSPEB (Parra, et al., 1998), but these determinations are not generally available. Since positive cultures are not always obtained, antistreptococcal antibody titers are usually used to demonstrate the existence of an antecedent streptococcal infection. Antistreptolysin O titers and anti-DNase B titers are the most frequently elevated in upper respiratory infections and pyodermitis, respectively. A streptozyme test that includes 4 major antigens

(DNase B, Streptolysin O, hyaluronidase and streptokinase) is reported to be positive in more than 80% of the cases (Rodríguez-Iturbe, 1984).

Renal biopsy is seldom performed in uncomplicated cases of APSGN in children with a typical clinical picture, particularly in epidemic situations. Biopsy is usually done in adult patients or when unusual features raise diagnostic doubts. These features generally include a normal serum complement early in the disease, or a persisting low complement more than one month after the onset of the acute nephritic syndrome. Clinical presentations with proteinuria in the nephrotic range or developing rapidly progressive renal failure are rare enough in APSGN that histopathological confirmation of the diagnosis is essential.

Pathology of Acute Post-Streptococcal Glomerulonephritis

Light microscopic findings

The majority of cases (including 72% in a recently published series of adult patients (Nasr, et al., 2008)) show diffuse proliferative and exudative glomerulonephritis (GN). The glomeruli in these cases are often enlarged and show global endocapillary hypercellularity with variable and often large numbers of neutrophils, as shown in Figures 1 and 2. Most of the remaining cases show focal proliferative and exudative GN, or predominantly mesangial proliferative GN, while a membranoproliferative GN (MPGN) pattern is rarely seen (Montseny, Meyrier, Kleinknecht, & Callard, 1995; Nasr, et al., 2008; Rosenberg, et al., 1985; Silva, 2005). When a Masson's trichrome stain is performed, fuschinophilic, red-orange subepithelial, and mesangial deposits may be evident. Crescents, primarily segmental cellular crescents, are present in up to half of cases, and may be accompanied by segmental fibrinoid necrosis with disruption of the glomerular basement membrane (GBM) that is evident on a silver methenamine stain (Nasr, et al., 2008; Montseny, Meyrier, Kleinknecht, & Callard, 1995).

After the first 1-2 weeks of the disease, there is a progressive decline in cellularity, initially from the loss of the neutrophils, which results in a combined mesangial and endocapillary proliferative GN. At this stage, the histologic appearance of the glomeruli may resemble that of an early MPGN. Over the ensuing weeks, endocapillary hypercellularity is lost, resulting in a predominantly mesangial proliferative GN that is visible by light microscopy.

In the acute phase of the disease, interstitial inflammation, which is typically comprised of a mixture of lymphocytes, monocytes, plasma cells, and neutrophils, is present in most cases. Focal intratubular neutrophils are not infrequent, with these cells coming from the inflamed glomeruli. In the study by Nasr et al. (Nasr, et al., 2008), histologic evidence of acute tubular injury, characterized by the flattening of proximal tubular epithelium with loss of brush borders and nuclear enlargement, was seen in 60% of cases. Mild to moderate arteriosclerosis was also seen in the majority of these adult cases; cases with underlying diabetic nephropathy tended to have more frequent and more severe arteriosclerosis, as well as arteriolar hyalinization and thickening (Nasr, et al., 2008).

Immunofluorescence Microscopy

Immunofluorescence findings in evolving stages of post-streptococcal GN have been elegantly defined by Sorger et al. (Sorger, et al., 1982). In the early phase of the disease (the initial 2–3 weeks), the glomeruli show finely granular deposits of C3 and usually IgG in the capillary walls and mesangial areas, in what has been termed a "starry sky" pattern (as shown in Figure 3. Later in the disease, with resorption of many of the capillary wall deposits, there is a predominantly mesangial pattern of staining with a predominance of C3. In reality, not all of the deposits that contribute to the mesangial pattern are actually within the mesangium; as discussed below, many are subepithelial deposits within the mesangial "waist" that are resorbed more slowly than deposits in peripheral portions of the glomerular capillaries. A third pattern of staining, characterized by coarse granular to confluent granular staining along the glomerular capillary walls and termed the "garland" pattern, is most often

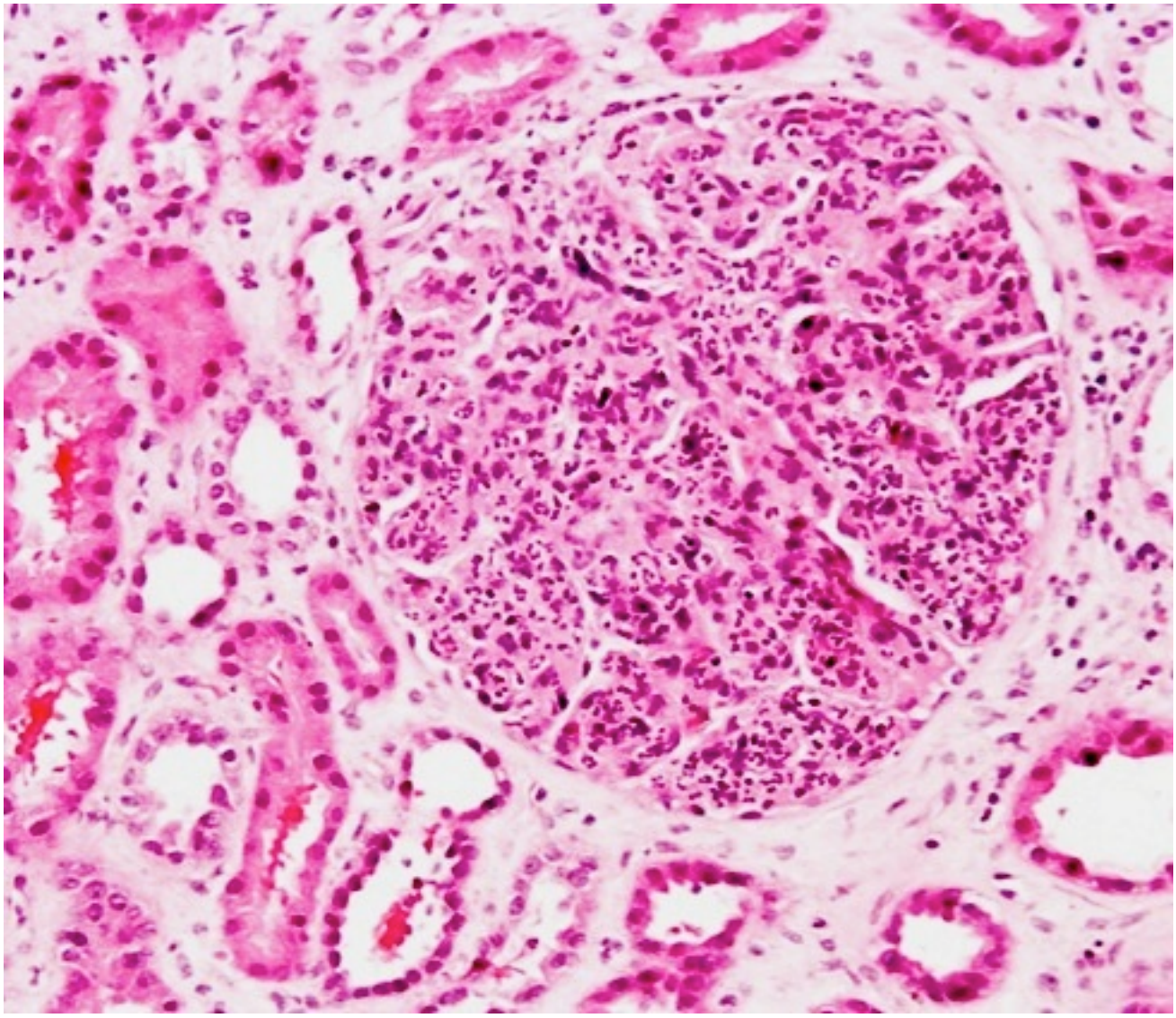

Figure 1. Acute post-streptococcal glomerulonephritis (GN) with severe proliferative and exudative GN. The glomerulus is enlarged and markedly hypercellular with a large number of neutrophils. Note the red blood cells in some tubular lumens. Hematoxylin and eosin (H & E) stain, original magnification x200.

seen early in the disease, but may be seen later as well (Sorger, et al., 1982). It is this pattern that best shows the individual subepithelial "humps" that are the characteristic ultrastructural feature of this disease.

In acute (and subacute) post-streptococcal GN, deposits of C3 are invariably present, accompanied by IgG in most cases, and by IgM in approximately 50%, although the latter staining tends to be of low intensity (Nasr, et al., 2008; Silva, 2005). IgA staining is uncommon and of low intensity when present, although IgA is often the dominant immunoglobulin present in post-staphylococcal GN (Nasr, et al., 2008; Haas, Racusen, & Bagnasco, 2008; Nasr, et al., 2003). Nasr et al. (Nasr, et al., 2008) found C1q staining, typically of low intensity, in approximately one-third of their cases. Staining for kappa and lambda light chains mirrors that for IgG, with respect to patterns that have similar staining intensity for both light chains. Focal and segmental blotchy to amorphous staining for fibrinogen, most typically at the periphery of glomerular tufts, is frequently noted within cellular crescents when these are present.

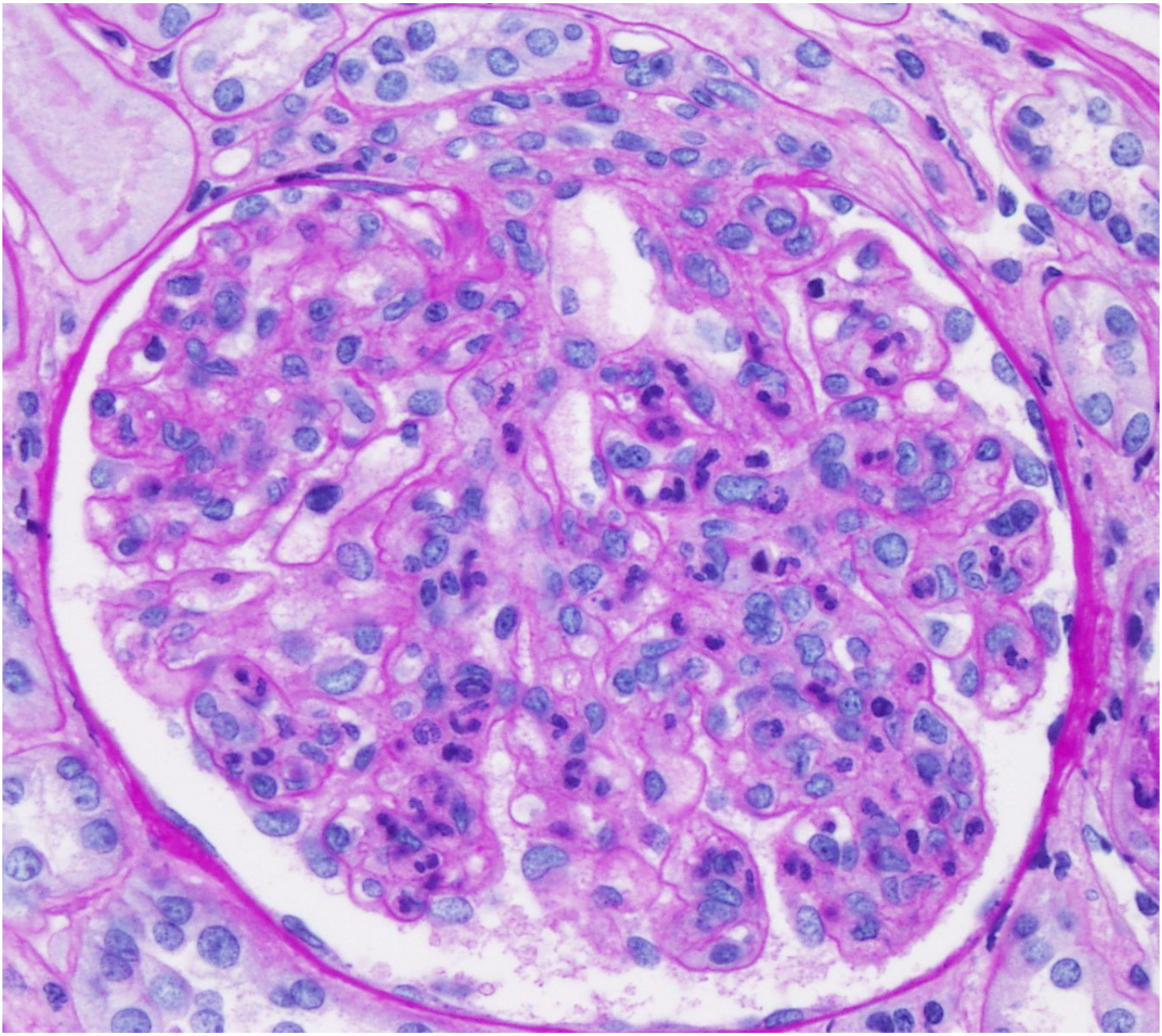

Figure 2. Acute post-streptococcal GN with proliferative and exudative GN. The glomerulus shows endocapillary hypercellularity with multiple neutrophils, although far fewer than the glomerulus in Figure 1. Periodic acid-Schiff (PAS) stain, original magnification x400.

Electron Microscopy

The characteristic ultrastructural finding of acute poststreptococcal GN is the presence of large subepithelial electron-dense deposits with a "hump-like" appearance, as shown in Figure 4. The number of these deposits varies considerably between different cases; they can be quite segmental or rather numerous, although not so much so as to suggest a membranous nephropathy. The size of the subepithelial "humps" may also vary considerably within any given glomerulus. In early post-infectious lesions, these deposits are distributed at various points along glomerular capillaries, although even at this stage, there is some tendency for the greatest number of deposits to be concentrated at or near the glomerular basement membrane reflection over mesangial areas (the mesangial "waist" or "notch," which can be seen in Figure 5), a finding first noted by Heptinstall (Heptinstall, 1974). In subacute and resolving cases, the fraction of subepithelial deposits localized to mesangial

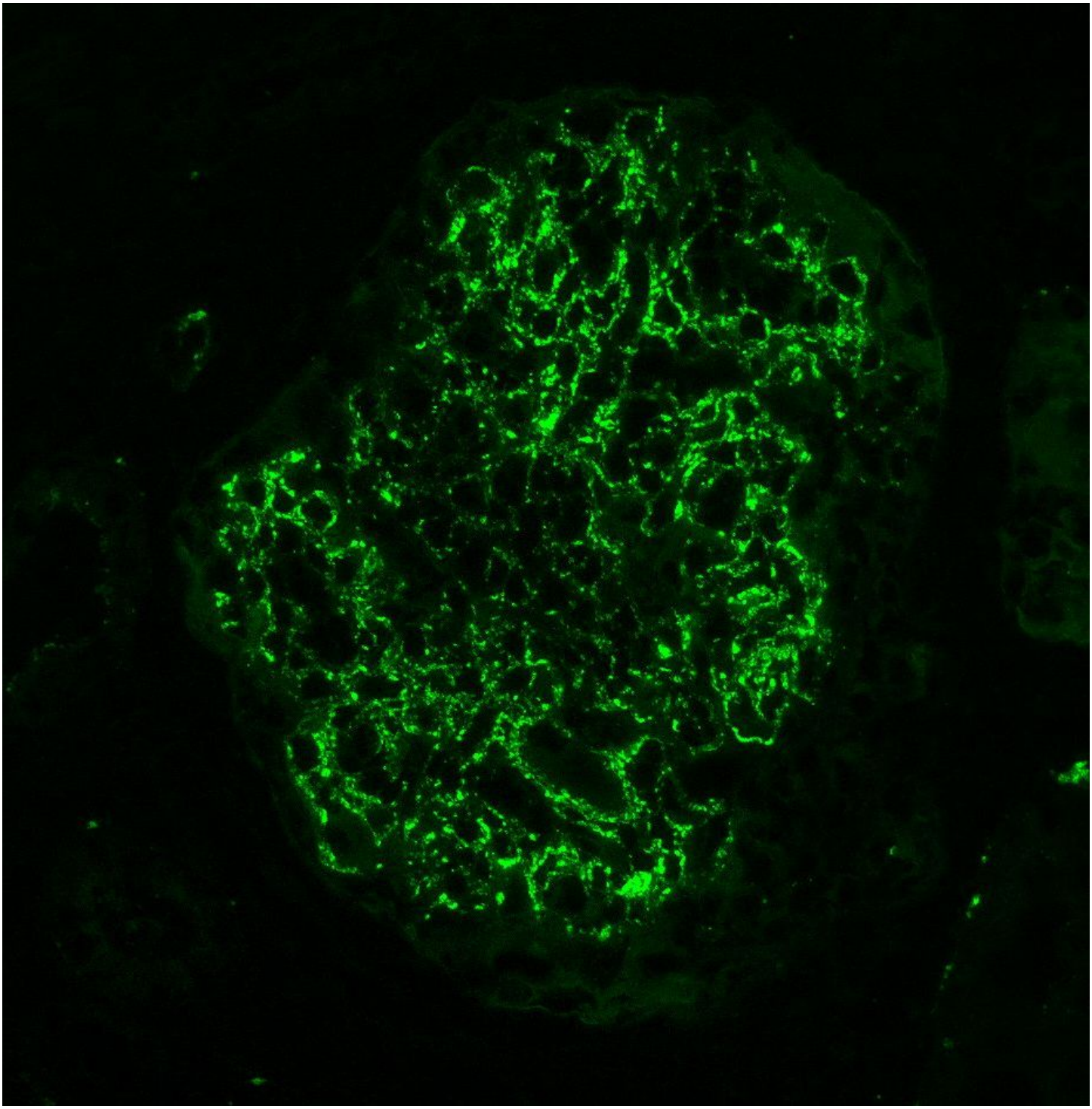

Figure 3. Immunofluorescence staining for C3 in acute post-streptococcal GN. There is granular staining in the glomerular capillary walls and mesangium, in a "starry-sky" pattern. Fluorescein isothiocyanate (FITC) conjugated anti-human C3, original magnification x400.

"waist" areas increases as more peripheral deposits are resorbed (Nasr, et al., 2008; Sorger, et al., 1982; Haas, 2003). Mesangial deposits are present in the great majority of cases of acute poststreptococcal GN and may be abundant, and show subendothelial deposits in most cases, although these tend to be small and segmental (Nasr, et al., 2008; Silva, 2005). Extraglomerular deposits are not a feature of this disease.

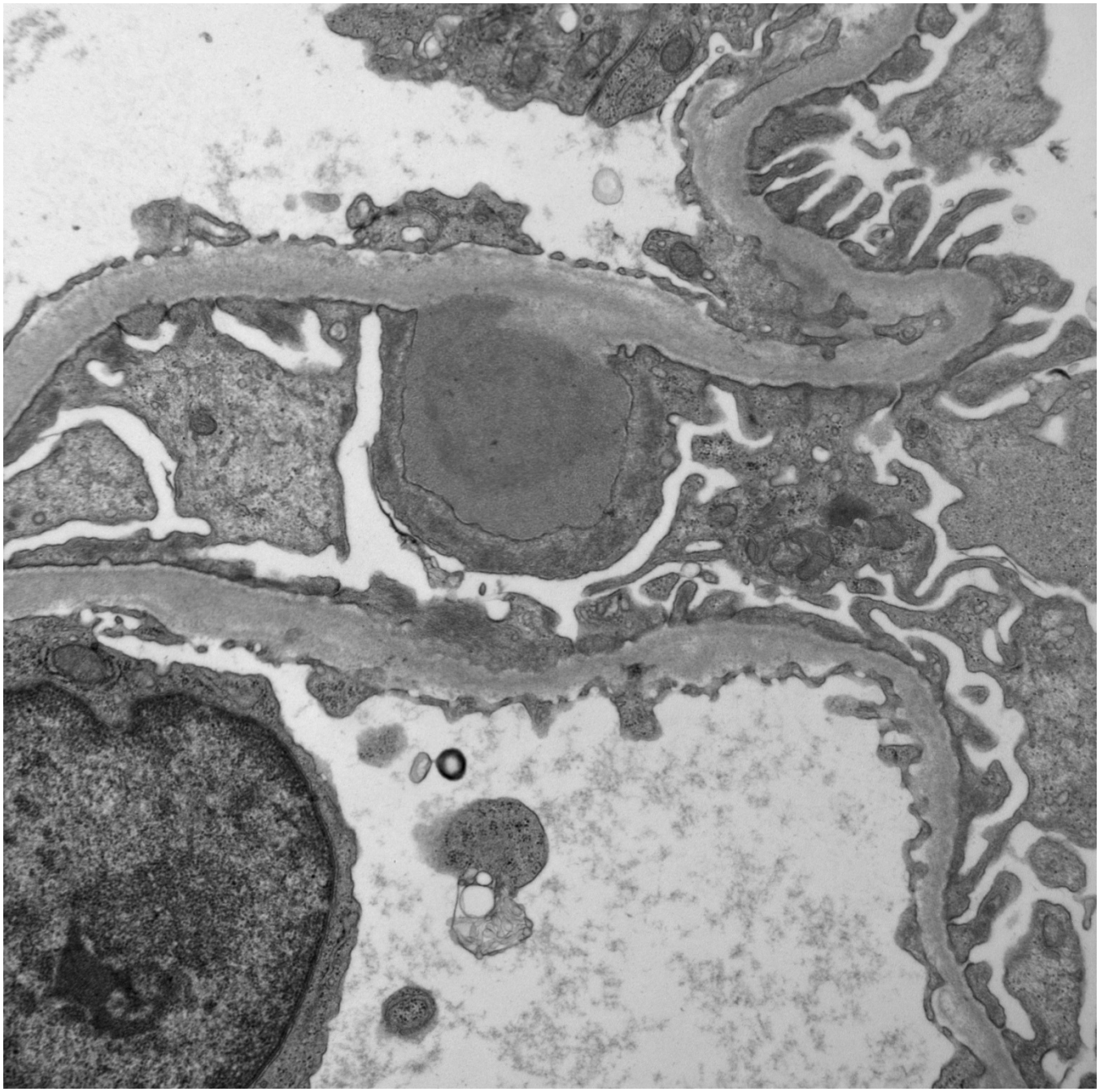

Figure 4. Electron-microscopy in acute post-streptococcal GN. Note the large, "hump"-like subepithelial deposit. The periphery of this deposit is slightly less electron-dense than its center, indicating very early resorption. The glomerular capillary with the subepithelial "hump" also contains a small subendothelial deposit. The glomerular basement membranes themselves are unremarkable and the podocyte foot processes are partially effaced. Uranyl acetate and lead citrate stain, original magnification x7500.

Treatment

If infection is present at the time of diagnosis, it should be treated. In epidemic situations and in high-risk communities, the administration of preventive antibiotic treatment to household members of index cases has been shown to decrease the number of cases of APSGN (Johnston, Carapetis, Patel, Wallace, & Spillane, 1999).

Patients with an acute nephritic syndrome require restriction of sodium and fluid intake. For more than three decades, loop diuretics have been known to accelerate the resolution of edema and improve hypertension

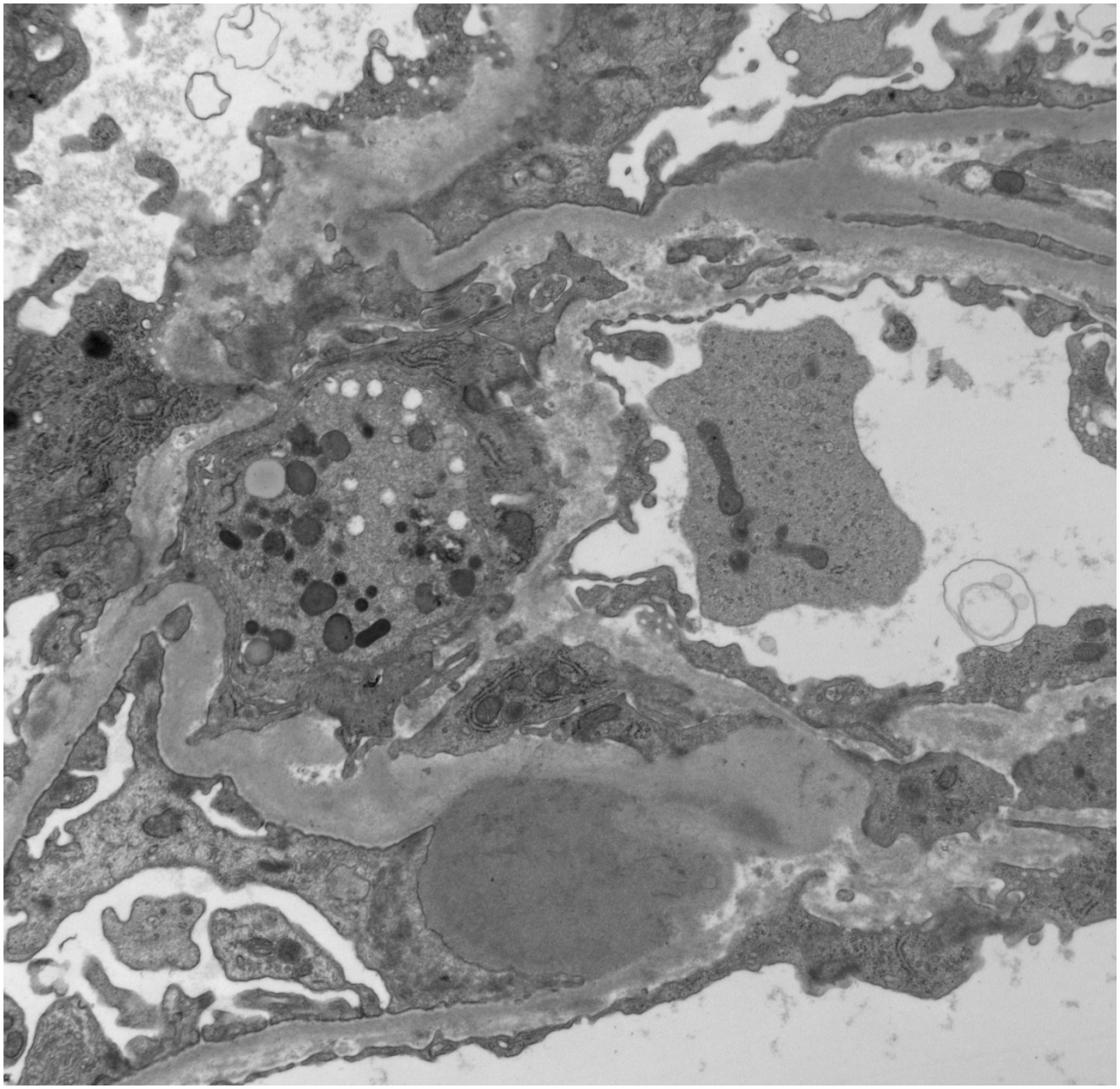

Figure 5. Electron microscopy in subacute post-streptococcal GN. A large, "hump"-like subepithelial deposit is present in a mesangial waist region; deposits are also present within the adjacent mesangial matrix. Uranyl acetate and lead citrate stain, original magnification x6000.

(Powell, McCredie, & Rotenberg, 1980). Thiazide diuretics are ineffective and aldosterone antagonists carry the risk of hyperkalemia. In cases with severe hypertension, nifedipine may be useful. Angiotensin-converting enzyme inhibitors and angiotensin receptor blockers carry the risk of hyperkalemia. Nitroprusside may be needed to treat hypertensive encephalopathy, but only in exceptional cases. Pulmonary edema may complicate the clinical course and should be treated with oxygen, loop diuretics, and rotating tourniquets. Digitalis is ineffective and carries an increased risk of intoxication. Rare complications in APSGN include posterior reversible leukoencephalopathy (Ahn & Ingulli, 2008) and immune-mediated pneumonitis (Wiles, et al., 2011). Hemodialysis and peritoneal dialysis may also be required to treat azotemia, hyperkalemia, or severe circulatory congestion.

Crescentic APSGN with a rapidly progressive clinical course has improved in isolated reports in association with the treatment with intravenous pulses of methylprednisolone; however, the real benefit of intravenous steroids, immunosuppression, or anticoagulation remains unproven (Zaffanello, Cataldi, Franchini, & Fanos, 2010).

Prognosis

The short-term prognosis of APSGN in children is excellent; but in adults, and particularly in debilitated adults, the mortality rate can be as high as 30%, as a consequence of a cardiovascular complication (Melby, Musick, Luger, & Khanna, 1987).

The long-term prognosis, as related to the development of chronic kidney disease, is also different in children and in adults. In a recent study of a specific outbreak of PSGN that resulted from the consumption of cheese contaminated with *Streptococcus zooepidemicus* and that affected mostly adults, there was an alarming incidence of chronic renal disease: impaired renal function was found in 30% of the patients after 2 years of follow-up (10% of them in chronic dialysis therapy) (Pinto, Sesso, Vasconcelos, Watanabe, & Pansute, 2001). In a particular subgroup of adult patients with APSGN that had massive proteinuria as the initial manifestation of the disease, the long-term prognosis was especially severe, with an incidence of chronic renal failure as high as 77% (Vogl, Renke, Mayer-Eichberger, Schmitt, & Bohle, 1986). The worse prognosis in adults has been attributed to age-related impairment of the Fc-receptor function of the mononuclear phagocyte system (Mezzano, et al., 1991). Deficiency of the complement factor H-related protein 5 has also been proposed as a factor that may result in a predisposition to the development of chronic renal disease (Vernon, et al., 2012).

Regarding the long-term prognosis of APSGN in children, initial reports in 1930 and 1940 indicated an excellent prognosis, but follow-up periods were relatively short. Subsequent studies have produced widely contrasting results, with the incidence of abnormal laboratory findings ranging from 3.5% (Potter, Lipschultz, Abidh, Poon-King, & Earle, 1982) to 60% (Baldwin, Gluck, Schacht, & Gallo, 1974). Discrepancies may partially result from the different prognosis of PSGN in adults and in children, which is not always taken into account in the reported series. Studies by Gallo et al. (Gallo, et al., 1980) in the 1980s reported that the incidence of glomerular sclerosis and fibrosis is nearly 50%, but the clinical relevance of these histological characteristics is uncertain. A revision of the follow-up studies in children 10-20 years after the acute episodes found that while approximately 20% of the patients had an abnormal urinalysis or creatinine clearance, less than 1% had developed end-stage kidney disease. Our own data (Rodríguez-Iturbe & Musser, 2008), which includes 110 children with epidemic and sporadic PSGN followed prospectively over 15–18 years after the acute episode, indicate an incidence of 7.2% of proteinuria, 5.4% of microhematuria, 3.0% of arterial hypertension, and 0.9% of azotemia. These values are essentially similar to those found in the general population. We have also followed 10 cases of subclinical PSGN for 10–11 years, and the prognosis is excellent. In specific communities, such as in Australian aboriginal groups, it has been found that patients who had APSGN have an increased risk for albuminuria (adjusted odds ratio (OR) of 6.1, 95% confidence interval (CI) of 2.2–16.9) and hematuria (OR of 3.7, 95% CI of 1.8–8.0) in relation to controls who did not have APSGN (Hoy, et al., 1998). Finally, the long-term prognosis of APSGN may be influenced by the coexistence of other risk factors of chronic renal failure. In patients with a history of APSGN, the association (two-hit) with both diabetes and metabolic syndrome are likely responsible for the high incidence of endstage renal disease in aboriginal communities in Northern Australia (White, Hoy, & McCredie, 2001; Hoy, et al., 2012). It has been reported that persisting arterial stiffness, as determined by brachial–ankle pulse wave velocity, is found in patients with APSGN who develop chronic renal disease (Yu, Yu, Yu, Lee, & Huang, 2011).

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Post-Streptococcal Autoimmune Sequelae: Rheumatic Fever and Beyond

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Introduction

Streptococcus pyogenes, the group A streptococcus, and its link to rheumatic fever, rheumatic heart disease, arthritis, and St Vitus dance are reported in the earliest historical accounts of post-streptococcal sequelae that occur anywhere from several weeks to several months after group A streptococcal infection (Stollerman, 2011; Stollerman, 1997; Wannamaker, 1973; Jones, 1944; Special Writing Group of the Committee on Rheumatic Fever, Endocarditis, and Kawasaki Disease of the Council on Cardiovascular Disease in the Young of the American Heart Association, 1992; Danjani, et al., 1988). Prior to the discovery of penicillin, hospital wards were filled with cases of children with rheumatic heart disease, which could be associated with polymigrating arthritis, the most common manifestation of rheumatic fever; or with Sydenham chorea, which is also known as St. Vitus dance in the early literature. The Jones criteria, which describe the onset and diagnosis of rheumatic fever, include major manifestations of the heart, brain, joints, and skin. Polymigrating arthritis, carditis associated with a heart murmur, erythema marginatum, a circinate skin rash, subcutaneous nodules, and the neurologic manifestation (Sydenham chorea) are all inflammatory reactions that may occur in acute rheumatic fever (Jones, 1944; Gerber, et al., 2009). Rheumatic fever or other sequelae generally follow group A streptococcal pharyngitis or other mucosal infections, and diagnosis requires elevated anti-streptolysin O and/or anti-DNAse B antibody titers that are increased over normal levels, or a positive throat culture or positive quick strep test for group A streptococci (Gerber, et al., 2009). Streptococcal infection of the throat, skin, or mucosa precedes autoimmune or inflammatory sequelae that are observed in acute rheumatic fever (Steer, Carapetis, Nolan, & Shann, 2002; Cunningham, 2000; Cunningham, 2012).

Post-streptococcal sequelae, such as rheumatic fever (Bisno, Brito, & Collins, 2003; Bisno, 2001; Bisno & Stevens, 1996; Bisno, Pearce, Wall, Moody, & Stollerman, 1970), occur primarily in childhood and adolescence. The primary age group most affected are children between the ages of 5 and 15 years old. Rheumatic fever is a global disease (Steer, Carapetis, Nolan, & Shann, 2002; McDonald, Currie, & Carapetis, 2004; Carapetis, Robins-Browne, Martin, Shelby-James, & Hogg, 1995; Carapetis, Currie, & Good, 1996; Carapetis, Walker, Kilburn, Currie, & MacDonald, 1997), and a resurgence of rheumatic fever in the United States has occurred since approximately 1983 in the intermountain region, specifically in Utah near Salt Lake City and the surrounding area (Kaplan, Johnson, & Cleary, 1989; Veasy, Tani, & Hill, 1994; Veasy, et al., 1987; Veasy, et al., 2004).

Some of the earliest reports of the immunology of rheumatic fever in humans reported that antibodies were bound to heart tissues, including valves and myocardia from rheumatic hearts (Kaplan & Dallenbach, 1961; Kaplan, Bolande, Rakita, & Blair, 1964; Zabriskie & Freimer, 1966), and animal studies suggested that antibodies against group A streptococcus might cross-react with the heart (Kaplan & Svec, 1964; Kaplan & Meyerserian, 1962; Kaplan & Suchy, 1964; Zabriskie, 1967). Heart-reactive antibodies were found in patients with rheumatic fever (Zabriskie, Hsu, & Seegal, 1970), and these antibodies would later be recognized to react with cardiac myosin (Galvin, Hemric, Ward, & Cunningham, 2000; Krisher & Cunningham, 1985). In Sydenham chorea, the neurologic manifestation of rheumatic fever, anti-neuronal antibodies were found in neurons in the basal ganglia of the brain (Husby, van de Rijn, Zabriskie, Abdin, & Williams, 1976). Both rheumatic carditis and

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Sydenham chorea have been extensively investigated for autoantibodies found in the blood against the heart and brain (Cunningham, 2000; Cunningham, 2012; Kaplan & Dallenbach, 1961; Husby, van de Rijn, Zabriskie, Abdin, & Williams, 1976; Dudding & Ayoub, 1968; Kaplan, Bolande, Rakita, & Blair, 1964). Pathogenic mechanisms of autoimmunity and inflammation, including both humoral and cellular autoimmunity, are continually under investigation in these streptococcal sequelae.

Both of these streptococcal sequelae rheumatic carditis and Sydenham chorea may occur due to molecular mimicry (Galvin, Hemric, Ward, & Cunningham, 2000; Kirvan, Swedo, Heuser, & Cunningham, 2003). Molecular mimicry is part of the normal immune response, including the response of the host to the group A streptococcus. Mimicry is the response against infectious microbes, which cross-react with host antigens and potentially lead to autoimmunity, which may produce inflammation in host tissues and lead to disease pathogenesis in susceptible hosts (Zabriskie & Gibofsky, 1986; Zabriskie, 1985). Mimicry and production of cross-reactive antibodies as well as cross-reactive T cells provide a "survival of the fittest" advantage to the host through immune recognition and immune responses against pathogens, due to the production of antibodies or responsive T cells that recognize both host and microbial antigens. In reference to T cell cross-reactivity, T cell receptor cross-reactivity between similar self and foreign peptides can reduce the size of foreign peptide-specific T cell populations and lead to the emergence of T cell populations that are specific for tissue restricted self-peptides, which can lead to autoimmunity and potentially disease following infection (Nelson, et al., 2015).

The determination of disease in humoral immunity and cross-reactivity is most likely related to the increasing avidity of the antibody, which must cause a cytotoxic reaction or a reaction that leads to inflammation or signaling to produce disease. The avidity of the antibody has also been shown to be important in germinal center reactions, in that antibody feeds back on the germinal center to shut down the production of a high avidity antibody (Zhang, et al., 2013). However, if such antibodies were trapped in immune complexes that were not cleared from the host either normally or quickly, they would prevent the attenuation of a high avidity antibody response, which may damage tissue. It has been known for some time that rheumatic fever is a disease with immune complexes that are probably not cleared normally (Read, Reid, Poon-King, Fischetti, Zabriskie, & Rapaport, 1977; Read, et al., 1986; Read & Zabriskie, 1977; Reddy, et al., 1990), and HLA B5 has been associated with immune complexes in acute rheumatic fever (Yoshinoya & Pope, 1980).

Evidence supports the hypothesis that molecular mimicry between the group A streptococcus and the heart or brain is important in the immune responses in rheumatic fever (Zabriskie & Freimer, 1966; Zabriskie, 1967; Galvin, Hemric, Ward, & Cunningham, 2000; Krisher & Cunningham, 1985; Kirvan, Swedo, Heuser, & Cunningham, 2003; Ellis, et al., 2010). Anti-streptococcal antibodies that are cross-reactive with the heart or brain that could recognize several types of epitopes that have already been defined (Cunningham, 2000; Krisher & Cunningham, 1985; Cunningham, 2003; Cunningham, 2014; Cunningham, Antone, Gulizia, McManus, Fischetti, & Gauntt, 1992). Other mechanisms may involve collagen or anti-collagen antibodies, and have recently been reviewed (Cunningham, 2014; Tandon, Sharma, Chandrasekhar, Kotb, Yacoub, & Narula, 2013). Collagen has not been found to play a direct role in molecular mimicry, but anti-collagen antibodies could be important in attacking collagen in host tissues—especially after a valve is damaged or exposed.

Rheumatic valvular heart disease is the most serious manifestation of rheumatic fever, and has been the focus of decades of research (Cunningham, 2000; Cunningham, 2012; Zabriskie, Hsu, & Seegal, 1970; Zabriskie, 1985; Reddy, et al., 1990; Veasy, 2004). Studies of Sydenham chorea (Kirvan, Swedo, Heuser, & Cunningham, 2003) and its related sequelae, such as pediatric autoimmune neurologic disorder associated with streptococci (PANDAS), have been investigated for anti-neuronal autoantibodies against the brain (Snider & Swedo, 2004; Swedo, 1994; Swedo, et al., 1998; Swedo, et al., 1997; Murphy, et al., 2007; Murphy, Storch, Lewin, Edge, & Goodman, 2012). The first 50 cases of PANDAS were described by Swedo and colleagues in children that presented with tics or obsessive compulsive symptoms and often particularly display small piano-playing choreiform movements of the fingers and toes (Swedo, 2002; Swedo, et al., 1998). A group of youth and young

adults with infections, as well as with acute and chronic tic and obsessive compulsive disorders (OCD), has also been investigated (Singer, Gause, Morris, Lopez, & Tourette Syndrome Study Group, 2008). The group of children with OCD/tics who demonstrate small choreiform movements, such as piano-playing movements of the fingers and toes, is immunologically similar to Sydenham chorea and is termed with the acronym PANDAS (Swedo, 1994; Swedo, 2002; Cox, et al., 2013). Acute onset tic and OCD symptoms can also follow other non-streptococcal infections and are considered to be pediatric acute onset neuropsychiatric syndrome, or PANS (Swedo, Leckman, & Rose, 2012). Another clinical research group has called for a broader concept of childhood acute neurologic symptoms, or CANS (Singer, Gilbert, Wolf, Mink, & Kurlan, 2012). The PANDAS subgroup is known to have the small choreiform movements, particularly of the fingers and toes that are usually not present in some of the other groups with acute or chronic tics, and OCD, which would be called PANS. Studies of anti-neuronal autoantibodies in Sydenham chorea and PANDAS with choreiform movements clearly identified a specific group of anti-neuronal antibodies that are present in both Sydenham chorea and PANDAS and identified specific antibody mediated neuronal cell-signaling mechanisms, which may partially lead to disease symptoms (Kirvan, Swedo, Snider, & Cunningham, 2006b; Kirvan, Swedo, Heuser, & Cunningham, 2003; Kirvan, Cox, Swedo, & Cunningham, 2007; Brimberg, et al., 2012; Ben-Pazi, Stoner, & Cunningham, 2013). The group of diseases associated with Sydenham chorea and the small choreiform movements generally seem to be related to antibodies against the dopamine receptors D1 and D2. Thus far, anti-D2 receptor antibodies are exclusively seen in Sydenham chorea and PANDAS with the small piano-playing choreiform movements of the fingers and toes (Cox, et al., 2013; Ben-Pazi, Stoner, & Cunningham, 2013). The combination of autoimmunity and behavior is a relatively new concept that links brain, behavior, and neuropsychiatric disorders to streptococcal infections.

Rheumatic Carditis: Anti-Streptococcal Humoral and Cellular Immunity against the Heart

Evidence supports mimicry and cross-reactivity between the group A streptococcal antigens and heart antigens (Zabriskie, 1967; Galvin, Hemric, Ward, & Cunningham, 2000; Kirvan, Swedo, Heuser, & Cunningham, 2003; Kaplan, 1963). Originally, mouse monoclonal antibodies (mAbs) produced against group A streptococci and heart reacted with striations in myocardium or mammalian muscle (Zabriskie, Hsu, & Seegal, 1970), as previously reported for human acute rheumatic fever sera or sera from animals immunized with group A streptococcal antigens (Kaplan, Bolande, Rakita, & Blair, 1964; Zabriskie & Freimer, 1966; Zabriskie, 1967; Zabriskie, Hsu, & Seegal, 1970). Early experiments were performed using human and animal sera that contain thousands of antibodies, and led to complicated studies that were difficult to interpret in order to determine cross-reactivity and molecular mimicry between the host and streptococcus. Mouse and human anti-streptococcal mAbs were developed soon after the discovery of B cell hybridoma production (Galvin, Hemric, Ward, & Cunningham, 2000; Krisher & Cunningham, 1985; Cunningham, Antone, Gulizia, McManus, Fischetti, & Gauntt, 1992; Cunningham & Swerlick, 1986; Shikhman & Cunningham, 1994; Shikhman, Greenspan, & Cunningham, 1994). The anti-streptococcal mAbs reacted with heart cells and identified cardiac myosin as one of the major proteins in heart cells, which was later found to cross-react with the dominant group A carbohydrate epitope, N-acetyl-beta-D-glucosamine (GlcNAc) or streptococcal M protein antigens (Cunningham, 2000; Galvin, Hemric, Ward, & Cunningham, 2000; Krisher & Cunningham, 1985). GlcNAc, the immunodominant epitope of the group A carbohydrate, is composed of a polyrhamnose backbone with side chains of N-acetyl-beta-D-glucosamine (GlcNAc) as the group A carbohydrate specificity that is recognized by many of the human cross-reactive anti-streptococcal antibodies directed against heart cells (Galvin, Hemric, Ward, & Cunningham, 2000). Responses against GlcNAc are strongly linked to antibody responses against cardiac myosin and other alpha helical coiled coil proteins (Shikhman & Cunningham, 1994; Shikhman, Greenspan, & Cunningham, 1994; Shikhman, Greenspan, & Cunningham, 1993; Malkiel, Liao, Cunningham, & Diamond, 2000).

Inflamed endothelium surrounding the valve allows T cells to enter the valve tissue and proliferate to produce inflammation and a damaged valve. Human mAb 3B6 derived from acute rheumatic carditis reacted with cardiac myosin, as well as with myocardium and valve endothelium (Figure 1), which may explain the reactivity of rheumatic carditis sera with striations in heart muscle as well as reactivity with the valve (Galvin, Hemric, Ward, & Cunningham, 2000). See Figure 1, which shows the reaction of mAb 3B6 with both myocardium striations as well as with the valvular endothelium. The rheumatic carditis derived mAb 3B6 antibody recognized laminin at the laminar basement membrane, as well as specific laminin peptide epitopes, as shown in Figure 2. The antibody cross-reacted with laminin epitopes, as well as with peptide epitopes, in human cardiac myosin (Figure 2), and similar anti-cardiac myosin antibodies were found in the sera of rheumatic heart disease (Galvin, Hemric, Ward, & Cunningham, 2000). Specifically, glycosylated proteins such as laminin or other extracellular matrix proteins glycosylated at the valve surface may also play a role in trapping antibodies at the surface of the valve and activating the endothelium and upregulating VCAM-1. Previous evidence demonstrated that glycosylated proteins and carbohydrate epitopes on the valve did, in fact, cross-react with the group A carbohydrate (Goldstein, Halpern, & Robert, 1967). Most important for the linkage between the group A carbohydrate and valvular heart disease in rheumatic fever, persistence of elevated antibody responses against the group A carbohydrate was correlated to a poor prognosis of valvular heart disease (Dudding & Ayoub, 1968). The evidence strongly links rheumatic valvular heart disease with the group A carbohydrate. Many human mAbs derived from rheumatic fever in humans recognized the group A carbohydrate epitope GlcNAc (Adderson, Shikhman, Ward, & Cunningham, 1998). Antibodies or immune complexes would generally be targeted to the valve surface and can lead to cellular infiltration of the valve.

Inflammation is often directly observed at the valve endothelium with upregulation of vascular cell adhesion molecule-1 (VCAM-1), as shown in valves from rheumatic heart disease (Roberts, et al., 2001) (Figure 3). Activation of VCAM-1 on the endothelium is an important first step that leads to valvular injury, edema, and infiltration of T cells that are reactive with the streptococcal M protein, as well as other streptococcal and host proteins. The valve is vulnerable to attack by the immune system following the activation of VCAM-1 on the valve endothelium that promotes subsequent cellular infiltration (Cunningham, 2012; Roberts, Kosanke, Terrence Dunn, Jankelow, Duran, & Cunningham, 2001). A diagram in Figure 4 shows the initial production of cross-reactive antibodies against the group A carbohydrate that attacks the valve endothelium with upregulation of VCAM-1 and subsequent infiltration of T cells from rheumatic carditis, which are specific for the streptococcal M protein and cardiac myosin, as well as other homologous epitopes (Ellis, Li, Hildebrand, Fischetti, & Cunningham, 2005).

In rheumatic carditis, cross-reactive antibodies may initially cause damage that leads to edema, annular dilation and chordal elongation. This prevents adequate surface coaptation of the valve leaflets (Veasy & Tani, 2005), which by itself does not directly impair myocardial function. Fibrinous vegetations in the rough zone of the anterior leaflet may be observed. After chordal elongation, the scarring of leaflets appears, which is the initial insult that leads to mitral regurgitation, and is then heard as a heart murmur.

Alpha helical coiled-coil protein structures such as the streptococcal M proteins, cardiac myosin, keratin, and laminin are responsible for some of the cross-reactivity between the group A carbohydrate epitope N-acetyl-beta-D glucosamine and the myocardium, skin, or valve (Cunningham, 2000; Shikhman & Cunningham, 1994; Shikhman, Greenspan, & Cunningham, 1994). Some of the cross-reactive antibodies found in rheumatic fever recognize the GlcNAc epitope and react with the myocardium and with valves. Human mAbs, which target the group A carbohydrate epitope GlcNAc, also react with well-defined peptide epitopes taken from alpha-helical coiled-coil proteins, and amino acid substitution experiments show that hydrophobic and aromatic amino acids are important to the interactions between cross-reactive antibody molecules (Shikhman, Greenspan, & Cunningham, 1994). Peptides from alpha-helical coiled-coil molecules have been described that mimic the group A carbohydrate epitope (Shikhman, Greenspan, & Cunningham, 1994).

Analysis of crystallized group A streptococcal M1 protein fragments leads to an explanation of how the alpha-helical coiled-coil structures and epitopes are recognized in alpha-helical proteins as a basis for molecular mimicry and cross-reactivity between streptococcal M proteins and cardiac myosin (McNamara, et al., 2008). Streptococcal M proteins are well known for their alpha-helical coiled-coil cross-reactive properties and cross-reactivity with cardiac myosin (Cunningham, 2000). M1 protein exhibited substantial irregularities and instabilities demonstrating a non-idealized alpha helix (McNamara, et al., 2008) similar to that seen in cardiac myosin and tropomyosin. When the coiled-coil alpha helix was idealized and had much fewer splayed regions that could interact with immune molecules such as antibodies, it had much less cross-reactivity with our cross-reactive mAbs (McNamara, et al., 2008). Mutations in M1 protein encoding an idealized alpha helix, stabilized the alpha-helical structure and diminished cross-reactive properties of the streptococcal M1 protein (McNamara, et al., 2008).

The aggregation of collagen by certain streptococcal serotypes, such as the M3 protein identified as a collagen-binding factor of M3 streptococci (Tandon, et al., 2013; Dinkla, et al., 2003a; Dinkla, et al., 2003b), may lead to autoantibodies against collagen I that are observed in human sera and are produced along with responses against cardiac myosin (Martins, et al., 2008). Immune responses against collagen I may also be due to the release of collagen from damaged valves during rheumatic heart disease (Cunningham, 2012). Antibody responses against collagen I are not cross-reactive, which suggests that the release of collagen from the valve could potentially be a source of exposure of collagen to the human immune system in rheumatic carditis. Streptococcal proteins with similarity to collagen have been reported (Lukomski, et al., 2000; Lukomski, et al., 2001), but apparently, cross-reactivity has not been reported in rheumatic heart disease. The collagen hypothesis as a possibility for the pathogenesis of rheumatic heart disease has been previously reviewed and discussed (Cunningham, 2014; Tandon, Sharma, Chandrasekhar, Kotb, Yacoub, & Narula, 2013). If the valve is damaged and a loss of collagen structure is observed, then collagen is clearly affected (Chopra & Narula, 1991). A loss of collagen structure in the damage to the valve does not preclude immune mediated damage by cross-reactive antibodies and T cells or the exposure of collagen to the immune system after the initial attack, due to cross-reactive immune responses.

There is no cardiac myosin directly in a valve that is attached in papillary muscle that contains cardiac myosin and is affixed to the myocardium (Tandon, et al., 2013; Roberts, et al., 2001). The cross-reactivity of cardiac myosin with a valve is due to the recognition of laminin or related proteins by autoantibodies against group A streptococci and cardiac myosin. Injury to the valve initially is proposed to be by the autoantibody response directed at valve endothelium and laminar basement membrane. Initially, chordae tendinae become edematous and elongated, which leads to abnormal valve leaflet coaptation and closure. Once VCAM-1 is elevated on the activated valve endothelium, lymphocytes and other immune cells extravasate into the valve (Roberts, et al., 2001).

Studies by Chopra et al. have shown that the endomyocardium was primarily infiltrated by T cells and that 56 percent of the specimens demonstrated characteristic Aschoff nodules (Chopra P. , Narula, Kumar, Sachdeva, & Bhatia, 1988) and electron microscopy showed a loss of normal arrangement of endothelial cells and architectural modifications of the valvular collagen (Chopra & Narula, 1991). In studies by Roberts et al., both CD4+ and CD8+ T cells infiltrated the valves in rheumatic fever (Roberts, et al., 2001), but the CD4+ T cell subset predominated over the CD8+ T cell subset in the rheumatic valve (Roberts, et al., 2001)(Figure 5). The granulomatous Th1 reaction is evident and the presence of gamma IFN has been reported in rheumatic valves (Guilherme, et al., 2004). Although less is known about Th17 responses in rheumatic heart disease, Th17 cells are important in group A streptococcal infections and have been identified in nasopharyngeal and tonsillar lymphoid tissues in streptococcal infection animal models (Dileepan, Linehan, Moon, Pepper, & Jenkins, 2011; Pepper, et al., 2010; Carapetis & Steer, 2010). The balance between T regulatory cells and Th17 cells was abnormal, which suggests that Th17 cells were increased in rheumatic heart disease.

The valve endocardium is the site of CD4+ T cell infiltration into the valve (Roberts, et al., 2001) (Figure 5). As Figure 2 shows, subendocardial infiltration is present and is shown near the papillary muscle attachment of the valve into the myocardium. Studies of T lymphocytes from both humans and Lewis rats (Lymbury, et al., 2003; Quinn, Kosanke, Fischetti, Factor, & Cunningham, 2001; Kirvan, Galvin, Hilt, Kosanke, & Cunningham, 2014) suggest a strong cross-reactivity between cardiac myosin and streptococcal M protein by the T lymphocytes isolated and cloned from blood (Ellis, Li, Hildebrand, Fischetti, & Cunningham, 2005) or heart valve (Ellis, Li, Hildebrand, Fischetti, & Cunningham, 2005; Guilherme, et al., 2004; Faé, Kalil, Toubert, & Guilherme, 2004; Faé, et al., 2006; Guilherme, Weidebach, Kiss, Snitcowsky, & Kalil, 1991; Guilherme, et al., 2000). Guilherme et al. reported that T cells cloned from peripheral blood in human rheumatic heart disease reflect similar specificities as T cells cloned from heart valves in rheumatic carditis (Guilherme, et al., 2001). Immunization with streptococcal recombinant M6 protein and the pepsin fragment of M protein (PepM5), as well as immunization with peptides from the A, B, and C repeat regions of the streptococcal M5 protein molecule, induced valvular heart disease in the Lewis rat model of valvulitis (Lymbury, et al., 2003; Kirvan, Galvin, Hilt, Kosanke, & Cunningham, 2014; Gorton, Blyth, Gorton, Govan, & Ketheesan, 2010; Gorton, Govan, Olive, & Ketheesan, 2009).

More detailed studies of T cell lines produced from Lewis rats immunized with streptococcal PepM5 protein induced valvulitis, and were strongly stimulated by specific M5 peptides (Kirvan, Galvin, Hilt, Kosanke, & Cunningham, 2014). Passive transfer of the M5 protein specific T cell lines transferred valvulitis as shown by infiltration of CD4+ T cells and upregulation of VCAM-1 on the valve endothelium (Kirvan, Galvin, Hilt, Kosanke, & Cunningham, 2014). It was notable that the passive transfer of a potentially pathogenic T cell line led to upregulation of the VCAM-1, while a control T cell line did not, and therefore did not infiltrate the valve. M protein-specific T cells may be important mediators of valvulitis in the Lewis rat model of rheumatic carditis (Quinn, Kosanke, Fischetti, Factor, & Cunningham, 2001). Mononuclear cells were infiltrated at the valve surface and inner valve in Lewis rats that were immunized with the group A streptococcal M5 serotype amino acid sequence residues 1–76 in the A repeat region (Kirvan, Galvin, Hilt, Kosanke, & Cunningham, 2014). Figure 6A shows infiltrated edematous valves from Lewis rats immunized with a peptide from a group A streptococcal M5 serotype, amino acid sequences in residues 59–115 found in the A repeat region of the M5 protein (Kirvan, Galvin, Hilt, Kosanke, & Cunningham, 2014). Edema of the valve structure at the chordae tendinae leads to loss of valve coaptation, regurgitation, and disease of the valve. Figure 6B illustrates a verrucous-type lesion, and Figure 6C shows an example of the cellular infiltration in a valve from Lewis rat immunized with recombinant M6 protein where 50% of the rats developed rheumatic valvulitis (3/6 rats studied) (Shikhman & Cunningham, 1994). In rheumatic heart disease, verrucae are seen in tissue sections of the valve (Stollerman, 2011; Stollerman, 1997; Wannamaker, 1973; Jones, 1944; Special Writing Group of the Committee on Rheumatic Fever, Endocarditis, and Kawasaki Disease of the Council on Cardiovascular Disease in the Young of the American Heart Association, 1992; Danjani, et al., 1988). VCAM-1 was observed on activated endothelium in the Lewis rat after administration of pathogenic T cell lines that targeted the valve and led directly to upregulation of VCAM-1 at the valve surface, which allowed for penetration of the T cell lines after their passive transfer (Kirvan, Galvin, Hilt, Kosanke, & Cunningham, 2014). VCAM-1 appeared on the Lewis rat valve (Kirvan, Galvin, Hilt, Kosanke, & Cunningham, 2014) (not shown), similar to that seen in human rheumatic valves, as shown in Figure 3.

Lewis rat T cells that recognize the M5 protein T cell peptide epitope DKLKQQRDTLSTQKETLE (NT5/6 ~ M5 peptide amino acid sequence from the A repeat region of M5 protein serotype of *Streptococcus pyogenes*) were reported to target the valve in the T cell passive transfer studies in the Lewis rat (Kirvan, Galvin, Hilt, Kosanke, & Cunningham, 2014). Most importantly, in studies of human rheumatic valves by Guilherme and colleagues, cloned T cells from the valves also recognized the DKLKQQRDTLSTQKETLE peptide sequence (Guilherme, et al., 2004; Faé, et al., 2006). Other T cell lines in the Lewis rat that recognized other epitopes were not pathogenic, and did not target the valve (Kirvan, Galvin, Hilt, Kosanke, & Cunningham, 2014). Guilherme et al. have shown the importance of the cytokines, such as gamma IFN production, in the valve, which would be associated with

the CD4+ cellular infiltrate (Guilherme, et al., 2004). Heart-infiltrating T cell clones from humans recognized both streptococcal and heart proteins in studies of human T cell clones isolated from rheumatic valves (Guilherme, et al., 2000; Guilherme, et al., 1995; Guilherme & Kalil, 2004).

The Lewis rat model suggests that the more pathogenic T cell clones releasing cytokines that affect the valve may actually lead to the activation of the VCAM-1 on the valve endothelium, which promotes the infiltration of particular clones (Kirvan, Galvin, Hilt, Kosanke, & Cunningham, 2014). Antibodies or immune complexes that bind the valve surface affect the surface endothelium, and lead to activation of the endothelium/endocardium on the valve and upregulation of VCAM-1. Antibodies against the valve, including those human mAbs derived from human rheumatic carditis, react with streptococcal group A carbohydrate and glycosylated proteins or cross-reactive sequences in alpha helical matrix proteins in the laminar basement membrane, such as laminin (Galvin, Hemric, Ward, & Cunningham, 2000). Once the valve endothelium is activated and collagen is exposed, the valve may continually be damaged by antibodies against laminin, as well as antibodies against collagen and other immunogenic valvular proteins (Martins, et al., 2008) (Figure 7). Anti-collagen antibodies can bind to M proteins or other collagen-binding proteins on the group A streptococcus and induce an immune response against the valve (Cunningham, 2014; Tandon, Sharma, Chandrasekhar, Kotb, Yacoub, & Narula, 2013). Initial damage of the valve chordae tendinae lead to annular dilation and chordal elongation, which prevents adequate surface coaptation of the valve leaflets (Veasy & Tani, 2005). Elevated troponin levels are not seen in rheumatic carditis, as the main damage is at and within the valve rather than in the myocardium.

Although cardiac myosin is not present in the valve, it serves as a biomarker of streptococcal cross-reactivity against the heart and is cross-reactive with other alpha-helical proteins in the valve, such as laminin and vimentin (Galvin, Hemric, Ward, & Cunningham, 2000; Goldstein, Halpern, & Robert, 1967; Galvin, Hemric, Kosanke, Factor, Quinn, & Cunningham, 2002; Guilizia, Cunningham, & McManus, 1992). Amino acid sequence homology between cardiac myosin and alpha-helical coiled coil proteins in the valve may be partially responsible for cross-reactivity with the valve (Gulizia, Cunningham, & McManus, 1991). Mimicry between the streptococcus and heart may result in initial damage to the valve, while more chronic disease could be related to the release of collagen I and other valvular proteins from damaged valves (Martins, et al., 2008). Cross-reactive anti-cardiac myosin antibodies may lead to initial valve inflammation at the endothelium, leading to edema, cellular infiltration, and fibrinous vegetations in the rough zone of the anterior leaflet. Scarring of the leaflets appears after chordal elongation, which is the initial cause of mitral regurgitation.

Repetitive streptococcal infections in children may lead to increased scar formation in the valve, and are responsible for recurrent rheumatic heart disease. Once the valve is infiltrated by T cells, scarring occurs, where neovascularization leaves the valve susceptible to subsequent attack. Elevated antibodies against the group A carbohydrate have long been associated with a poor prognosis and poor recovery from of rheumatic carditis until the diseased valve is replaced (Dudding & Ayoub, 1968).

In rheumatic carditis, disease activity is associated with immune responses against specific peptide epitopes of the human cardiac myosin heavy chain (Ellis, et al., 2010). Disease progression and monitoring effective treatment can potentially be followed by responses against human cardiac myosin peptides in the S2 hinge region of human cardiac myosin (Gorton, et al., 2011). The reactivity against cardiac myosin epitopes in rheumatic carditis were similar in children from different global regions, such as the US, India, and Hawaii. The underlying basis for the recognition of similar cardiac myosin epitopes in rheumatic carditis suggests that antigen-specific B cells are allowed to proliferate in susceptible individuals and pathogenic B cells are not eliminated by clonal deletion, receptor editing, or anergy. T cell clones were found in human rheumatic carditis that demonstrated strong avidity to cardiac myosin. Cardiac-myosin–specific T cells would normally be deleted or rendered anergic by normal tolerance mechanisms, but may be stimulated by streptococcal M proteins during infections. The T cell clones with the highest avidity for cardiac myosin, as compared to other host proteins, were isolated from rheumatic carditis (Ellis, Li, Hildebrand, Fischetti, & Cunningham, 2005) and were strongly

stimulated by peptides of streptococcal M protein and cardiac myosin. T cells isolated from human rheumatic valves were similar to those found in peripheral blood, and also proliferated to peptides of streptococcal M protein and cardiac myosin (Faé, et al., 2006).

Guilherme has proposed several other antigens in the valve that are of importance, such as vimentin, which are recognized by T cells cloned from valves (Guilherme, Köhler, Postol, & Kalil, 2011). Vimentin in human valve tissues was previously reported to be recognized by the mouse cross-reactive anti-streptococcal mAbs (Gulizia, Cunningham, & McManus, 1991), and the mouse mAbs were also reactive with DNA, similar to antibodies in systemic lupus erythematosus (Cunningham & Swerlick, 1986). Studies of anti-idiotypic antibodies developed against the anti-cardiac myosin idiotype My1 in rheumatic fever demonstrated the presence of the rheumatic fever idiotype My1 in rheumatic fever and acute glomerulonephritis, and also in systemic lupus erythematosus and Sjogrens syndrome (McCormack, Crossley, Ayoub, Harley, & Cunningham, 1993).

Human antibody responses against human cardiac myosin have identified peptide epitopes in acute rheumatic carditis (Ellis, et al., 2010). Immune responses to cardiac myosin were similar among a small sample of worldwide populations, in which immunoglobulin G targeted the S2 subfragment hinge region within S2 peptides that contained human cardiac myosin heavy chain amino acid residues 842–992 and 1164–1272. The human cardiac myosin S2 fragment epitopes were also found to be similar among populations with rheumatic carditis worldwide, regardless of the infecting group A streptococcal M protein serotype. Homologous epitopes shared among different rheumatogenic streptococcal M protein serotypes could prime the immune system against the heart during repeated streptococcal infections, and eventually lead to breaking tolerance, epitope spreading, and initiating rheumatic heart disease in susceptible individuals.

Susceptibility to ARF and RHD may result from variations in host genes, which may be potential risk factors for disease. Thus, identifying genetic associations may be important in understanding the immune responses which can be misdirected toward heart, joints, or the brain, and that lead to carditis, arthritis, or Sydenham's chorea. In some studies of ARF, familial associations were found to be inherited with limited penetrance and concordance among dizygotic twins, which suggests an inherited susceptibility, but not classical Mendelian genetics (Bryant, Robins-Browne, Carapetis, & Curtis, 2009). Genetic associations and polymorphisms have been found in several immune related genes, as described herein. It is likely that ARF is immunologically related to systemic lupus erythematosus and Sjogren's syndrome, since the My1 idiotype, identified in rheumatic fever, was present only on antibodies in these two diseases, but not in others, such as rheumatoid arthritis or IgA nephropathy (McCormack, Crossley, Ayoub, Harley, & Cunningham, 1993).

In ARF and RHD, susceptibility to disease may be linked to HLA predisposition, as is the case for many autoimmune-related diseases. In rheumatic heart disease, the DR-7 haplotype has been associated with mitral valve disease in a large Latvian population (>1200 children), along with several other haplotypes that conferred risk or protection (Stanevicha, et al., 2003), and HLA haplotype DR-7 has been linked to rheumatic heart disease in the Brazilian population (Guilherme, Weidebach, Kiss, Snitcowsky, & Kalil, 1991; Guilherme, Kalil, & Cunningham, 2006) as well as in the Egyptian and Turkish populations (Guilherme, Köhler, Postol, & Kalil, 2011). Other genes such as polymorphisms in cytokine genes, which have been associated with rheumatic fever and rheumatic heart disease, and a more detailed discussion of HLA haplotypes has been performed by Guilherme et al. (Guilherme, Köhler, Postol, & Kalil, 2011). Genes that were associated with rheumatic fever included a TLR 2 polymorphism. Berdeli et al. reported that the common TLR-2 Arg to Gln polymorphism at position 753 was significantly ($p<0.0001$) associated with acute rheumatic fever in Caucasian Turkish population compared to controls. It is interesting that our recent study in humans reported the tendency of human cardiac myosin or its fragments to bind to human TLR 2, which stimulated monocytes to produce proinflammatory cytokines (Zhang, Cox, Alvarez, & Cunningham, 2009). Human cardiac myosin epitopes may link innate and adaptive immunity, which leads to chronic inflammation in the heart (Zhang, Cox, Alvarez, & Cunningham, 2009).

Additional genes linked to rheumatic fever include the mannose-binding lectin gene O allele, which was associated with aortic regurgitation in rheumatic heart disease. The mannose binding lectin allele encodes for a lower production of the protein and is associated with increased risk of rheumatic fever and rheumatic heart disease (Guilherme, Köhler, Postol, & Kalil, 2011; Ramasawmy, et al., 2008). Mannose-binding lectin is important in clearance of bacteria and is associated with the complement system (Guilherme, Köhler, Postol, & Kalil, 2011). The FcRγIIA receptor gene may express a polymorphism, which causes differences in binding of IgG2 in humans. Turkish children with a high risk of developing rheumatic fever possessed the *131R/R* genotype of FcRγIIA, and the *131H/R* allele of the FcRγIIA was associated with an intermediate risk (Berdeli, Celik, Ozyürek, & Aydin, 2004). Failure to clear immune complexes from the blood may potentially lead to the continued development of high-affinity antibodies (Zhang, et al., 2013) that would attack the heart, brain, or other tissue sites in rheumatic fever. HLA B5 has been associated with immune complexes in acute rheumatic fever (Yoshinoya & Pope, 1980). These genetic associations are important to note as they are involved in inflammatory and immune responses against the streptococcus, and potentially predispose those who possess them to rheumatic heart disease.

Although host susceptibility may be a result of host genetic predisposition, the environmental influence exerted by *S. pyogenes* on host-streptococcal interactions is important in the development of rheumatic fever and RHD. Although the role of specific streptococcal strain variations in acute rheumatic fever may not be well characterized in epidemics of rheumatic fever, there have been reports of a relationship between rheumatic-fever–associated strains that were isolated from the great acute rheumatic fever epidemics of the World War II era where the *S. pyogenes* were rich in M protein, heavily encapsulated by hyaluronic acid, and highly virulent in mice (Stollerman, 2001). These highly mucoid strains primarily infected the throat rather than the skin and were also seen with the rheumatic fever outbreak in Utah in the United States (Veasy, et al., 2004). In the past, certain M protein serotypes were associated with rheumatic fever outbreaks, such as the well-known M5 protein serotype (Bisno, 1995; Bisno, Pearce, Wall, Moody, & Stollerman, 1970). *S. pyogenes* strains isolated in more tropical climates do not have the characteristics of these earlier strains isolated in North America. The epidemiology suggests a greater diversity of *S. pyogenes* strains, and skin-associated strains may dominate in ARF in the more tropical regions (Bryant, Robins-Browne, Carapetis, & Curtis, 2009). Both skin and throat strains are evident in rheumatic fever in the tropics, and there is increasing evidence of skin-associated strains linked to cases of rheumatic fever (Bryant, Robins-Browne, Carapetis, & Curtis, 2009).

The host-streptococcal interaction may have evolved from years of penicillin therapy and prophylaxis, which might lead to rheumatic fever outbreaks which do not have the same characteristic "rheumatogenic" strains that have been reported in previous outbreaks in the United States over the past 50 years (Bisno, 1995). Children in the great rheumatic fever epidemics who did not survive also did not transfer the most severe genetic predisposition to rheumatic fever to another generation. Changes in both the streptococcus and host over the past 100 years may have attenuated the host and the streptococcus such that we may not see severe ARF outbreaks, similar to those reported in the preantibiotic era.

Sydenham Chorea and Pediatric Autoimmune Neuropsychiatric Disorder Associated with Streptococci (PANDAS): Anti-Streptococcal Humoral Immunity against the Brain

Sydenham chorea is well established as the primary neurologic manifestation of acute rheumatic fever (Taranta & Stollerman, 1956). The symptoms of Sydenham chorea include involuntary movements and neuropsychiatric behaviors that predate the involuntary movements. Initially in Sydenham chorea, IgG was observed to be associated with neurons in caudate and putamen regions of the basal ganglia (Husby, van de Rijn, Zabriskie, Abdin, & Williams, 1976). In later studies, human mAbs were derived from Sydenham chorea (Kirvan, Swedo, Heuser, & Cunningham, 2003) and were shown to cross-react with the group A streptococcal carbohydrate

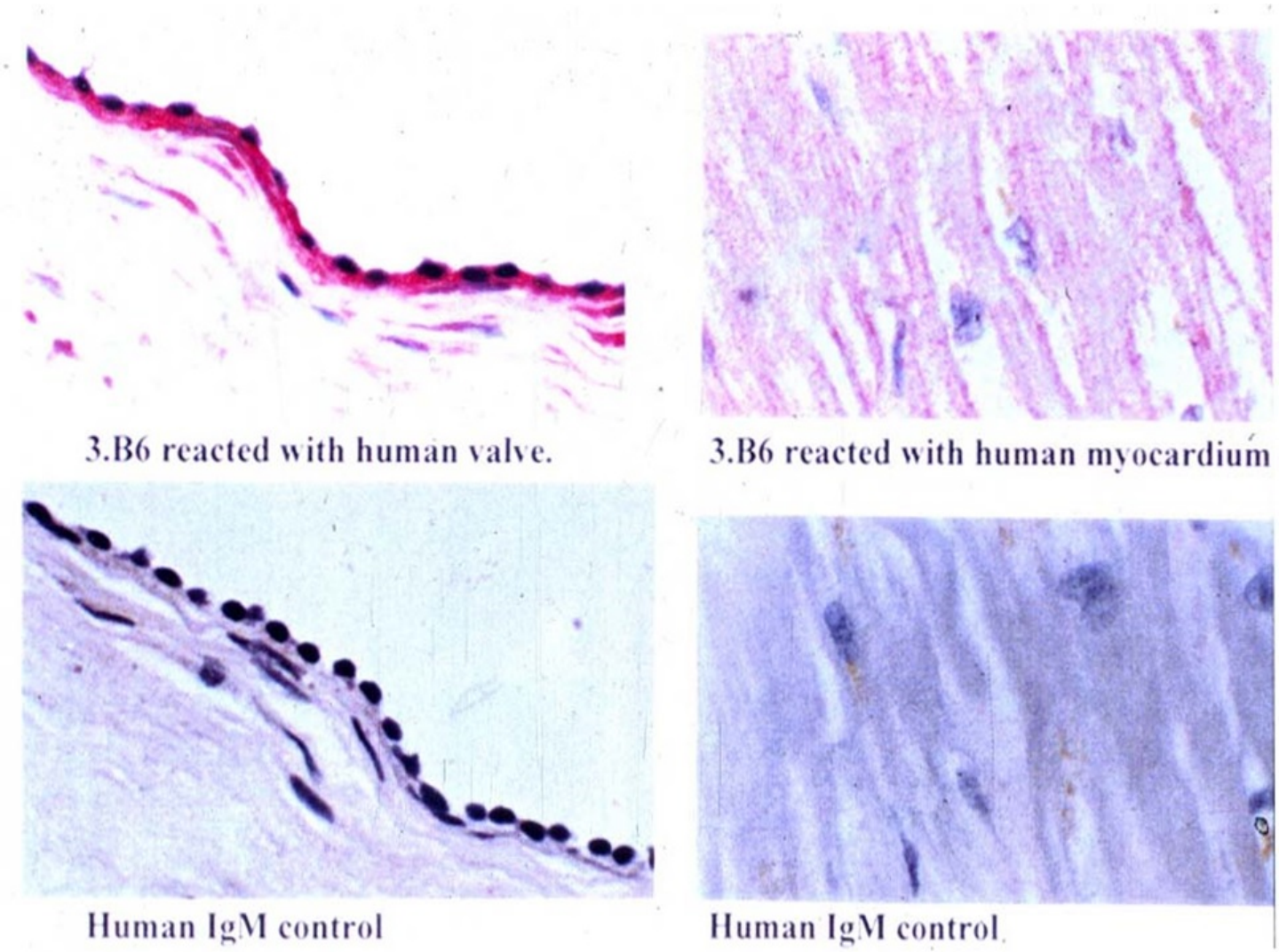

Figure 1. Reactivity of rheumatic carditis-derived human IgM monoclonal antibody (mAb) 3.B6 with a normal human valve. Formalin-fixed human mitral valves were treated with human mAb 3.B6 at 10 μg/mL. mAb 3.B6 binding was detected using secondary biotin-conjugated anti-human IgG antibody and alkaline phosphatase-labeled streptavidin, followed by phosphatase substrate Fast Red. (**b**) Control valve sections were negative after reaction with human IgM and secondary antibody conjugate at 10 μg/mL. The figure is from Galvin et al. (Galvin J. E., Hemric, Ward, & Cunningham, 2000) and is used with permission.

epitope N-acetyl-beta-D-glucosamine (GlcNAc) and brain antigens lysoganglioside (Kirvan, Swedo, Heuser, & Cunningham, 2003) and tubulin (Kirvan, Cox, Swedo, & Cunningham, 2007), which are both enriched in the brain. Evidence for the autoantibody cross-reactivity between streptococci and brain is shown by the reaction of the chorea-derived human mAbs with group A streptococcal wall-membranes, as well as the group A carbohydrate epitope GlcNAc and the brain (Kirvan, Swedo, Heuser, & Cunningham, 2003; Kirvan, Cox, Swedo, & Cunningham, 2007; Kirvan, Swedo, Kurahara, & Cunningham, 2006a). IgG antibodies in sera or cerebrospinal fluid or the chorea-derived mAbs from Sydenham chorea reacted with human neuronal cells (SKNSH cell line) at the cell membrane and also activated the calcium calmodulin-dependent protein kinase II (CaMKII) (Kirvan, Swedo, Heuser, & Cunningham, 2003) in human neuronal cells. The activation of CaMKII led to increased dopamine release from the human neuronal cell line, as shown in experiments using tritiated dopamine (Kirvan, Swedo, Kurahara, & Cunningham, 2006a). In addition, intrathecal transfer of the chorea-derived mAb 24.3.1 into brains of Lewis rats induced elevated tyrosine hydroxylase activity in dopaminergic neurons (Kirvan, Swedo, Heuser, & Cunningham, 2003; Kirvan, Swedo, Kurahara, & Cunningham, 2006a). CaMKII activation and signaling activity by Sydenham chorea sera was abrogated by removal of IgG from the sera (Kirvan, Swedo,

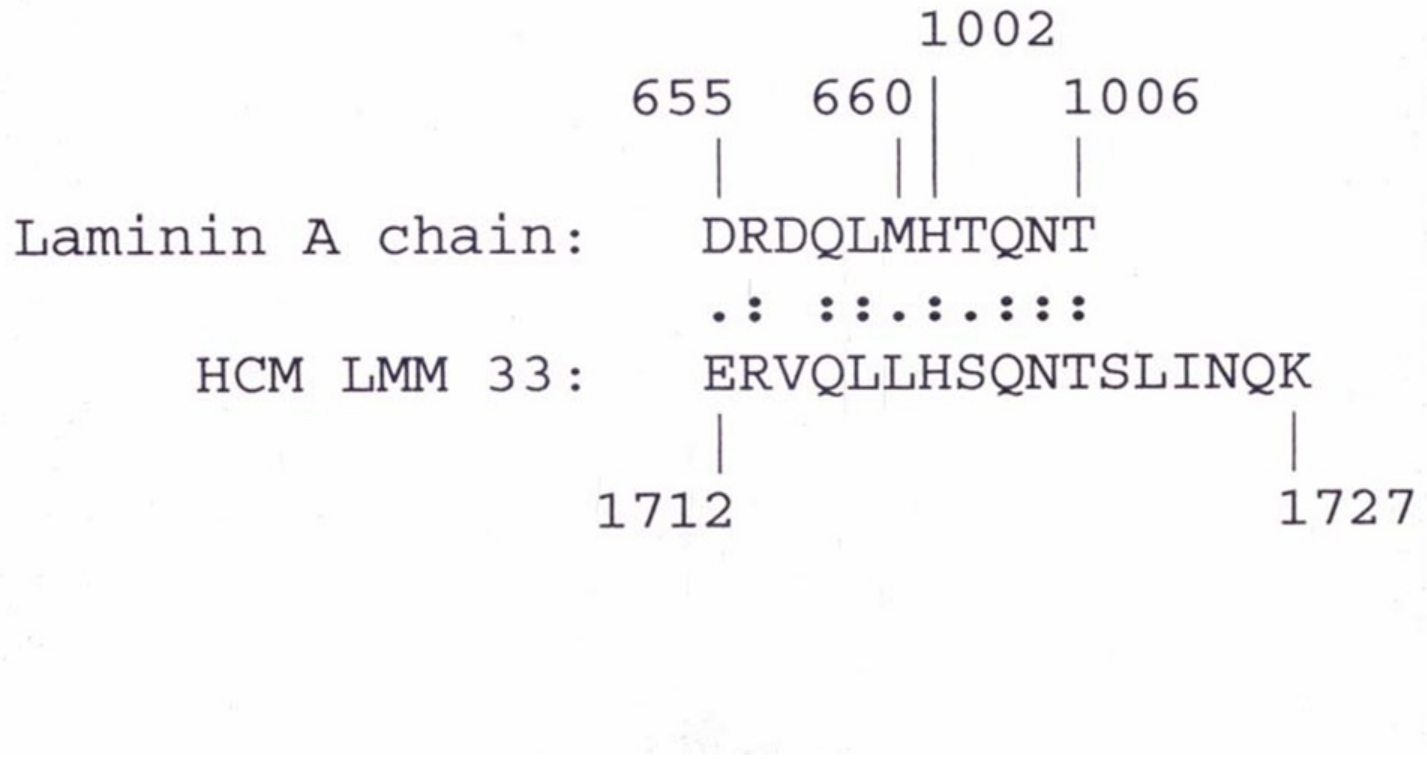

Figure 2. Amino acid sequence alignment of laminin A chain and human cardiac myosin LMM peptide demonstrating sequence homology with epitopes that are recognized by human mAb 3B6 derived from rheumatic heart disease. Amino acid sequence designations of two homologous regions in the laminin A chain and human cardiac myosin (HCM). The sequences shown are 64% identical and 91% homologous. (:), Identity; (.), conserved amino acid substitution. The figure is from Galvin et al. (Galvin J. E., Hemric, Ward, & Cunningham, 2000) and is used with permission.

Heuser, & Cunningham, 2003; Brimberg, et al., 2012), and plasmaphoresis led to improvement of symptoms (Perlmutter, et al., 1999; Garvey, Snider, Leitman, Werden, & Swedo, 2005). Antibody-mediated neuronal cell CaMKII activation and signaling by IgG antibodies in serum or cerebrospinal fluid from Sydenham chorea was also associated with symptoms (Kirvan, Swedo, Heuser, & Cunningham, 2003; Ben-Pazi, Stoner, & Cunningham, 2013). Antibody-mediated neuronal cell signaling in Sydenham chorea is a novel pathogenic mechanism, which may lead to symptoms of involuntary movements with associated neuropsychiatric behaviors in acute rheumatic fever (Kirvan, Swedo, Heuser, & Cunningham, 2003). Sydenham chorea may serve as a model for other movement and neuropsychiatric disorders, such as pediatric autoimmune neuropsychiatric disorder associated with streptococcal infections, or PANDAS (Swedo, et al., 1998).

To investigate the in vivo targets of the human mAbs derived from Sydenham chorea, the antibody V genes of Sydenham chorea mAb 24.3.1 (Cox, et al., 2013) were expressed in transgenic (Tg) mice. The Tg mice demonstrated chorea antibody V gene expression in serum, and upon breaking the blood brain barrier, the human-mouse chimeric IgG antibody targeted dopaminergic tyrosine hydroxylase positive neurons in the basal ganglia (Cox, et al., 2013) as shown in merged Figure 8. mAb 24.3.1 derived from Sydenham chorea was shown to react with and signal the human dopamine D2 receptor (Cox, et al., 2013). Evidence suggested that chorea-derived human mAb reacted with the dopamine receptor D2. mAb 24.3.1 reacted with a flag-tagged dopamine D2 receptor and functionally signaled the human D2 receptor that was expressed in transfected cell lines. The

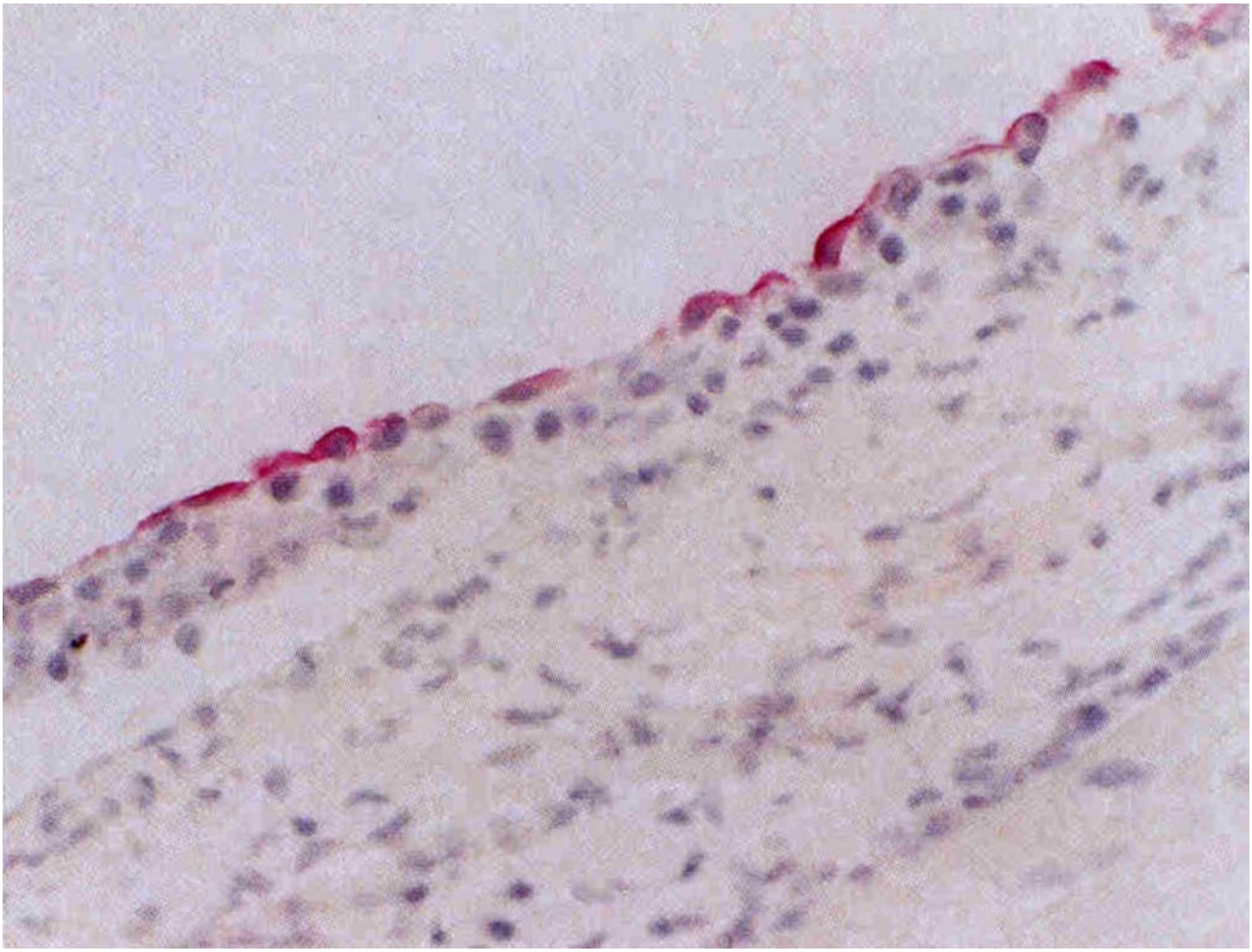

Figure 3. Vascular cell adhesion molecule-1(VCAM-1) expressed on rheumatic valve. Tissue stained with anti-human IgG conjugated to alkaline phosphatase and developed using Fast Red. The figure is from Roberts et al. (Roberts, et al., 2001) and is used with permission.

data show that the human mAb and human Sydenham chorea sera IgG targeted the dopamine D2 receptor (Cox, et al., 2013). Symptoms measured by the USCRS Sydenham chorea rating scale were correlated to the ratio of anti-D1R/D2R antibodies (Ben-Pazi, Stoner, & Cunningham, 2013). Cox et al. further described that anti-D1 receptor and anti-D2 receptor antibodies (IgG) were significantly elevated in serum from Sydenham chorea, as well as from PANDAS (Cox, et al., 2013).

For years, little attention was given to neuropsychiatric obsessive-compulsive symptoms, which can predate chorea as the primary neurologic manifestation of acute rheumatic fever (Ben-Pazi, Stoner, & Cunningham, 2013). Small choreiform piano-playing movements of the fingers and toes were reported in the first 50 cases of PANDAS reported by Swedo et al. (Swedo, et al., 1998). PANDAS, which has similarities to Sydenham chorea (including a similar immunologic profile of anti-neuronal antibodies), is characterized by the abrupt onset of tics and obsessive-compulsive disorder (OCD). The fine choreiform movements in PANDAS are not as obvious as the choreoathetoid involuntary movements seen in Sydenham chorea (Garvey, Snider, Leitman, Werden, & Swedo, 2005; Garvey & Swedo, 1997). The fine choreiform movements may go unnoticed in PANDAS, and cause a child to have poor handwriting skills (which are generally associated with learning and behavioral regression), enuresis, separation anxiety and night-time fears; anorexia may appear in approximately 17 percent of cases

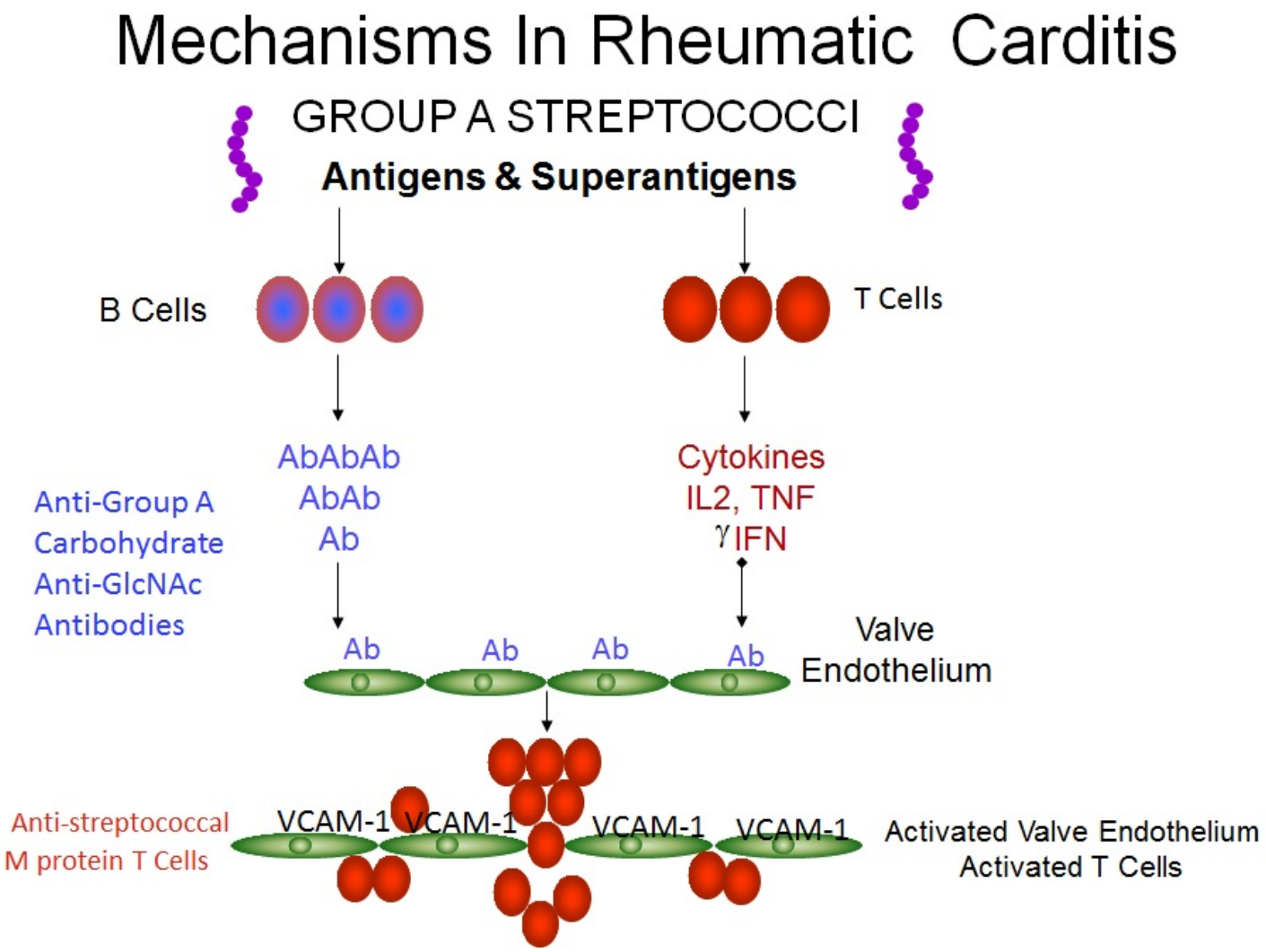

Figure 4. Diagram illustrating the B cell and T cell responses against the group A streptococcal antigens and superantigens and the proposed pathogenesis in rheumatic carditis. Antibodies against the dominant group A carbohydrate epitope N-acetyl-beta-D-glucosamine (GlcNAc) are shown to target the valve endothelium, as shown. The valve endothelium becomes inflamed and activated and expresses vascular cell adhesion molecule 1 (VCAM-1), as shown in Figure 3, and activated T cells cross-reactive to group A streptococcal M protein and human cardiac myosin epitopes extravasate through the endothelium into the valve. The figure is from (Cunningham M. W., 2012) and is used with permission.

(Swedo, et al., 1998). PANDAS symptoms and pathogenesis, which is also covered in great detail in a separate chapter in this book, may be observed in other types of infections and are not always associated with group A streptococcal infections. In the presence of other infections or in the absence of streptococcal infection, the disease is referred to as pediatric acute onset neuropsychiatric syndrome, or PANS (Swedo, Leckman, & Rose, 2012). The clinical evaluation consensus of PANDAS and PANS has recently been published (Chang, et al., 2015). More chronic types of tics and OCD may or may not be associated with streptococcal infections, and may be associated with *Mycoplasma* infections, influenza, or Lyme disease, but few studies have methodically considered the infections that may exacerbate PANS. More chronic tics and OCD do not appear to display the small choreiform piano-playing movements of the fingers and toes, and are not similar to Sydenham chorea in their anti-neuronal antibody patterns of antibodies against the dopamine D2 receptor. Chronic forms of tics and OCD do not appear to have the IgG antibodies against the D2 receptor (Cox, et al., 2015; Singer, et al., 2015). The PANDAS cases that have the small choreiform piano-playing movements of the fingers and toes appear to share antibodies against both D1 and D2 receptors, and may also have elevated antibodies against tubulin and

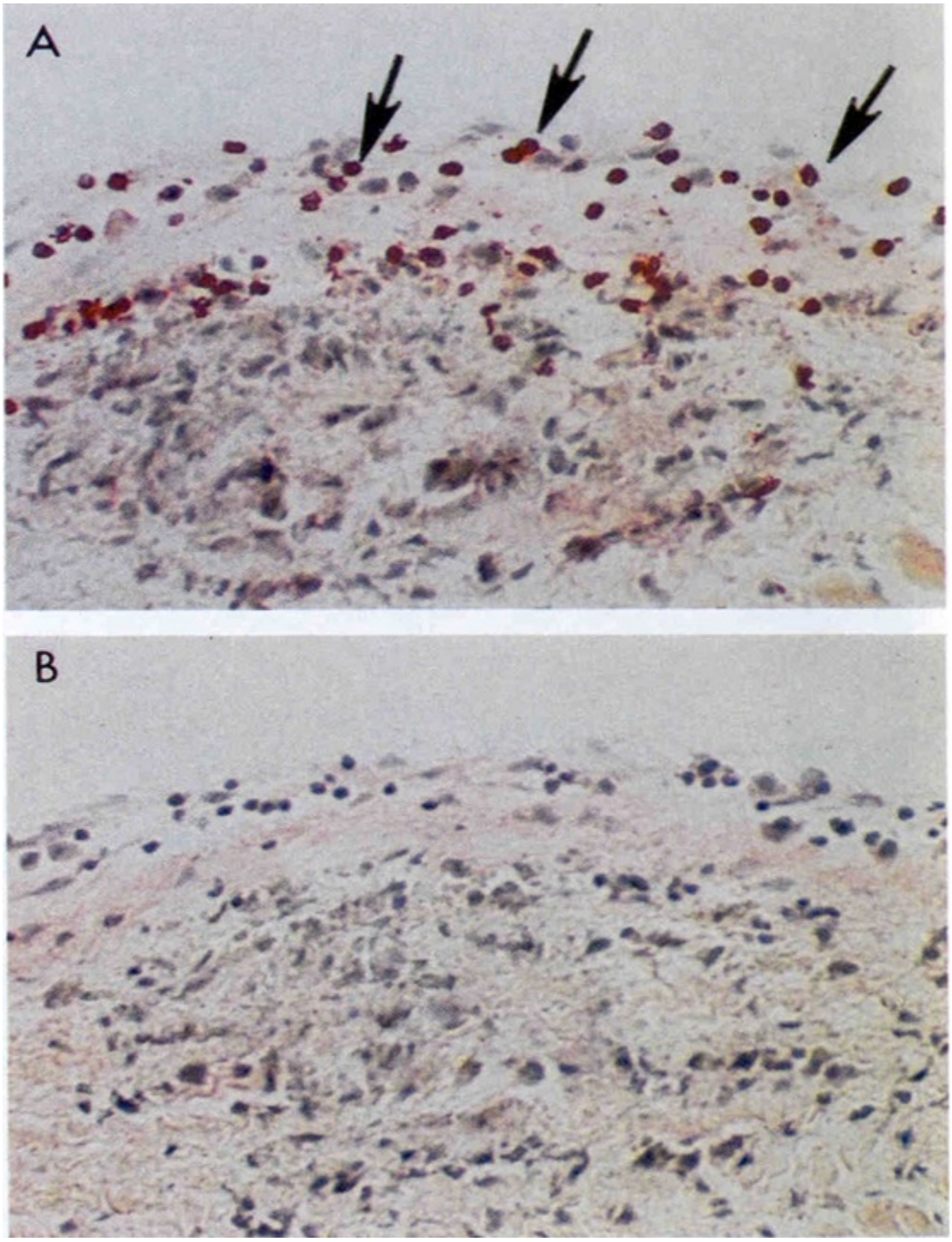

Figure 5. Extravasation of CD4+ lymphocytes (arrows) into valve above Aschoff's body in the subendocardium of the left atrial appendage. A) Tissue stained with anti-CD4+ monoclonal antibody reagent and developed with the alkaline phosphatase and Fast Red to detect the binding of the antibody reagent to the CD4+ T cells entering the valve. B) Control valve tissue shown did not react with the secondary antibody conjugate. Figure is from Quinn et al. (Quinn, Kosanke, Fischetti, Factor, & Cunningham, 2001) with permission.

lysoganglioside (Kirvan, Cox, Swedo, & Cunningham, 2007; Cox, et al., 2013; Brimberg, et al., 2012; Ben-Pazi, Stoner, & Cunningham, 2013). Studies suggest that more chronic forms of tics and OCD appeared to have anti-neuronal antibodies that were significantly elevated against only the dopamine D1 receptor and/or antibodies against lysoganglioside (Cox, et al., 2015; Singer, et al., 2015). Most cases of PANDAS and/or PANS notably

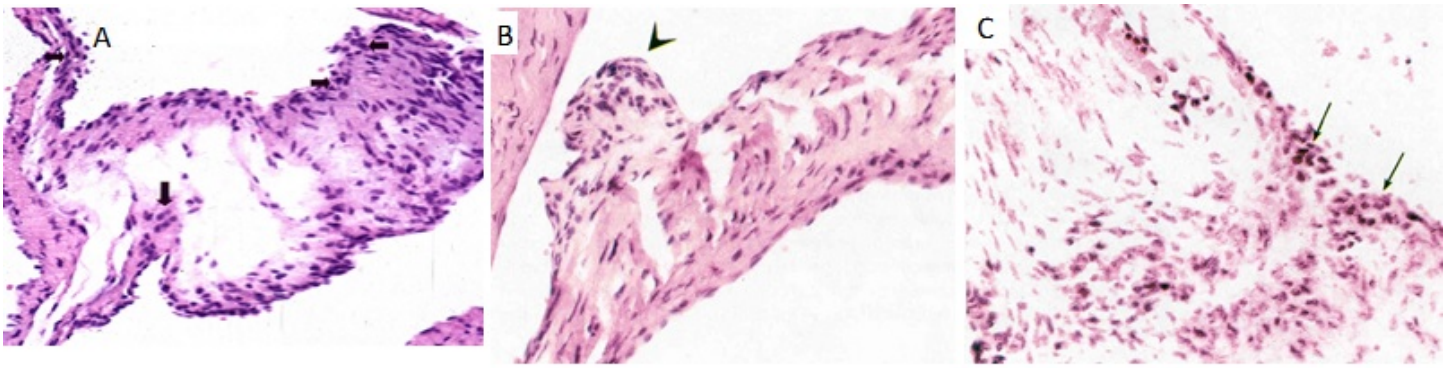

Figure 6. A) Induction of valvulitis, edema and cellular infiltration (arrows) in hematoxylin and-eosin-stained heart valves from Lewis rats immunized with group A streptococcal M5 peptides as a group including peptides NT1-NT4/5 [AVTRGTINDPQRAKEALD amino acid (aa) residues 1–18; NT-2 KEALDKYELENHDLKTKN aa residues 14–31; NT-3 LKTKNEGLKTENEGLKTE aa residues 27–44;NT-4 GLKTENEGLKTENEGLKTE aa residues 40–58; NT-4/5 GLKTEKKEHEAENDKLK aa residues 54–70. Figure is from Kirvan et al. (Kirvan, Galvin, Hilt, Kosanke, & Cunningham, 2014) with permission. B) Verrucous nodule (arrow) observed on valve and C) cellular infiltration (arrows) after immunization with group A streptococcal rM6 protein. Tissues stained with hematoxylin and eosin. Figures B and C are from Quinn et al. (Quinn, Kosanke, Fischetti, Factor, & Cunningham, 2001) with permission.

appeared to have elevated serum CamKII activation and signaling of a human neuronal cell line (SKNSH), regardless of the presence of one or both of the anti-D2 or anti-D1 receptor antibodies (Cox, et al., 2015).

Animal models of Sydenham chorea and PANDAS have been successful in showing that immunization with group A streptococcal antigens or passive transfer of purified anti-streptococcal IgG antibodies in both a mouse model (Yaddanapudi, et al., 2010; Hoffman, Hornig, Yaddanapudi, Jabado, & Lipkin, 2004) and a Lewis rat model (Brimberg, et al., 2012; Lotan, et al., 2014) are associated with behavioral changes. To be more specific, immunization of a mouse model (Hoffman, Hornig, Yaddanapudi, Jabado, & Lipkin, 2004) with a streptococcal antigen led to antibody deposits in several brain regions, including deep cerebellar nuclei (DCN), globus pallidus, and the thalamus, and led to a display of behavioral alterations, such as increased rearing behavior and obsessive responses (Hoffman, Hornig, Yaddanapudi, Jabado, & Lipkin, 2004). Evidence suggested that immune responses against group A streptococci were associated with motoric and behavioral disturbances, and also suggested that anti-streptococcal anti-neuronal antibodies that are potentially cross-reactive with brain components may lead to movement and obsessive behaviors following streptococcal infections (Hoffman, Hornig, Yaddanapudi, Jabado, & Lipkin, 2004). Passive transfer of purified IgG anti-streptococcal antibodies from the immunized mice into naïve recipients led to behavior changes and antibody deposits in brain tissues (Yaddanapudi, et al., 2010).

Studies in the Lewis rat model (Brimberg, et al., 2012) demonstrated that exposure to group A streptococcal antigens led to the inability of the rats to hold a food pellet for a normal length of time, and immunized rats could not traverse a narrow beam as well as control rats (Brimberg, et al., 2012). Group A streptococcal immunized rats demonstrated compulsive grooming behavior, as compared to normal rats, after spray treatment with a water mist (Brimberg, et al., 2012). Deposits of IgG were found in the striatum, thalamus, and frontal cortex of group A streptococcal immunized rats. Study of the cortex and basal ganglia revealed alterations in dopamine and glutamate levels, which is consistent with the pathophysiology of Sydenham chorea and its related neuropsychiatric disorder. Anti-streptococcal sera from rats immunized with *S. pyogenes* antigen activated CaMKII in SKNSH human neuronal cells (Brimberg, et al., 2012), in a manner similar to sera from Sydenham chorea (Kirvan, Swedo, Heuser, & Cunningham, 2003; Kirvan, Swedo, Snider, & Cunningham, 2006b). Antibody removal with anti-igG beads removed the signaling activity of the sera (Brimberg, et al., 2012), which further demonstrated its association with IgG antibodies. The fact that plasmaphoresis improves the symptoms in

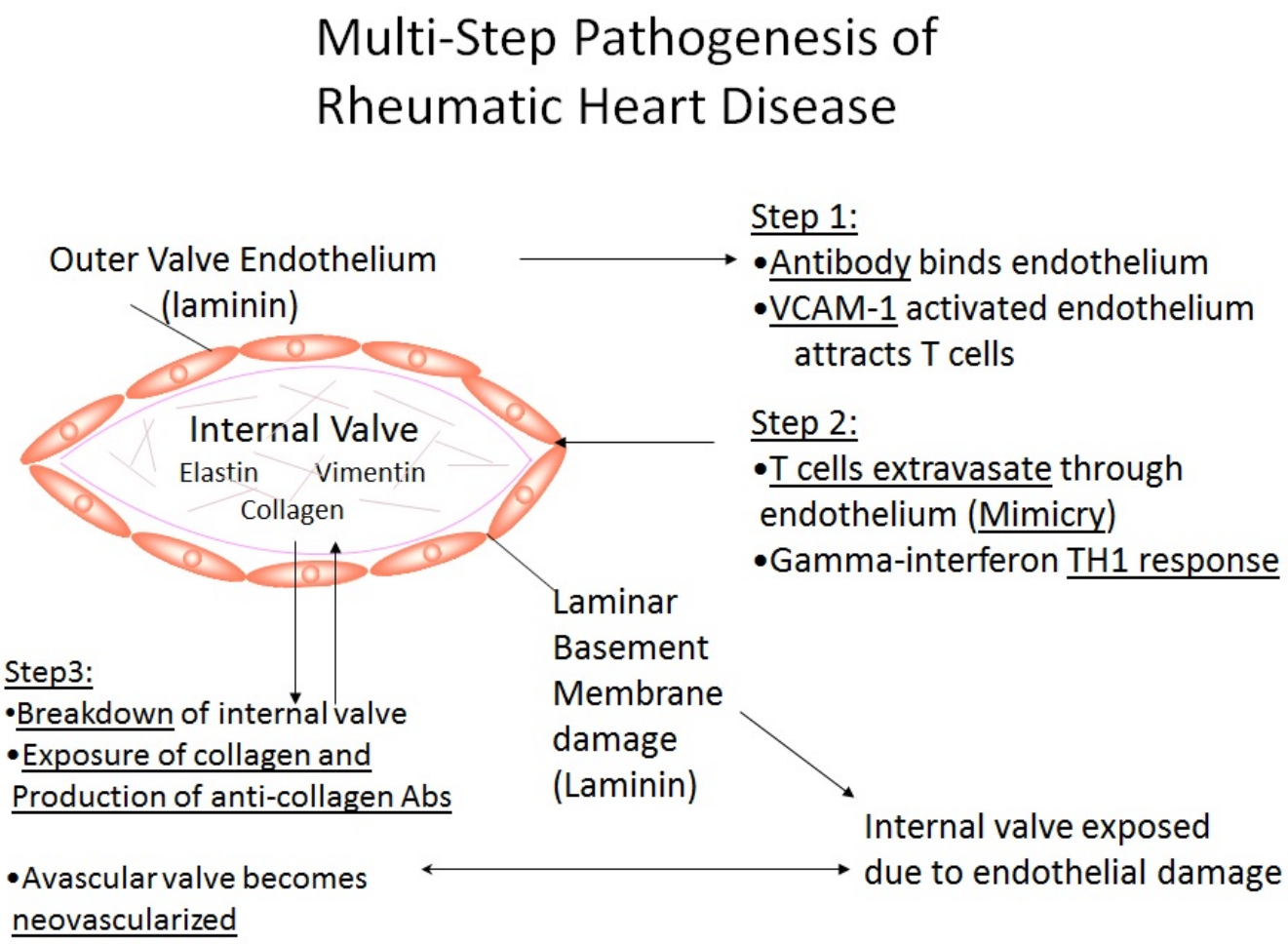

Figure 7. Multistep hypothesis of development of rheumatic carditis and heart disease. Diagram illustrates the process of initial mimicry that leads to granuloma formation, gamma interferon production and fibrosis/scarring in the valve. After the initial process of inflammation has developed in the valve, other proteins in the valve may then be recognized by the immune cells leading to epitope spreading and additional responses against other valve proteins such as vimentin and collagen. The figure is from (Cunningham M. W., 2012; Guilherme, Kalil, & Cunningham, 2006; Cunningham M. W., 2006) and is used with permission.

Sydenham chorea and in PANDAS is consistent with the hypothesis that antibodies are mediating the symptoms of disease (Perlmutter, et al., 1999; Perlmutter, et al., 1998).

To summarize, the antineuronal antibodies found in Sydenham chorea, PANDAS and PANS, anti-lysoganglioside (Kirvan, Swedo, Snider, & Cunningham, 2006b), anti-tubulin (Kirvan, Cox, Swedo, & Cunningham, 2007), anti-dopamine D2 receptor (Cox, et al., 2013; Brimberg, et al., 2012; Ben-Pazi, Stoner, & Cunningham, 2013), and anti-dopamine D1 receptor (Ben-Pazi, Stoner, & Cunningham, 2013) antibodies have all been reported and studied. The anti-dopamine D2 receptor/anti-dopamine D1 receptor antibody ratio was correlated with the UFMG-Sydenham-Chorea-Rating-Scale (USCRS) of neuropsychiatric symptoms (Ben-Pazi, Stoner, & Cunningham, 2013). Most importantly, the functional signaling activity of the anti-neuronal antibodies was observed when human chorea derived mAbs or sera from Sydenham chorea or PANDAS was reacted with dopamine D2 receptor-expressing transfected cell lines (Cox, et al., 2013). Further evidence that Sydenham chorea is antibody-mediated includes the study of human Sydenham chorea-derived mAbs, which activated CaMKII signaling in human neuronal cells (Cox, et al., 2013). The signaling activity in the human neuronal cell line also led to the overproduction of dopamine (Kirvan, Swedo, Kurahara, & Cunningham, 2006a), which would affect central dopamine pathways.

A model diagram of the potential immunological and physiological mechanism of Sydenham chorea, the major neurologic manifestation of rheumatic fever, is shown in Figure 9 (Cunningham, 2012). In theory, this mechanism may also apply to PANDAS and PANS. The effects of antineuronal antibodies on the brain may include: 1) the binding of antibody to the D1 and D2 dopamine receptors with subsequent signaling of the receptors, which are often found as heterodimers with each other or with other G protein coupled receptors in the membrane; 2) the potential effects of binding to lysoganglioside in the membranes of neurons; and 3) the release of excess dopamine, which can affect receptor density and potentially sensitivity, as well as the possible effects of the excess dopamine and CaMKII signaling on other receptors expressed in the membranes of the neurons and their projections. Excess dopamine released from the SKNSH cell line was observed when treated with a human mAb 24.3.1 or acute Sydenham chorea sera (Kirvan, Swedo, Kurahara, & Cunningham, 2006a). As described above, evidence in animal models and humans strongly suggests that antibodies mediate inflammatory consequences in Sydenham chorea, PANDAS, and PANS (Perlmutter, et al., 1999). It is highly possible that other brain antigens are targeted by autoantibodies in PANDAS/PANS or other related autoimmune neuropsychiatric and mental disorders that may affect memory, learning, and behavior (Yaddanapudi, et al., 2010; Hoffman, Hornig, Yaddanapudi, Jabado, & Lipkin, 2004; Huerta, Kowal, DeGiorgio, Volpe, & Diamond, 2006; Kowal, et al., 2004; DeGiorgio, et al., 2001).

Summary and Perspective

Molecular mimicry between the group A streptococcus, heart, and brain is supported by evidence from studies of human mAbs, human T cell clones, and serum IgG antibodies derived from streptococcal sequelae and rheumatic fever (Galvin, Hemric, Ward, & Cunningham, 2000; Kirvan, Swedo, Heuser, & Cunningham, 2003). Human mAbs derived from rheumatic carditis and Sydenham chorea have supported the hypothesis that antibodies against the group A streptococcal carbohydrate epitope GlcNAc recognize cross-reactive structures in the heart and brain, which can lead to rheumatic carditis/rheumatic heart disease and Sydenham chorea, respectively (Galvin, Hemric, Ward, & Cunningham, 2000; Kirvan, Swedo, Heuser, & Cunningham, 2003). The rheumatic valve has been linked to humoral immune responses that attack the endocardium and allow activated T cells cross-reactive with cardiac myosin and streptococcal M protein epitopes to enter through activated VCAM-1+ endothelium at the valve surface (Roberts, et al., 2001). Although a Th1 response was observed by Guilherme et al. in the valve (Guilherme, et al., 2004), Th17 cells have also been recognized to have the potential for importance in the pathogenesis of rheumatic heart disease, where the balance between Th17 and T regulatory lymphocyte subsets was altered in disease that favored the Th17 subset. (Bas, et al., 2014). As early as 1989, Bhatia et al. reported changes in the lymphocyte subsets (Bhatia, et al., 1989). Changes in the subsets may lead to a Th17 predominance in disease with excess antibody production, including the cross-reactive antibodies, as well as the formation of immune complexes. Th17 cells have been established in responses against group A streptococci (Dileepan, Linehan, Moon, Pepper, & Jenkins, 2011; Wang, et al., 2010) and extracellular pathogens. Antibody-mediated neuronal cell signaling may be an important mechanism of antibody pathogenesis in Sydenham chorea, as well as in diseases such as PANDAS and PANS.

The emerging theme in mimicry suggests that cross-reactive autoantibodies target intracellular antigens, but to be pathogenic, antibodies must also target the surface antigens of neuronal cells or valve endothelial cells by targeting extracellular matrix proteins on the heart valve, such as laminin (Galvin, Hemric, Ward, & Cunningham, 2000), or binding to receptors such as the dopamine receptors which signal neurons (Cox, et al., 2013) or by other inflammatory effects caused by immune complexes of cross-reactive antibodies (Nelson, et al., 2015; Zhang, et al., 2002).

The avidity of the cross-reactive antibodies is important in dictating antibody-mediated cell signaling in the brain (Kirvan, Swedo, Heuser, & Cunningham, 2003), or complement-mediated cytotoxicity on heart cells (Cunningham, et al., 1992; Mertens, Galvin, Adderson, & Cunningham, 2000). For example, see Figure 10, where cross-reactive mouse anti-streptococcal/anti-myosin mouse mAbs were compared for their binding

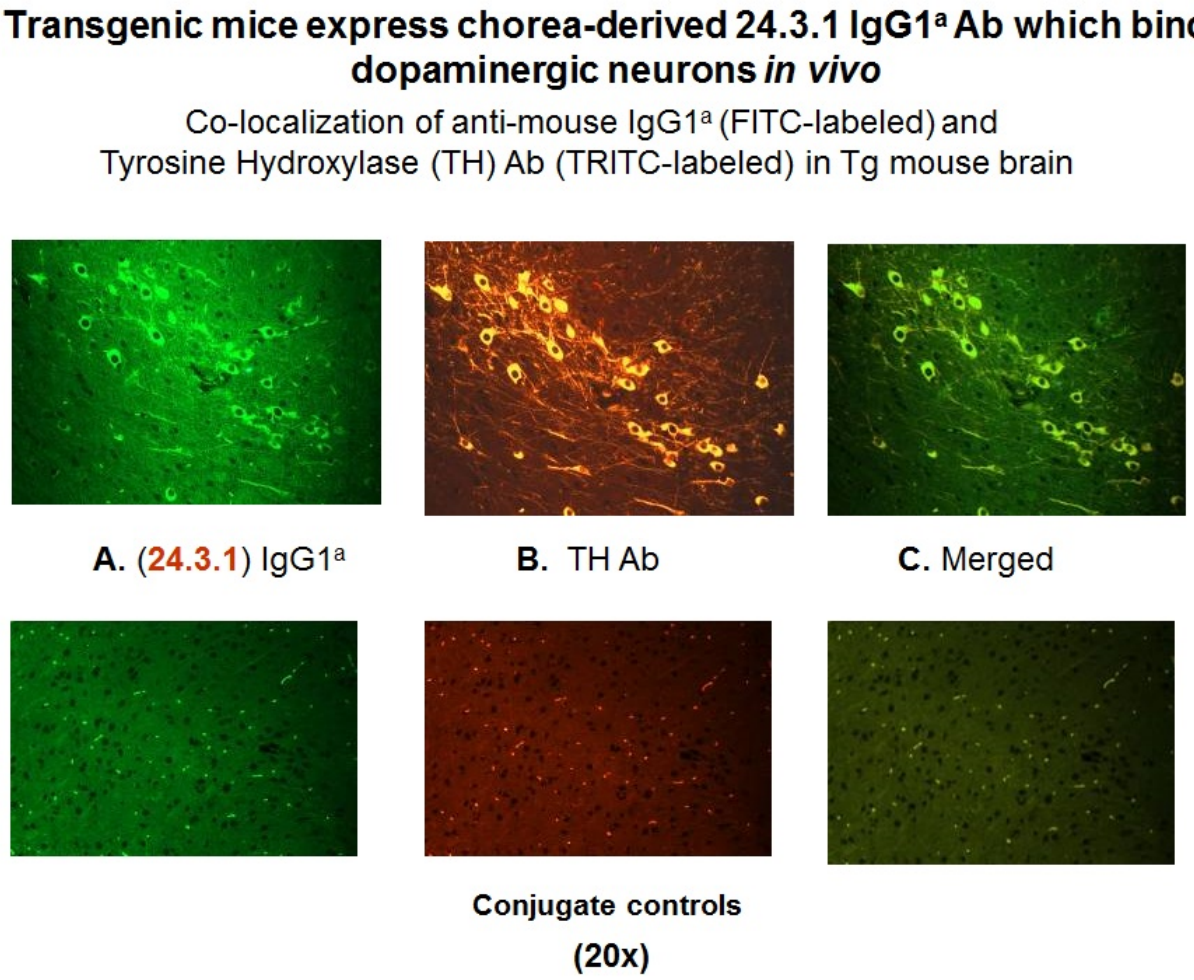

Figure 8. Human Sydenham chorea 24.3.1 V gene expressed as a human V gene-mouse IgG1a constant region in Transgenic(Tg) mice targets dopaminergic neurons. Chimeric Tg24.3.1 VH IgG1a Ab expressed in Tg mouse sera penetrated dopaminergic neurons in Tg mouse brain in vivo. Colocalization of Tg 24.3.1 IgG1a (anti-IgG1a Ab, green left panel) and tyrosine hydroxylase antibody (anti-TH Ab, yellow middle panel). TH is a marker for dopaminergic neurons. The left panel shows IgG1a (FITC labeled), the center panel shows TH Ab (TRITC labeled), and the right panel is a merged image (FITC-TRITC). Brain sections (basal ganglia) of VH24.3.1 Tg mouse (original magnification 320), showing FITC labeled anti-mouse IgG1a (A), TRITC-labeled anti-TH Ab(B), and merged image (C). Controls treated with secondary antibody are negative. The figure is from Cox et al. (Cox, et al., 2013) and is used with permission.

avidity. The two mAbs that were cytotoxic for heart cells (mAb36.2.2 and mAb54.2.8) were found to have the highest avidity for cardiac myosin as shown in Figure 10 (mAbs 36.2.2 and 54.2.8 as shown farthest to the left in Figure 10). Antibodies can be cross-reactive without rendering disease consequences in the host, if their avidity is not strong enough to dictate functional signaling, cytotoxicity of a host cell, or deposit in tissues. Most of the mAbs shown in Figure 10 are cross-reactive, but are without consequences (no cytotoxicity of heart cells) and may not be associated with disease (Mertens, Galvin, Adderson, & Cunningham, 2000), but the two most avid mAbs were cytotoxic for heart cells in culture (Cunningham, et al., 1992).

The presence of cross-reactive T cells in the host repertoire has been related to the recognition of antigen in the thymus where T cells that are too reactive with self antigens are deleted. Recent studies by Jenkins and colleagues suggest that cross-reactive T cells that recognize self and foreign epitopes with lower affinity are not deleted and remain in the T cell repertoire of the host, which predisposes the host to autoimmune disease after exposure to microbial antigens (Nelson, et al., 2015). Figure 11 shows the cross-reactivity of a T cell clone G4s from rheumatic carditis, which recognized M protein more avidly than cardiac myosin (Ellis, Li, Hildebrand,

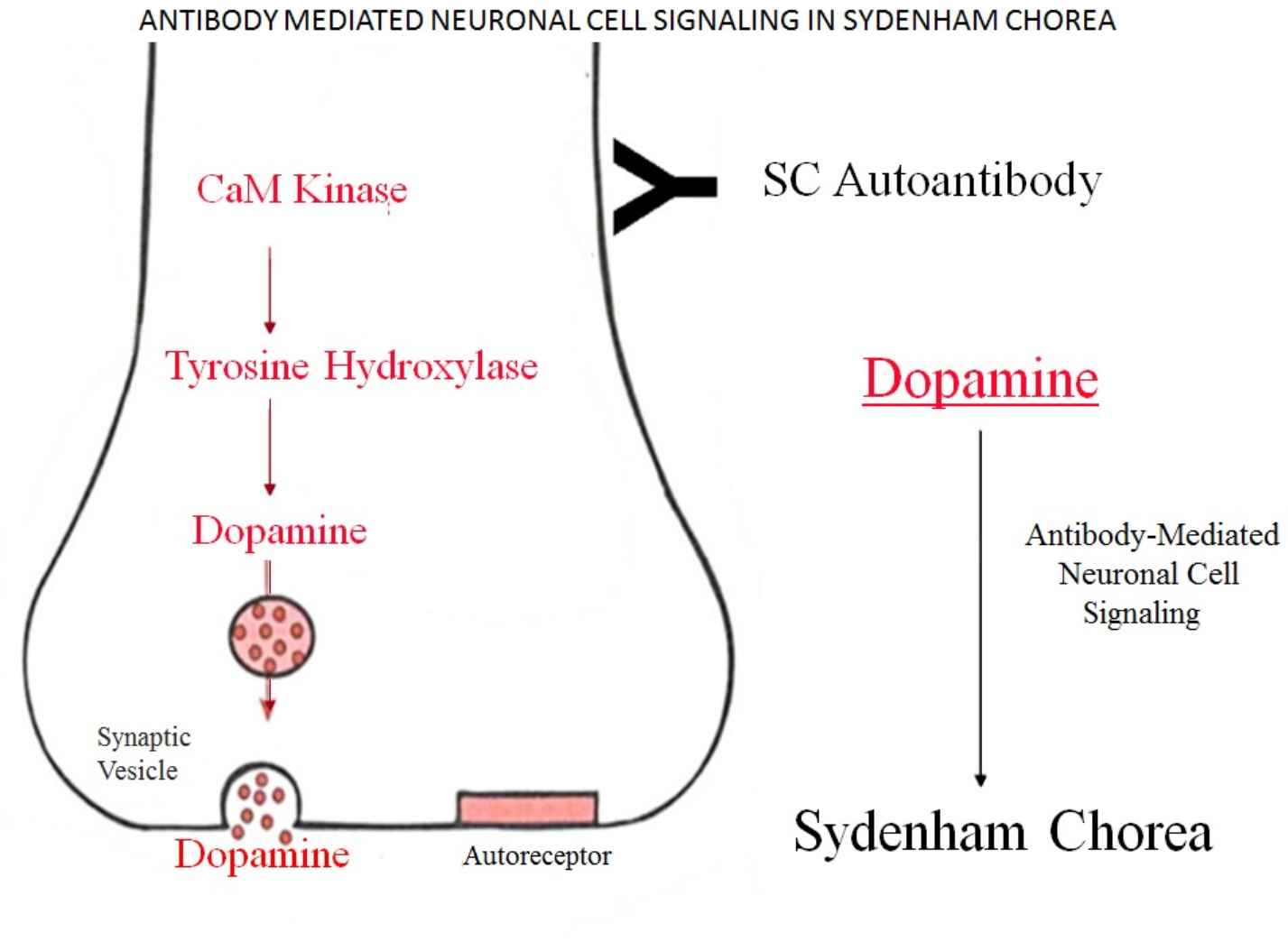

Figure 9. Simplified illustration of a potential pathogenic mechanism in Sydenham chorea. An antineuronal antibody (IgG) may bind to receptors on neuronal cells and trigger the signaling cascade of CaMKII, tyrosine hydroxylase and dopamine release which may potentially lead to excess dopamine and the manifestations of Sydenham chorea. The figure is from (Cunningham M. W., 2012; Cunningham M. W., 2006) and is used with permission.

Fischetti, & Cunningham, 2005). The recognition of the streptococcal antigen more avidly than the host antigen is expected—but T cell clones which bind host antigen more avidly will become activated and produce the cytokines that lead to autoimmune consequences and disease in the host, as described above for the T cell clones passively transferred into Lewis rats (Kirvan, Galvin, Hilt, Kosanke, & Cunningham, 2014).

Acute rheumatic fever and its related autoimmune sequelae associated with group A streptococcal infections is complicated by several risk factors that contribute to disease, including repeated streptococcal infections, a host's genetic susceptibility (such as HLA haplotype), and environmental aspects, which can include the interaction of host and streptococcus determining the outcome and disease. The same three risk factors are important in autoimmune diseases. The study of molecular mimicry in rheumatic fever has revealed interesting and plausible mechanisms and host-microbe relationships for both B and T cell responses in disease. Pathogenic mechanisms have been suggested and supported by disease-derived human mAbs (Galvin J. E., Hemric, Ward, & Cunningham, 2000; Kirvan, Swedo, Heuser, & Cunningham, 2003; Shikhman & Cunningham, 1994), human T cell clones (Ellis, Li, Hildebrand, Fischetti, & Cunningham, 2005), animal models of both rheumatic valvulitis (Quinn, Kosanke, Fischetti, Factor, & Cunningham, 2001) and Sydenham chorea (Brimberg, et al., 2012; Lotan,

et al., 2014), and translation of the disease models back to the human to apply the findings directly to rheumatic fever and streptococcal sequelae.

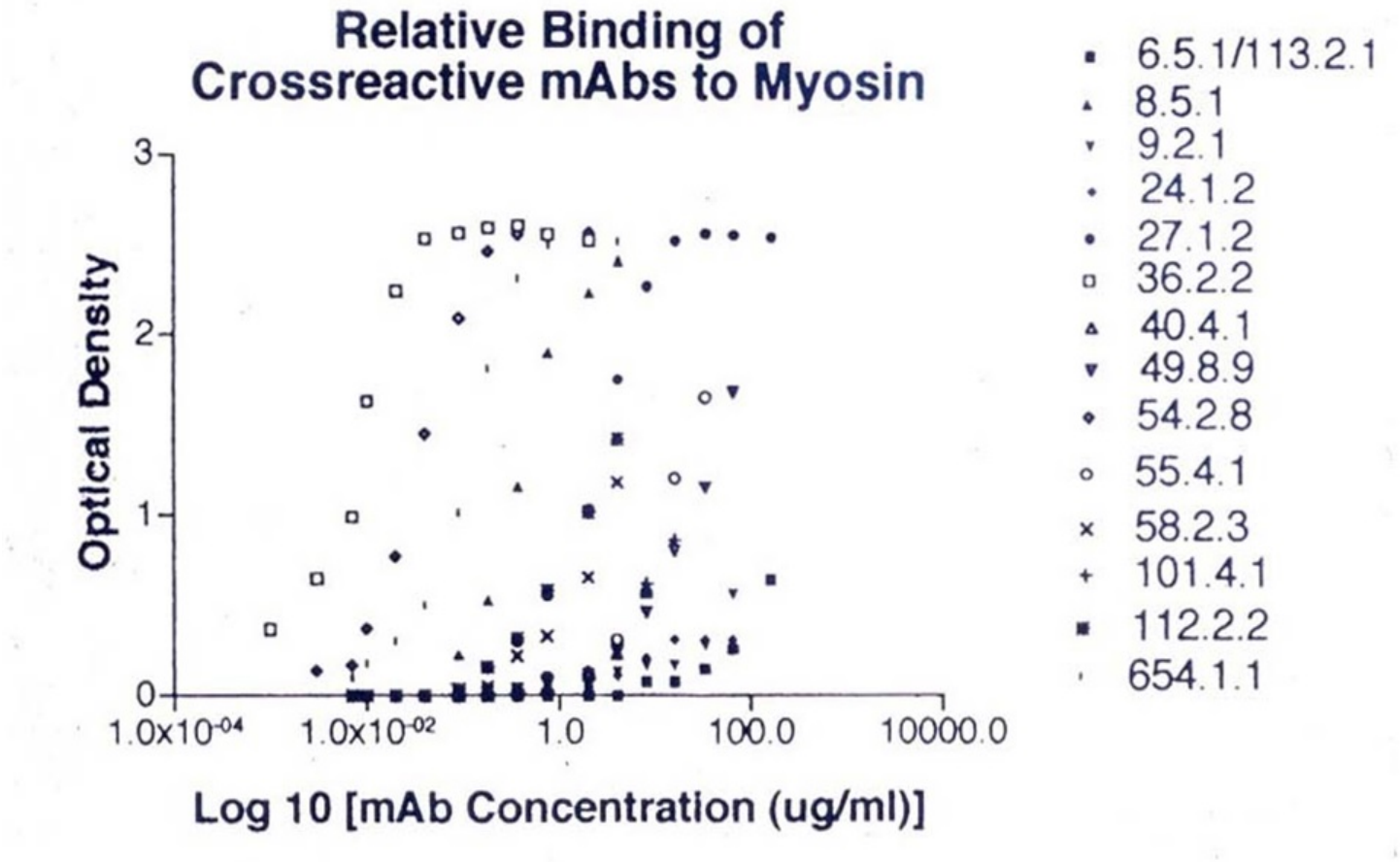

Figure 10. Comparison of binding avidity of antistreptococcal antibodies reactive to human cardiac myosin in the enzyme-linked immunosorbent assay. Highest avidity mAbs (36.2.2 and 54.2.8) were cytotoxic for rat primary heart cells (ATCC) in culture in the presence of complement (Cunningham, et al., 1992; Mertens, Galvin, Adderson, & Cunningham, 2000). The figure is from Cunningham et al. (Cunningham, et al., 1992; Mertens, Galvin, Adderson, & Cunningham, 2000) and is used with permission.

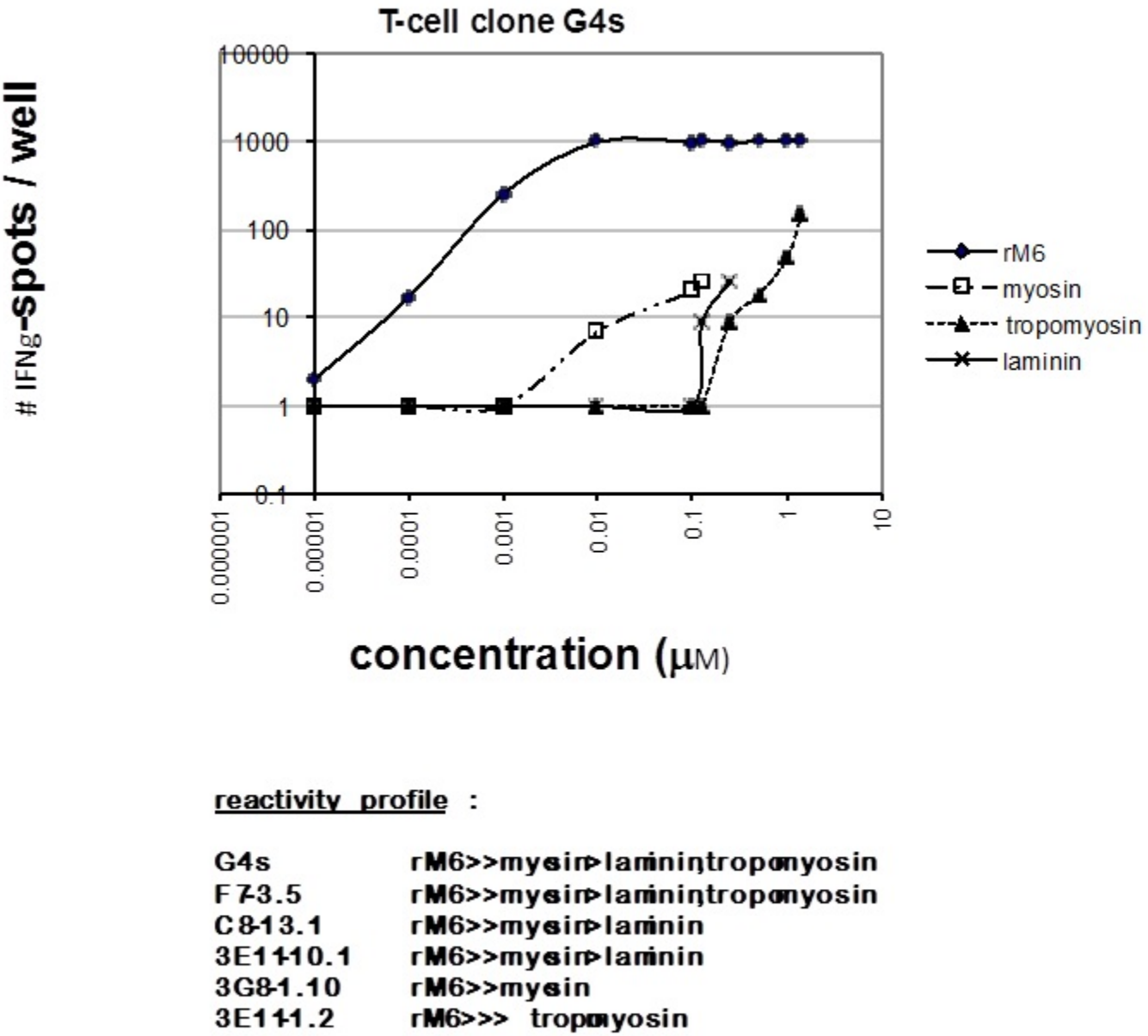

Figure 11. Dose response of cross-reactive T cell clone G4s. A cross- reactive Ag dose-dependent IFN-gamma response curve is shown for 1000 cells/ well G4s cells at 24 h after stimulation with rM6 protein, human cardiac myosin, laminin, and tropomyosin. The response was greatest for rM6 protein, which was approximately100-fold > human cardiac myosin. The cardiac myosin response was 10-fold > laminin and tropomyosin for T cell clone G4s. Other T cell clones not shown had a similar responsive profile to M protein and cardiac myosin. The figure is from Ellis et al. (Ellis, Li, Hildebrand, Fischetti, & Cunningham, 2005) and is used with permission.

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Laboratory Diagnosis of *Streptococcus pyogenes* (group A streptococci)

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Introduction

Historically, *Streptococcus pyogenes* (group A streptococci) was first cultured and identified as the cause of erysipelas by Friedrich Fehleisen in 1883, and it received its species designation from Rosenbach in 1884. Today, laboratory diagnosis of group A streptococcal infections still largely relies on culturing bacteria from clinical specimens. To detect streptococci in clinical samples (and especially *S. pyogenes*), the material is most often cultured on blood agar plates, which facilitates an easy preliminary screen for β-hemolytic colonies. Subsequent confirmation of suspicious colonies as *S. pyogenes* can be achieved by several easy, rapidly performed laboratory tests that are still widely applied in clinical microbiology, despite the increasing use of automated identification systems. In contrast to the diagnosis of acute *S. pyogenes* infections, the diagnosis of poststreptococcal diseases, such as glomerulonephritis, acute rheumatic fever, and cerebral disorders relies on the determination of specific antibodies. For epidemiological studies and outbreak investigations, different typing methods have been developed. In addition to classical serology based typing methods, well-established molecular typing systems are available, which provide large databases of already characterized strains. This chapter will try to give a comprehensive overview of classical microbiology and serology tests, molecular methods, automated systems, as well as both molecular and conventional typing methods that are used for the identification and characterization of *S. pyogenes*.

Culturing techniques

Streptococci are generally grown on agar media supplemented with blood. This technique allows the detection of β-hemolysis, which is important for subsequent identification steps, and enhances the growth of streptococci by the addition of an external source of catalase. Selective media for culturing Gram-positive bacteria (such as agar media that contains phenylethyl alcohol, or Columbia agar with colistin and nalidixic acid) also provide adequate culturing conditions for *S. pyogenes*. Optimal incubation conditions for the vast majority of streptococcal strains include a temperature range of 35°C to 37°C in the presence of 5% CO_2 or under anaerobic conditions. These conditions are optimized for culturing streptococcal species that belong to the viridans group, but they may not be ideal for growing *S. pyogenes*.

Special procedures have been developed to optimize the identification of *S. pyogenes* in throat cultures. When properly performed and interpreted, culturing throat swabs on a 5% sheep blood agar with trypticase soy base incubated in air remains the gold standard and reference method for the diagnosis of *S. pyogenes* acute pharyngitis (Murray, Wold, Schreck, & Washington, 1976; Shulman, et al., 2012). These conditions represent reliable and well-accepted methods with a sensitivity of 90% or higher, as shown with studies using duplicate throat cultures (Bisno, 2001; Murray, Wold, Schreck, & Washington, 1976). In most cases of acute streptococcal pharyngitis, ample growth of typical colonies can be observed after 24 hours of incubation at 35-37°C. If only a few colonies of *S. pyogenes* appear after incubation under these conditions, interpretation becomes more difficult, and these patients are most likely to be streptococcal carriers, rather than acutely infected individuals

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(Bisno, 2001). Differentiation between a carrier and an acutely infected individual could also reflect inadequate specimen collection, a lack of optimal conditions for incubation, and inaccurate reading of plates. False negative results with small numbers of organisms most likely occur because of overgrowth of upper respiratory tract microorganisms that produce toxic materials to mask hemolysis. To increase detection rates after the initial 18 to 24 hours of incubation, negative cultures should be re-examined after an additional 24 hours of incubation. For presumptive identification of *S. pyogenes,* cultures should be tested for bacitracin susceptibility and PYR activity (as described below). A definitive diagnosis should include a positive Lancefield group A antigen test. Negative results can be confirmed after a total culture time of 48 hours.

A number of studies have been performed to enhance *S. pyogenes* isolation, including analysis of incubation conditions in anaerobic or CO_2 enriched atmospheres, as well as the use of various media selective for β-hemolytic streptococci (Kellogg, 1990; Kurzynski & Meise, 1979; Welch, Hensel, Pickett, & Johnson, 1991; Milatović, 1981). In view of existing cost limitations and uncertain benefits, these additional efforts are not generally recommended. Incubation in anaerobic or a CO_2-enriched atmosphere more frequently leads to the isolation of non-*S. pyogenes* β-hemolytic streptococci (Altun, Almuhayawi, Ullberg, & Özenci, 2013). To suppress the growth of commensal respiratory microbiota, including other β-hemolytic streptococci, Streptococcus-selective media may be used, which is highly sensitive for the isolation of *S. pyogenes* (Welch, Hensel, Pickett, & Johnson, 1991). Another option is the addition of a blood agar plate that contains sulfamethoxazole-trimethoprim to inhibit the normal respiratory microbiota (Kurzynski & Meise, 1979). Details of these study results have been summarized in several publications (Bisno, Gerber, Gwaltney, Kaplan, & Schwartz, 1997; Kellogg, 1990).

A concern rarely addressed when culturing pharyngeal specimens for *S. pyogenes* on blood agar plates is the role of nonhemolytic *S. pyogenes* isolates. Culture based screening relies on the detection of β-hemolytic colonies and subsequent identification steps. However, clinical nonhemolytic *S. pyogenes* isolates that carry deletions of SLS genes have been published (Yoshino, et al., 2010). Moreover, nonhemolytic *S. pyogenes* strains have repeatedly been implicated as causing pharyngitis, as well as invasive infections (James & McFarland, 1971; Cimolai, Trombley, & Bhanju, 2002; Dierksen & Tagg, 2000; Jantsch, et al., 2013). Standard throat cultures will not detect these strains and it is currently unknown if there is a true burden of disease caused by nonhemolytic *S. pyogenes* strains.

Morphology

To identify *S. pyogenes* in clinical samples, blood agar plates are screened for the presence of β-hemolytic colonies. The typical appearance of *S. pyogenes* colonies after 24 hours of incubation at 35-37°C is dome-shaped with a smooth or moist surface and clear margins. They display a white-greyish color and have a diameter of ≥ 0.5 mm, and are surrounded by a zone of β-hemolysis that is often two to four times as large as the colony diameter. Microscopically, *S. pyogenes* appears as Gram-positive cocci, arranged in chains (Figure 1).

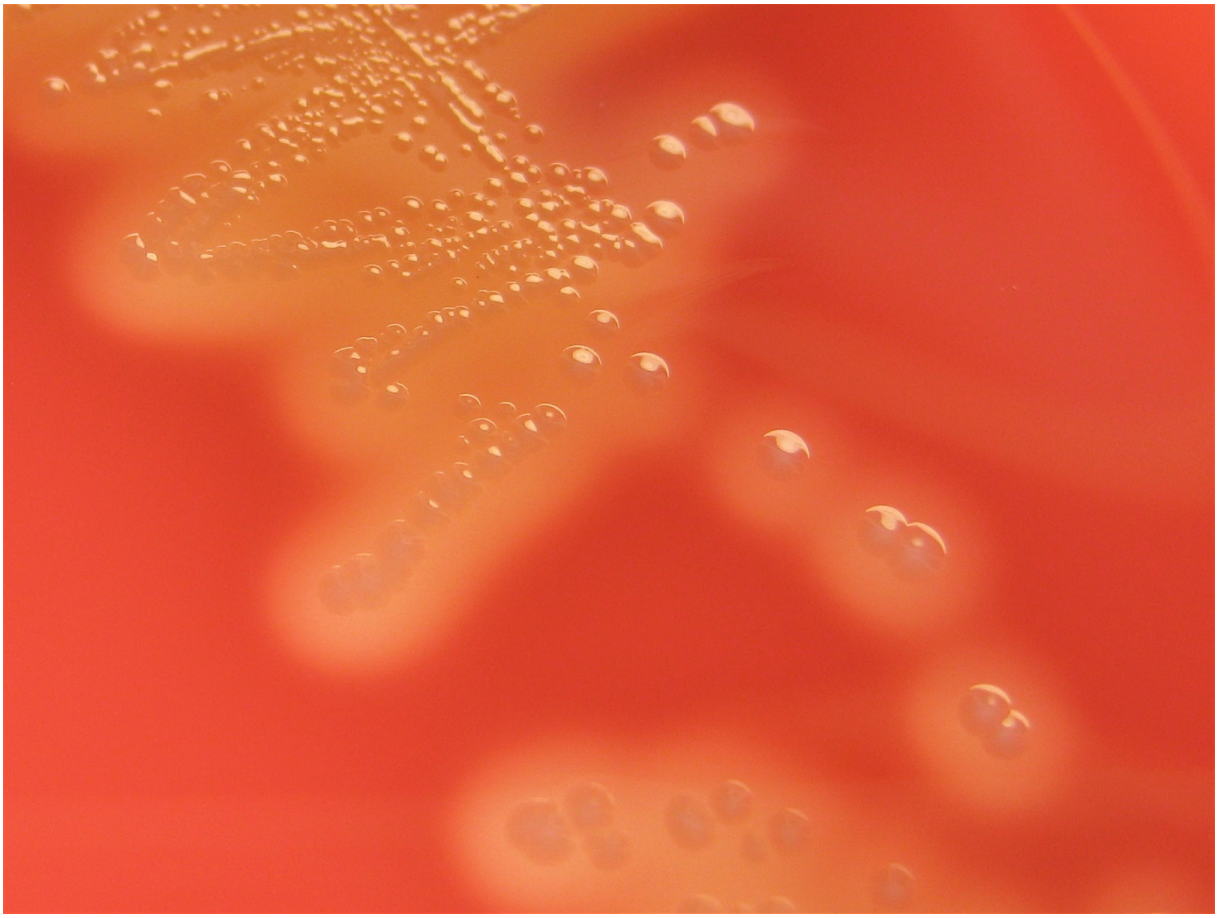

Figure 1: Typical appearance of *S. pyogenes* on sheep-blood agar plates, following 24 hour incubation under aerobic conditions.

Conventional identification tests

After the detection of β-hemolytic colonies displaying a typical *S. pyogenes* morphology, catalase testing confirms that the isolates represent streptococci. A few easy, rapidly performed laboratory tests can then be applied for definite species identification. Since each of the tests, which are detailed below, has some limitations, the most reliable identification results can be achieved by combining two of the following methods.

Lancefield antigen determination

Rebecca Lancefield was the first to develop a method for distinguishing β-hemolytic streptococci into different species by determining the presence of Lancefield antigens on streptococcal surfaces through antibodies (Lancefield, 1933). Currently, commercially available Lancefield antigen grouping sera, obtained from many different suppliers, are still widely applied in microbiology laboratories for the differentiation of β-hemolytic streptococci. The commercial kits provide substrates for rapid antigen extraction and subsequent agglutination by specific antibodies, and are typically directed towards Lancefield antigens A, B, C, F, and G. While there is a good correlation between the presence of certain Lancefield antigens with specific streptococcal species, this correlation is not 100% in the cases of Lancefield group A, C, or G antigens. Except for rare mutations, all *S. pyogenes* strains harbor the Lancefield group A antigen on their surface, but the presence of the group A antigen is not limited to *S. pyogenes.* It has also been found in species from the *Streptococcus anginosus* group (Facklam, 2002), as well as in rare *Streptococcus dysgalactiae* subsp. *equisimilis* isolates (Brandt, Haase, Schnitzler, Zbinden, & Lütticken, 1999). Therefore the detection of Lancefield group A necessitates further testing for a reliable species diagnosis of *S. pyogenes*, which can be achieved by bacitracin susceptibility discs or a PYR determination test.

PYR test

The PYR test is a rapid colorimetric method often used to distinguish *S. pyogenes* from other β-hemolytic streptococci with a similar morphology (such as *S. dysgalactiae* subsp. *equismilis)* and tests for the presence of the enzyme pyrrolidonyl aminopeptidase. This enzyme hydrolyzes L-pyrrolidonyl-β-naphthylamide (PYR) to β-naphthylamide, which produces a red color when a cinnamaldehyde reagent is added. The test can be performed on paper strips that contain dried chromogenic substrates for the pyrrolidonyl aminopeptidase within a few minutes (Kaufhold, Lütticken, & Schwien, 1989). PYR spot tests are available from a number of commercial vendors. For standard laboratory identification procedures, PYR positive β-hemolytic streptococci that display the typical morphology of *S. pyogenes* can be presumptively identified as *S. pyogenes*. Other PYR positive β-hemolytic streptococcal species, such as *Streptococcus iniae* and *Streptococcu porcinus*, are primarily animal-associated species and are rarely identified in human clinical specimens. To avoid potential misidentification, it is important to distinguish Streptococcus from Enterococcus prior to PYR testing, and to keep in mind that species and strains from other closely related genera may be PYR-positive (including the genera Abiotrophia, Aerococcus, Enterococcus, Gemella, Staphylococcus, and Lactococcus). Enterococci presenting with β-hemolysis are occasionally found on blood agar plates; however, PYR-positive β-hemolytic enterococcal isolates display a different colonial morphology, and when combined with other phenotypic characteristics, are easily distinguished from streptococci. To avoid false positive reactions caused by other PYR-positive bacterial species, which may be present in mixed cultures, this test should only be performed on pure cultures.

Bacitracin susceptibility

Streptococcus pyogenes can be differentiated from other non-group A β-hemolytic streptococci by their increased sensitivity to bacitracin. The bacitracin test, along with the Lancefield antigen A test, is used for greater specificity in the identification of *S. pyogenes*, since other β-hemolytic strains of streptococci that may contain the group A antigen are resistant to bacitracin. The bacitracin test is also used to distinguish *S. pyogenes* from other β-hemolytic streptococci that are PYR-positive, such as *S. iniae* and *S. porcinus*. To perform a bacitracin susceptibility test, it is important to make a subculture of the strain to be tested on a sheep blood agar plate (SBA), since placing the bacitracin disc on a primary plate could give variable results. The strain being tested is streaked with several individual colonies of a pure culture on an SBA agar plate and a disk containing 0.04 U of bacitracin is placed on the SBA plate. After overnight incubation at 35°C in 5% CO_2, a zone of inhibition surrounding the disc indicates the susceptibility of the strain. It is noteworthy that bacitracin-resistant strains of *S. pyogenes* have been observed in a number of European countries (Malhotra-Kumar, Wang, Lammens, Chapelle, & Goossens, 2003; Mihaila-Amrouche, Bouvet, & Loubinoux, 2004; James & McFarland, 1971); however, bacitracin resistance has not yet been reported in the US to date.

Species determination of *S. pyogenes* in automated identification systems

During the last decade, automated bacterial identification systems have gained more and more importance in the clinical laboratory. Currently, a variety of products that incorporate batteries of physiologic tests are commercially available for species identification of streptococci. These products generally perform well with commonly isolated pathogenic streptococci, such as *S. pyogenes*. Although automated bacterial identification by MALDI-TOF (Bruker Corporation, 2015; bioMérieux, Inc., 2015) has limitations in identifying several streptococcal species (including *S. dysgalactiae* subsp. *equisimilis,* which may be misidentified as *S. pyogenes*), the results for *S. pyogenes* correspond well to species identification by conventional tests (Schulthess, et al., 2013). New commercial systems for the identification of streptococci include the FDA approved Verigene Gram-positive blood culture (BC-GP) nucleic acid test (Nanosphere, Inc., 2014) and the FilmArray platform (BioFire Diagnostics, LLC, 2015) for the direct identification of bacterial pathogens from blood culture bottles (Altun, Almuhayawi, Ullberg, & Özenci, 2013; Wojewoda, et al., 2013). Highly favorable results from the application of these systems were obtained for major bacterial pathogens, including *S. pyogenes* and *S. agalactiae*. The direct

identification of *S. pyogenes* from blood culture bottles enables the rapid administration of a suitable antibiotic treatment, which offers a considerable advantage for patients suffering from life-threatening invasive diseases. However, for the majority of *S. pyogenes* isolates encountered in the clinical laboratory, serologic tests, in conjunction with presumptive physiologic tests (as described above) still offer an acceptable and cost-effective alternative to commercially available identification systems.

Antibiotic resistance testing

Penicillin remains the drug of choice for the empirical treatment of *S. pyogenes* infections, despite over sixty years of use. *S. pyogenes* has also remained uniformly susceptible to penicillin and resistance testing for penicillins or other β-lactams approved for treatment of *S. pyogenes* is not necessary for clinical purposes, in accordance with CSLI recommendations. In contrast to *Streptococcus agalactiae*, for which rare isolates with reduced susceptibility to penicillin have been reported and mutations in the cell wall synthesis enzyme Pbp2x were found, *S. pyogenes* isolates with altered penicillin susceptibilities that could be confirmed by a reference laboratory have not yet been encountered. Nevertheless, more than 10% of patients report suspected or confirmed allergies to penicillins, which frequently leads to the use of macrolides as an alternative treatment. Since macrolide resistance rates among *S. pyogenes* isolates have been increasing in North America as well as in Europe (Desjardins, Delgaty, Ramotar, Seetaram, & Toye, 2004), resistance testing is mandatory for these substances. *S. pyogenes* macrolide resistance rates correlate with the use of macrolides in clinical practice, and geographic differences in resistance rates are often due to differences in macrolide use. Susceptibility testing for macrolides should be performed using erythromycin, since resistance and susceptibility of azithromycin, clarithromycin, and dirithromycin can be predicted by testing erythromycin. To detect inducible clindamycin resistance in *S. pyogenes*, CLSI recommends a double-disc diffusion assay. Similar to the resistance situation for penicillins, a reduced susceptibility to glycopeptides has not yet been found in *S. pyogenes.* Currently, resistance testing will most often be performed by automated systems that provide ready-made panels of antibiotics for different bacterial species. Further discussion of antibiotic resistance can be found in a subsequent chapter.

Direct antigen detection of *S. pyogenes* from throat specimens

S. pyogenes infections represent the most common cause of acute pharyngitis, and account for up to 37% of pediatric cases (Shaikh, Leonard, & Martin, 2010) and 5 to 15% of cases in adults (Shulman, et al., 2012). If a diagnosis can be provided rapidly, prompt initiation of antibiotic therapy will relieve symptoms, avoid sequelae, and reduce transmission rates. This is most often achieved through the application of so-called "rapid antigen tests". Numerous assays for direct detection of the group A-specific carbohydrate antigen in throat swabs by agglutination methods or immunoassays (enzyme, liposome, or optical) have become commercially available during the past two decades. A list of FDA-cleared tests is accessible online (U.S. Department of Health and Human Services, 2015). Although these tests provide rapid results to allow early treatment decisions, culturing throat swabs for *S. pyogenes* remains the gold standard. The sensitivities of rapid antigen tests range from 58% to 96%, but have never equaled that of culture tests (Facklam, 1987; Uhl, et al., 2003). Therefore, national advisory committees continue to recommend confirmation of negative rapid test results with a throat culture in children and adolescents (Shulman, et al., 2012). However, a routine back-up throat culture is dispensable in adult patients, due to the low incidence of streptococcal pharyngitis and rheumatic fever in this age group. The specificity of rapid antigen tests is generally high, even though false-positive antigen results can be seen from patients previously diagnosed and/or treated for *S. pyogenes* (Chapin, Blake, & Wilson, 2002), or patients colonized with non-*S. pyogenes* streptococcal species that carry the Lancefield group A antigen. Despite the obvious advantages of a rapid diagnosis, it must be noted that the positive predictive value of rapid group A antigen tests is currently low in the adult population, and can frequently result in unnecessary antimicrobial therapy (Peterson & Thomson, 1999).

Nucleic acid detection techniques

Several years ago, a method based on nucleic acid detection was first introduced for direct *S. pyogenes* diagnosis from clinical throat swabs. The GASDirect test identifies specific rRNA sequences of *S. pyogenes* in pharyngeal specimens by a single-stranded chemiluminescent nucleic acid probe (Hologic, Inc., 2015; Steed, Korgenski, & Daly, 1993; Pokorski, Vetter, Wollan, & Cockerill, 1994). The test has performed well in comparison to standard streptococcal culture methods and has received FDA clearance. Sensitivity and specificity ranged from 89%–95% and 98%–100%, respectively, as compared to culture results, which reached a sensitivity of 98%–99% (Chapin, Blake, & Wilson, 2002; Steed, Korgenski, & Daly, 1993; Pokorski, Vetter, Wollan, & Cockerill, 1994). The GASDirect test can be applied for primary testing, has also been used as a backup test to negative antigen tests (Nakhoul & Hickner, 2013), and is suitable for batch screening of throat cultures.

A commercial polymerase chain reaction (PCR) method for the direct detection of *S. pyogenes* using the illumigene system (Meridian Bioscience, Inc., 2015) has recently received FDA clearance. Excellent sensitivity (99%) and specificity (99.6%) were demonstrated for the illumigene test in a multicenter evaluation study (Anderson, et al., 2013; Buchan & Ledeboer, 2014). This test relies on loop-mediated isothermal amplification (LAMP) technology with *S. pyogenes* specific primers. In 2015, two point of care tests for the detection of *S. pyogenes* in throat swabs using rapid automated PCR technology received FDA clearance. Both the cobas Strep A test, running on the cobas Liat platform (F. Hoffmann-La Roche Ltd., 2015) and the Simplexa Group A Strep Direct Test (Focus Diagnostics, Inc., 2013) provide PCR results for individual samples within 20 minutes. Apart from FDA-released documents, however, scientific publications on the performance of these tests concerning *S. pyogenes* detection, are not yet publicly available.

Serologic tests

The diagnosis of poststreptococcal diseases, such as rheumatic fever or glomerulonephritis, can be aided by the detection of certain streptococcal antibodies. Such a diagnosis is rarely useful in acute infections, since antibody development takes about one to two weeks after the onset of acute infection to be detectable in serum samples. Rising antibody levels only occur in patients suffering from *S. pyogenes* infections and streptococcal carriers do not experience an increase of antibody titers (Shet & Kaplan, 2002), when acute and convalescent sera are compared. A fourfold rise in antibody titers is regarded as a definitive proof of antecedent streptococcal infection. While the measurement of a definite antibody rise is the more reliable detection method, serum of patients suffering from glomerulonephritis or rheumatic fever may have reached peak antibody levels at the onset of symptoms and thus will not experience any further rises in these levels. Multiple variables influence antibody levels: these include the site of infection, time since the onset of symptoms, age of the patient, the background prevalence of streptococcal infections in a particular region, seasonal changes, and patient comorbidities (Ayoub, et al., 2003). Age is an especially important determinant of streptococcal antibody levels. Due to the frequent exposure to *S. pyogenes,* children between 6 and 15 years display the highest antibody titers, as compared with very young infants and adults: thus, "normal levels" in children may considerably exceed regular background titers of adults. Prompt antibiotic treatment of acute infections can reduce the magnitude of, but will not abolish the immune response to streptococcal antigens. The most widely used antibodies for the diagnosis of poststreptococcal diseases are anti-streptolysin O and anti-DNase B.

Streptolysin O is a cholesterol-dependent hemolysin of *S. pyogenes* that belongs to the group of thiol-activated cytolysins. Antibody levels against streptolysin O (ASO) start rising after one week of infection and reach maximum levels at about three to six weeks of infection. The upper limits of normal (ULN) of ASO are 240–320 in the pediatric age group 6–15 years (Shet & Kaplan, 2002). While the ASO response following streptococcal upper respiratory tract infection is usually high, pyoderma caused by *S. pyogenes* does not elicit a strong ASO response. *Streptococcus dysgalactiae* subsp. *equisimilis* can also produce streptolysin O; thus, elevated ASO titers are not specific to *S. pyogenes* infections. The classical test for measuring ASO titers is a neutralization assay,

where hemolysis through streptolysin O is inhibited by patient serum that harbors antistreptolysin O antibodies. Results are expressed as Todd units, which is the reciprocal of the highest titer not showing any hemolysis. Newer tests based on latex agglutination and nephelometric measurements are also available.

S. pyogenes produces several nucleases that are important for the escape of bacteria from neutrophil extracellular traps (Sumby, et al., 2005). Among the four streptococcal deoxyribonucleases (DNase), the immunologic host response is most consistent against DNase B. DNase B titers start appearing at two weeks after the onset of infection, and may not reach maximum titers for six to eight weeks. Similar to ASO titers, the upper limits of normal for pediatric patients (6–15 years) is much higher, and the ULN for Anti DNase B in this age group is 640 (Shet & Kaplan, 2002). Anti-DNase B titers tend to remain elevated longer than the ASO titers and are more reliable than ASO for the confirmation of a preceding streptococcal skin infection. Moreover, since only 80–85% of patients with rheumatic fever will present with elevated ASO titers, additional DNase titers may be helpful. Since DNase B is specific for *S. pyogenes* and not present in *Streptococcus dysgalactiae* subsp. *equisimilis,* increased ASO levels without changes in the anti-DNase B titers may indicate *Streptococcus dysgalactiae* subsp. *equisimilis* infections. The classic anti-DNase B assay is a neutralization assay, and is based on the inhibition of nuclease activity through antibodies present in patient serum. Other less standardized assay techniques that are available include a latex agglutination test.

A more historical test is the hemagglutination-based streptozyme test that was developed to detect antibodies against multiple extracellular streptococcal products as a supplementary test in the clinical laboratory. However, variabilities in the standardization of the test and an inconsistent specificity have been reported (Gerber, Wright, & Randolph, 1987). Tests for the detection of antibodies against the group A carbohydrate, as well as serotype-specific antibodies, are measured for research purposes only and usually have no clinical use in the diagnosis of streptococcal infection.

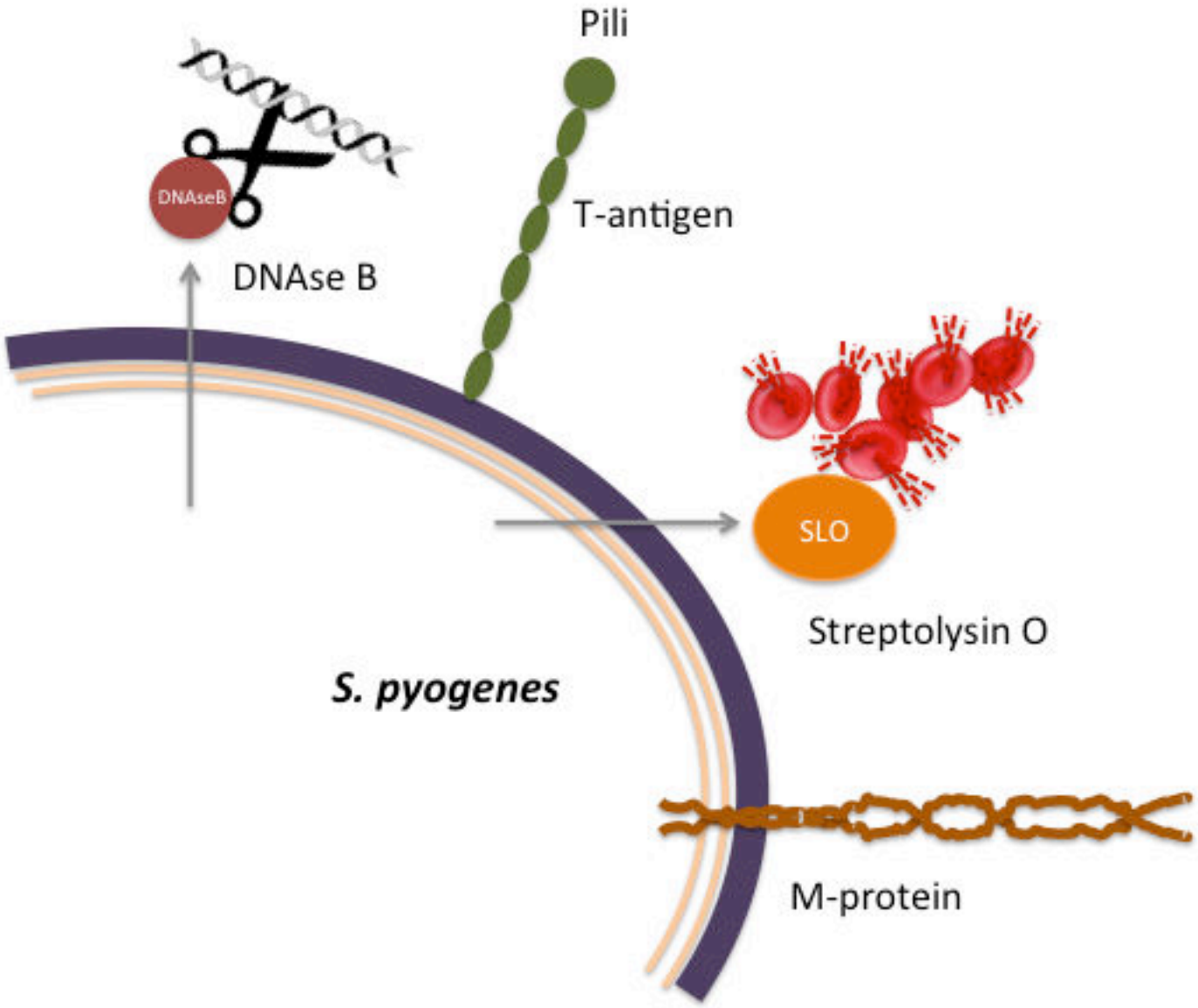

Figure 2. Common antigenic proteins of *S. pyogenes* used for diagnostic and typing purposes.

Typing of *Streptococcus pyogenes*

In most clinical cases of acute infections, subtyping of group A streptococcal strains has no immediate diagnostic or therapeutic consequences. Such typing is typically performed by reference laboratories for epidemiologic surveys or in outbreak situations, and may provide important information about the evolutionary relatedness of various strains. Although classical antibody-dependent typing systems of surface proteins have been used for many years, molecular methods have become more and more prevalent, since they do not require the maintenance of rarely used large antibody panels or the establishment of specialized techniques. As an additional advantage, the determination of DNA sequences is independent from culture conditions and gene expression.

Conventional typing of *S. pyogenes* is based upon the antigenic specificity of the surface-expressed T and M proteins. (Johnson & Kaplan, 1993). The trypsin-resistant T protein is part of the pilus structures (Mora, et al., 2005). T type identification can be achieved by commercially available assays that use approximately 20

recognized anti-T sera. Molecular analysis has successfully established an association between pilus *genes* and recognized T-serotypes (Falugi, et al., 2008). M proteins are major antiphagocytic virulence factors of *S. pyogenes* (Fischetti, 1989). The different antigenic specificities are based on N-terminal sequence variations in the M-proteins, which are detected by precipitation typing, using M-protein specific antisera. 83 M serotypes are currently validated and internationally recognized as serologically unique. They are designated as M1 to M93, in accordance with their Lancefield classifications (Facklam, et al., 2002). M serotypes that are not included are from non-*S. pyogenes* organisms, such as *Streptococcus dysgalactiae* subsp. *equisimilis*, or are duplicates of an already existing M serotype.

A molecular serotyping system has been established on the basis of the nucleotide sequence variations that encode the amino termini of M proteins. This system is based on the amplification and subsequent nucleotide sequencing of an *emm* gene fragment by a conserved primer pair. The *emm* genes encode M proteins and correlate with the Lancefield M serotypes. This methodology allows an assignment to a validated M protein gene sequence (*emm*1 through *emm*124) and easy identification of new *emm*-sequence types and subtypes. Molecular serotyping through *emm* sequencing has evolved into the "gold standard" molecular methodology of *S. pyogenes* typing (Facklam, et al., 2002). A large database of more than 200 distinct *emm* gene sequences from strains originally used for Lancefield serotyping, including *emm* sequences from β-hemolytic groups C, G, and L streptococci, is accessible at the CDC website (Centers for Disease Control and Prevention, 2014). The current type definition is based upon the sequence of the 90 nucleotides that encode the N terminal 30 amino acid residues of the processed M protein. This annotation is favored, as it is most consistent with the classical serology-based typing scheme. Subtypes can be assigned by sequencing 150 nucleotides that encode the N terminal 50 residues of the mature M protein. Due to the rapidly increasing availability of newly sequenced *S. pyogenes* strains, the database is constantly growing. However, it has not yet been determined if the newer M-proteins that are defined solely through sequencing are functional. In most instances, the potential antiphagocytic or opsoninogenic properties of these proteins have not been experimentally tested.

Additional molecular typing techniques applicable for *S. pyogenes* have been developed. In analogy to *emm* typing, a nucleotide based T-typing system by determination of the *tee* gene has been published (Falugi, et al., 2008). Furthermore, several years ago, an MLST scheme was developed for *S. pyogenes*. In population genetic studies, a stable association between *emm* type and MLST could be demonstrated for isolates obtained decades apart and/or from different continents (Enright, Spratt, Kalia, Cross, & Bessen, 2001). In *S. pyogenes* outbreaks, the restriction digestion of *emm* gene PCR amplicons may be a valuable tool for the rapid identification of isolates that carry identical or highly similar *emm* genes (Beall, et al., 1998). For clusters of isolates that share the same *emm* type, PFGE profiles may be helpful in further distinguishing these strains (Musser, et al., 1995). Several *emm*-types can be shared between different clonal groups of *S. pyogenes*.

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License

Mechanisms of Antibiotic Resistance

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Introduction

Streptococcus pyogenes, or group A streptococcus, is a major human pathogen that causes over 600 million infections annually (Lynskey, Lawrenson, & Sriskandan, 2011). This species is able to colonize the upper respiratory tract and skin of asymptomatic people, but is also responsible for a wide range of diseases, including suppurative infections and non-suppurative complications, which may occur either endemically or as outbreaks (Cunningham, 2000; Efstratiou, 2000). The types of infections can be divided into three groups: superficial infections (such as pharyngotonsillitis, impetigo, erysipelas, vaginitis, or post-partum infections), deep infections (such as bacteremia, cellulitis, myositis, necrotizing fasciitis, puerperal sepsis, pericarditis, meningitis, pneumonia, or septic arthritis), and toxin-mediated diseases (such as scarlet fever or streptococcal toxic shock syndrome [STSS]) (Efstratiou, 2000). These infections also play a significant role in the development of post-infection immune sequelae, including rheumatic fever, acute glomerulonephritis, and reactive arthritis (Cunningham, 2000). Clinical isolates of *S. pyogenes* were classically differentiated into M serotypes, based on structural differences of the M protein (encoded by the *emm* gene), which is a fibrillar cell-wall protein involved in adherence to human cells and prevention of opsonophagocytosis (Lynskey, Lawrenson, & Sriskandan, 2011; Cunningham, 2000). This method was replaced in the late 1990s by the typing system based on sequencing of the 5' end of the *emm* gene, and is referred to as *emm* typing (Facklam, et al., 1999). Even though there were significant differences in the *emm*-type distribution that depended on geographical area or clinical disease state, the most common *emm* types found in a large systematic review were *emm*1 (18.3%), *emm*12 (11.1%), *emm*28 (8.5%), *emm*3 (6.9%), and *emm*4 (6.9%) (Steer, Law, Matatolu, Beall, & Carapetis, 2009). As far as antimicrobial resistance, *S. pyogenes* has remained highly susceptible to almost all classes of antibiotics [Table 1] (Bourbeau & Campos, 1982; Kayser, 1994; Chin, Gu, Yu, Zhang, & Neu, 1991; Cohen, et al., 1991; Bouanchaud, 1997; Blondeau, Church, Yaschuk, & Bjarnason, 1999). Among *S. pyogenes* clinical isolates in some geographic regions, only resistance to macrolides (and related compounds) and tetracyclines are commonly found.

Table 1. In vitro activity of 31 antimicrobial agents against wild-type isolates of *S. pyogenes* (Bourbeau & Campos, 1982; Kayser, 1994; Chin, Gu, Yu, Zhang, & Neu, 1991; Cohen, Huband, Mailloux, Yoder, Roland, & Heifetz, 1991; Bouanchaud, 1997; Blondeau, Church, Yaschuk, & Bjarnason, 1999; Amábile-Cuevas, Hermida-Escobedo, & Vivar, 2001; King & Phillips, 2001; Gemmell, 2001; Cantón, Loza, Morosini, & Baquero, 2002; Blondeau & Sanche, 2002; Noviello, Ianniello, Leone, & Esposito, 2003; Carpenter & Chambers, 2004; Keating & Scott, 2004; Brown & Rybak, 2004; Brauers, Kresken, Hafner, & Shah, 2005; Bradford, Weaver-Sands, & Petersen, 2005; Pankey, 2005; Rubinstein & Vaughan, 2005; Al-Lahham, De Souza, Patel, & Reinert, 2005; Lynch, File, & Zhanel, 2006; Ziglam, 2007; Hair & Keam, 2007; Zhanel, et al., 2007; Mazzariol, Koncan, Vitali, & Cornaglia, 2007; Morrissey, Ge, & Janes, 2009; Pfaller, Castanheira, Sader, & Jones, 2010; Biek, Critchley, Riccobene, & Thye, 2010; Jones, Mendes, Sader, & Castanheira, 2011; Karlowsky, Adam, Poutanen, Hoban, Zhanel, & Canadian Antimicrobial Resistance Alliance, 2011; Pérez-Trallero, Tamayo, Montes, Garcia-Arenzana, & Iriarte, 2011; Jones, Sader, & Flamm, 2013).

Antibiotic	MIC values (µg/ml)		EUCAST breakpoints[a] (µg/ml)	
	Range of MIC_{50}	Range of MIC_{90}	S ≤	R >
β-lactams				
Penicillin G	≤0.01	≤0.01-0.03	0.25	0.25

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Antibiotic	MIC values (µg/ml)		EUCAST breakpoints[a] (µg/ml)	
	Range of MIC_{50}	Range of MIC_{90}	S ≤	R >
Ampicillin/Amoxicillin	≤0.01	≤0.01-0.03	-	-
Cefaclor	0.06-0.5	0.12-0.5	-	-
Cefuroxime	≤0.01	≤0.01-0.03	-	-
Cefixime	0.12-0.25	0.12-0.25	-	-
Cefpodoxime	0.01	0.01	-	-
Ceftriaxone/Cefotaxime	≤0.01-0.03	≤0.01-0.06	-	-
Cefepime	≤0.01	0.03	-	-
Ceftaroline	≤0.01	≤0.01	-	-
Imipenem	≤0.01	≤0.01	-	-
Aminoglycosides				
Gentamicin	4	4	-	-
Macrolides and related compounds				
Erythromycin	≤0.01-0.06	0.03-0.12	0.25	0.5
Clarithromycin	0.03	0.03-0.06	0.25	0.5
Spiramycin	0.25	0.5	-	-
Clindamycin	≤0.06-0.12	≤0.06-0.12	0.5	0.5
Quinupristin-dalfopristin	≤0.12-0.5	≤0.12-0.5	-	-
Telithromycin	0.01	0.01-0.03	0.25	0.5
Fluoroquinolones				
Ciprofloxacin	≤0.25-0.5	0.5-2	-	-
Levofloxacin	0.25-0.5	0.5-1	1	2
Moxifloxacin	0.06-0.12	0.12-0.25	0.5	1
Tetracyclines				
Tetracycline	≤0.25-0.5	0.25-1	1	2
Doxycycline	0.12	0.5	1	2
Tigecycline	0.01-0.12	0.03-0.25	0.25	0.5
Glycopeptides				
Vancomycin	0.25-0.5	0.25-1	2	2
Teicoplanin	0.06-0.5	0.12-1	2	2
Others				
Daptomycin	≤0.03-0.06	0.06-0.25	1	1
Linezolid	0.5-1	1-2	2	4
Cotrimoxazole	0.06-0.25	0.25-0.5	1	2
Rifampin	0.12	0.12	0.06	0.5
Bacitracin	1	1-2	-	-
Chloramphenicol	2	4	8	8

Table 1. continued from previous page.

Antibiotic	MIC values (µg/ml)		EUCAST breakpoints[a] (µg/ml)	
	Range of MIC_{50}	Range of MIC_{90}	S ≤	R >
Fusidic acid	4	4-8	-	-

[a] Available at http://www.eucast.org/clinical_breakpoints/.

Beta-Lactams

Even though *S. pyogenes* has remained universally susceptible to β-lactams [Table 1] since the 1940s, a significant number of treatment failures have been reported (Gillespie, 1998). For instance, a meta-analysis of therapeutic studies showed that the bacteriological treatment failure rate in streptococcal pharyngotonsillitis was around 12% from 1953 to 1993 (Markowitz, Gerber, & Kaplan, 1993). In the past 15 years, the rate of penicillin failure has dramatically increased to almost 40% in some regions of the world (Brook, 2013). The main explanations for penicillin failure include: (i) intracellular persistence of *S. pyogenes*, due to the poor penetration of penicillin into tonsillar tissues, including tonsillar epithelial cells; (ii) protection of *S. pyogenes* by β-lactamase-producing bacteria (namely *Staphylococcus aureus*, *Haemophilus* spp., *Moraxella catarrhalis*, and anaerobes) that are commonly part of the oral microbiota; (iii) coaggregation between *M. catarrhalis* and *S. pyogenes*, which may enhance *S. pyogenes* colonization through the facilitation of its adherence to human epithelial cells; and (iv) alteration of the commensal bacterial microbiota, which can compete for nutrients (Brook, 2013; Kaplan, Chhatwal, & Rohde, 2006; Pichichero & Casey, 2007; Schaar, Uddbäck, Nordström, & Riesbeck, 2014).

Although penicillin is generally the agent of choice for the treatment of tonsillopharyngitis caused by *S. pyogenes*, a meta-analysis demonstrated that oral cephalosporins (such as cefadroxil and cefpodoxime proxetil) seemed to be more efficient than oral penicillin, with two times fewer bacteriological and clinical failures (Casey & Pichichero, 2004). Also, failure with penicillin is highest in retreatment cases where cephalosporins are more effective (Casey & Pichichero, 2004). The superior activity of cephalosporins, which is likely related to a more important ability for *S. pyogenes* eradication, may be due to their higher efficacy in killing ingested bacterial cells (Kaplan, Chhatwal, & Rohde, 2006).

Mechanistically, β-lactam antibiotics inhibit the last steps of peptidoglycan synthesis by binding to high-molecular-weight penicillin-binding proteins (PBPs) (Rice, 2012). In *Streptococcus pneumoniae* and viridans group streptococci, resistance to β-lactams is mediated by alterations in the PBP binding site by the generation of low-affinity enzymes. By contrast, such a mechanism of β-lactam resistance has not yet been reported in β-hemolytic streptococci, despite the extensive use of penicillins for the treatment of *S. pyogenes* infections (Horn, et al., 1998). One possible explanation for this difference is the limited ability of *S. pyogenes* to exchange genetic material and acquire new resistance determinants. Unlike pneumococci, *S. pyogenes* is not intrinsically competent and does not readily acquire exogenous DNA—although it does produce numerous types of extracellular DNases (Horn, et al., 1998). In addition, gene transfer by conjugation in *S. pyogenes* seems to be very unusual, since clinical isolates rarely contain plasmids (Horn, et al., 1998). Finally, penicillin-resistant and penicillin-tolerant laboratory mutants of *S. pyogenes* were isolated in vitro after treatment by ethyl methane sulfonate (Gutmann & Tomasz, 1982). These strains, which express low-affinity PBPs, exhibited a 32-fold increase in penicillin G MICs (from 0.006 to 0.2 µg/ml), but showed severe physiological defects with extremely poor growth rates and gross morphological abnormalities (Gutmann & Tomasz, 1982). This finding suggests that these strains have a low probability to develop as clinical isolates, while it seems that tolerance observed in such mutants does not have clinical relevance. In contrast to *Enterococcus faecalis* strains (Murray, 1992), no β-lactamase genes have been identified among any strains of *S. pyogenes*.

Aminoglycosides

Aminoglycosides are bactericidal antimicrobial agents that primarily inhibit bacterial protein biosynthesis by binding to the 16S rRNA of the 30S small ribosomal subunit (Becker & Cooper, 2013). Other metabolic perturbations caused by these molecules include interference with the respiratory chain electron transport system, induction of ionic disorders, disruption of bacterial cell membrane integrity, and disturbances of DNA/RNA synthesis (Kotra, Haddad, & Mobashery, 2000). Aminoglycosides are active against a large spectrum of aerobic Gram-negative bacilli and Gram-positive cocci, while anaerobes are highly resistant. Like enterococci, streptococci are intrinsically resistant to low antibiotic concentrations (MICs ranging from 4 to 64 µg/ml), which is due to their limited drug uptake [Table 1]. However, the combination of aminoglycosides with cell-wall-active agents (such as penicillins and glycopeptides) results in a significant bactericidal synergy. Indeed, the inhibition of peptidoglycan synthesis mediated by such molecules would increase the uptake of aminoglycosides and induce the formation of reactive oxygen species (Zembower, Noskin, Postelnick, Nguyen, & Peterson, 1998; Barnes, Herrero, & Albesa, 2005). High-level resistance to aminoglycosides (MICs >2,000 µg/ml) that entirely abolishes synergistic bactericidal activity is often due to the enzymatic inactivation mediated by aminoglycoside-modifying enzymes (AMEs), while a less common mechanism corresponds to ribosomal alterations (Jana & Deb, 2006). Note that the genes that encode for AMEs are often located on plasmids. There are three different classes of AMEs, which depend on the reaction catalyzed: aminoglycoside acetyltransferases (AACs), aminoglycoside phosphotransferases (APHs), and aminoglycoside nucleotidyltransferases (ANTs) (Chow, 2000). In enterococci (which are closely related to streptococci), the major AME enzymes are: APH(3')-IIIa, which mediates high-level resistance to kanamycin; ANT(4')-Ia, which confers resistance to kanamycin, amikacin, and tobramycin; and AAC(6')-Ie-APH(2")-Ia, a bifunctonial enzyme that mediates resistance to virtually all the clinically available aminoglycosides, including kanamycin, amikacin, tobramycin, gentamicin, and netilmicin, but except streptomycin (Chow, 2000). In *S. pyogenes*, high-level resistance to aminoglycosides appears to be rare, and only a few strains resistant to both kanamycin and streptomycin have been reported (Horodniceanu, Buu-Hoï, Delbos, & Bieth, 1982; Lakshmi & Kim, 1989). This resistance was due to the production of both APH(3')-IIIa and ANT(6)-Ia enzymes, and has been demonstrated to be transferable by conjugation (Lakshmi & Kim, 1989; van Asselt, Vliegenthart, Petit, van de Klundert, & Mouton, 1992). No gentamicin-resistant clinical isolates have been described to date.

Macrolides-Lincosamides-Streptogramins-Ketolides

Macrolides, lincosamides, and streptogramins (MLS) are recommended as alternate antibiotics for the treatment of *S. pyogenes* infections in patients who are allergic to β-lactams or in cases of penicillin failure (Bisno, Gerber, Gwaltney, Jr., Kaplan, & Schwartz, 2002). Although MLS compounds are chemically distinct, they are considered to be a unique group, since they share a similar mode of action through binding to (or in the vicinity of) the ribosomal peptidyl transferase center (PTC) (Schlünzen, et al., 2001). Also, they present an overlapping spectrum of activity within the same MIC ranges [Table 1]. Practically, macrolides are classified according to the number of atoms that form the lactone ring, and there are 14- (e.g. erythromycin or clarithromycin), 15- (e.g. azithromycin), or 16-membered macrolides (e.g. spiramycin or josamycin). Notably, a new class of molecules has been recently developed that have evolved from macrolides, and these are known as ketolides (e.g. telithromycin). Lincosamides comprise only two members: lincomycin and its semisynthetic derivative, clindamycin. Streptogramins are actually composed of a mixture of two compounds that act synergistically: streptogramin A (e.g. dalfopristin) and streptogramin B (e.g. quinupristin) (Dang, Nanda, Cooper, Greenfield, & Bronze, 2007).

The first macrolide-resistant *S. pyogenes* isolate was reported in the USA in 1968 (Sanders, Foster, & Scott, 1968). In the late 1990s and early 2000s, the incidence of macrolide resistance dramatically increased in several European countries, such as Finland, France, Greece, Italy, Portugal, and Spain, with erythromycin resistance

rates usually exceeding 20% (Seppälä, et al., 1997; Granizo, Aguilar, Casal, Dal-Ré, & Baguero, 2000; Bingen, et al., 2004; Silva-Costa, Ramirez, & Melo-Cristino, 2005; Creti, et al., 2007; Richter, et al., 2008). Despite some geographical variations, rates of macrolide resistance in the USA have remained relatively low (around 5%) since the 1990s (Brown & Rybak, 2004; Tanz, et al., 2004; Richter, et al., 2005). More recently, some European studies have described a significant decrease in erythromycin resistance in *S. pyogenes*, such as in France or Spain (d'Humières, et al., 2012; Montes M. , et al., 2014). From an epidemiological point of view, it was frequently demonstrated that the prevalence of erythromycin-resistant *S. pyogenes* correlated well with the total consumption of macrolide antibiotics in particular geographical areas (Seppälä, et al., 1997; Granizo, Aguilar, Casal, Dal-Ré, & Baguero, 2000; Seppälä, Klaukka, Lehtonen, Nenonen, & Huovinen, 1995; Albrich, Monnet, & Harbarth, 2004; Bergman, et al., 2004; Hsueh, Shyr, & Wu, 2005). Besides antibiotic consumption, changes in the clonal composition of the *S. pyogenes* population may also be an important cause for fluctuations in macrolide resistance rates (Montes, Tamayo, Mojica, García-Arenzana, Esnal, & Pérez-Trallero, 2014; Silva-Costa, Friães, Ramirez, & Melo-Cristino, 2012).

Notably, a relationship between virulence and macrolide resistance has emerged. Specifically, erythromycin resistance is associated with the increased cell invasiveness of *S. pyogenes* (Facinelli, Spinaci, Magi, Giovanetti, & Varaldo, 2001). Indeed, this association could be due to the presence of the *prtF1* gene, which is more frequently found among macrolide-resistant strains. The *prtF1* gene encodes the fibronectin-binding protein F1, an adhesion that allows *S. pyogenes* to be efficiently internalized by and survive within human respiratory cells (Facinelli, Spinaci, Magi, Giovanetti, & Varaldo, 2001; Haller, Fluegge, Arri, Adams, & Berner, 2005). By contrast, biofilm formation could be an important factor to explain therapeutic failures and recurrences due to macrolide-susceptible *S. pyogenes* clinical isolates (Baldassarri, et al., 2006). Additionally, erythromycin-susceptible strains form a significantly thicker biofilm than resistant isolates, while those harboring *erm*-class genes have a less organized biofilm than *mef*(A)-positive strains (see below). Finally, the presence of *prtF1* appears to be negatively associated with the ability to form biofilm (Baldassarri, et al., 2006).

MLS resistance may be due to several different mechanisms: (i) post-transcriptional target site modifications caused by rRNA methylases (*erm*-class genes); (ii) target mutations (in 23S rRNA or ribosomal proteins L4 and L22); or (iii) the acquisition of active efflux (*mef* genes) (Leclercq & Courvalin, 1991; Weisblum, 1995a).

All genes of the *erm* family encode methyltransferases that specifically add one or two methyl residues on the N^6 amino group of the highly conserved adenine A2058 in domain V of the 23S rRNA, which corresponds to the ribosomal PTC (Lai & Weisblum, 1971). This ribosomal methylation conveys cross-resistance to macrolides-lincosamides-streptogramins B (MLS_B) that can be constitutively expressed (the so-called $cMLS_B$ phenotype) or inducibly expressed (the so-called $iMLS_B$ phenotype) [Figure 1] (Weisblum, 1995b). In streptococci, resistance is commonly mediated by two classes of methylases encoded by *erm*(B) and *erm*(TR) genes, and both are usually located chromosomally (Leclercq, 2002). The *erm*(B) gene (previously known as *ermAM*) was first identified in *Streptococcus sanguinis* (Horinouchi, Byeon, & Weisblum, 1983). Because *erm*(TR) shows 82.5% nucleotide identity with the original *erm*(A) gene (Seppälä, Skurnik, Soini, Roberts, & Huovinen, 1998), it has been proposed that *erm*(TR) belongs to the *erm*(A) class (Roberts, et al., 1999). However, the *erm*(A) subclass *erm*(TR) will be referred to as *erm*(TR) throughout this work.

Whereas *erm*(B) is primarily associated with a $cMLS_B$ phenotype and rarely with a $iMLS_B$ phenotype, macrolide resistance conferred by *erm*(TR) in streptococci is usually expressed inducibly even if some $cMLS_B$ *erm*(TR)-positive strains have occasionally been reported [Figure 1] (Leclercq, 2002). In *S. pyogenes*, there is a substantial heterogeneity of susceptibility patterns among inducibly-resistant isolates, which have been subdivided into three distinct subtypes, designated i-MLS_B-A, $iMLS_B$-B, and $iMLS_B$-C [Table 2] (Arpin, Canron, Noury, & Quentin, 1999; Giovanetti, Montanari, Mingoia, & Varaldo, 1999; Giovanetti, Montanari, Marchetti, & Varaldo, 2000; Betriu, et al., 2000; Malbruny, et al., 2002; Bingen, et al., 2002). In contrast to cML_B isolates that are highly resistant to lincosamides, all types of iMLSB are associated with susceptibility to lincosamides [Figure 1 and

Table 2]. Phenotypically, iMLS$_B$-A strains are highly resistant to all macrolides; iMLS$_B$-B strains are highly resistant to 14- and 15-membered macrolides, but are susceptible to 16-membered macrolides; and iMLS$_B$-C strains present low-level resistance to 14- and 15-membered macrolides, but remain susceptible to 16-membered macrolides [Table 2]. cMLS$_B$ and iMLS$_B$-A isolates usually harbor the *erm*(B) gene, while iMLS$_B$-B and iMLS$_B$-C isolates possess the *erm*(TR) gene (Giovanetti, Montanari, Mingoia, & Varaldo, 1999). Notably, resistance to ketolides is observed in cMLS$_B$ and iMLS$_B$-A *S. pyogenes* isolates, while resistance is correlated to the degree of dimethylation by Erm(B) of the A2058 of the 23S rRNA (Douthwaite, Jalava, & Jakobsen, 2005). Even though the Erm(TR) methylase in *S. pyogenes* is not inducible with lincosamides, clindamycin resistance (MIC, 64 mg/L) due to constitutive expression can easily be obtained in vitro (at a frequency of ca. 10^{-7}) from inducible *erm*(TR)-harboring strains [Figure 2] (Fines, Gueudin, Ramon, & Leclercq, 2001). This is due to alterations in the structure of regulatory sequences, which are composed of two leader peptides (15 and 19 amino acids) involved in post-transcriptional regulation (the so-called translational attenuation) that controls the expression of the methylase gene, in a manner similar to that found for *erm*(C) in staphylococci (Weisblum, 1995b; Horinouchi & Weisblum, 1980). These modifications in the promoter sequence (including base changes, insertions/duplications, or deletions) have also been described in clinical isolates (Doktor & Shortridge, 2005; Malhotra-Kumar, et al., 2009). Mechanistically, these modifications are responsible for modifications in the mRNA secondary structure that result in changes in accessibility of the ribosome-binding site and the initiation codon of the methylase to the ribosomes, and subsequently for the translation of the *erm*(TR) transcripts [Figure 3] (Fines, Gueudin, Ramon, & Leclercq, 2001; Doktor & Shortridge, 2005; Malhotra-Kumar, et al., 2009).

Originally identified as part of Tn*917* on a non-conjugative plasmid from *E. faecalis* (Tomich, An, & Clewell, 1980), the *erm*(B) gene in *S. pyogenes* is carried by different elements, depending on whether it is expressed constitutively or inducibly (Varaldo, Montanari, & Giovanetti, 2009). When constitutively expressed, this gene is carried by Tn*916* family elements, such as Tn*3872* (ca. 24 kb) or Tn*6002* (ca. 21 kb) [Figure 4] (Varaldo, Montanari, & Giovanetti, 2009; Brenciani, Bacciaglia, Vecchi, Vitali, Varaldo, & Giovanetti, 2007; Brenciani, Tiberi, Morici, Oryasin, Giovanetti, & Varaldo, 2012; Brenciani, Tiberi, Morroni, Mingoia, Varaldo, & Giovanetti, 2014). Tn*3872* results from the insertion of Tn*917* into *orf9* of Tn*916*, with *erm*(B) thus physically linked to *tet*(M), while conjugal transfer has been demonstrated from *S. pyogenes* to *S. pyogenes* [Figure 4] (Brenciani, Bacciaglia, Vecchi, Vitali, Varaldo, & Giovanetti, 2007). Tn*6002* corresponds to the insertion of the *erm*(B) element (ca. 3 kb) between *orf20* and *orf19* of Tn*916*, which also leads to an *erm*(B)/*tet*(M) linkage, and for which intraspecific conjugal transfer has been demonstrated in *S. pyogenes* (Brenciani, Bacciaglia, Vecchi, Vitali, Varaldo, & Giovanetti, 2007). When inducibly expressed, *erm*(B) is carried by an element originally named Tn*1116* (ca. 48 kb), but now renamed ICE*Sp1116*, since it has been demonstrated to belong to the Tn*GBS* family of integrative and conjugative elements (ICEs) (Brenciani, Bacciaglia, Vecchi, Vitali, Varaldo, & Giovanetti, 2007; Brenciani, Tiberi, Morici, Oryasin, Giovanetti, & Varaldo, 2012; Brenciani, Tiberi, Morroni, Mingoia, Varaldo, & Giovanetti, 2014). This element presents a unique mosaic structure related to the Tn*Gallo*1 from *Streptococcus gallolyticus* with two inserted fragments separated by an IS*1216*: the *erm*(B)-containing fragment (derived from the plasmid pSM19035) and the right-hand portion of *Clostridium difficile* Tn*5397* that contains a truncated *tet*(M) gene [Figure 4] (Brenciani, Tiberi, Morici, Oryasin, Giovanetti, & Varaldo, 2012).

Different *erm*(TR)-carrying ICE elements have been described in *S. pyogenes*: ICE 10750-RD.2 (ca. 49 kb) and ICE*Sp1108* (ca. 45 kb) in tetracycline-susceptible strains, and ICE*Sp2905* (ca. 66 kb) in tetracycline-resistant strains, due to the presence of the *tet*(O) gene [Figure 5] (Varaldo, Montanari, & Giovanetti, 2009; Brenciani, Tiberi, Bacciaglia, Petrelli, Varaldo, & Giovanetti, 2011; Giovanetti, Brenciani, Tiberi, Bacciaglia, & Varaldo, 2012). While the element ICE 10750-RD.2 is integrated into an *hsdM* chromosomal gene-encoding host DNA restriction/modification methyltransferase, both ICE*Sp1108* and ICE*Sp2905* are integrated in the chromosome at the 3' end of the conserved RNA uracil methyltransferase (*rum*) gene (Varaldo, Montanari, & Giovanetti, 2009; Brenciani, Tiberi, Bacciaglia, Petrelli, Varaldo, & Giovanetti, 2011). The ICE*Sp2905* results from one ICE (ICE*Sp2907*) being integrated into another (ICE*Sp2906*), with the former containing *erm*(TR) and the latter containing *tet*(O), and the whole inserted into a scaffold of clostridial origin [Figure 5] (Giovanetti, Brenciani,

Tiberi, Bacciaglia, & Varaldo, 2012). Notably, all these structures share an almost identical conserved core sequence (ca. 2 kb) that includes *erm*(TR) and two adjacent antibiotic resistance (tetronasin and spectinomycin) genes [Figure 5] (Brenciani, Tiberi, Bacciaglia, Petrelli, Varaldo, & Giovanetti, 2011). Note that *erm*(TR) can be transferred by conjugation to susceptible recipients of *S. pyogenes* and other Gram-positive bacteria (Giovanetti, et al., 2002). In addition, it has been shown that *Peptostreptococcus* spp. may serve as an important reservoir for *erm*(TR)-mediated macrolide resistance (Reig, Galan, Baquero, & Perez-Diaz, 2001).

Another methylase gene, *erm*(T) (previously named *ermGT*), has also been identified in *S. pyogenes* that expresses an $iMLS_B$ resistance phenotype (Woodbury, et al., 2008). Originally described in *Lactobacillus reuteri* in a chromosomal location (Tannock, et al., 1994), this gene is borne on a small mobilizable plasmid (ca. 5 kb) in *S. pyogenes* (Woodbury, et al., 2008; DiPersio, DiPersio, Beach, Loudon, & Fuchs, 2011).

Macrolide resistance can also be due to ribosomal mutations, either in the domain V of 23S rRNA (*rrn*) operons or in the ribosomal proteins L4 and L22 (*rplD* and *rplV* genes, respectively) (Leclercq, 2002). In *S. pyogenes*, two clinical isolates with such target mutations were described for the first time in 2002 (Malbruny, et al., 2002). The former strain exhibited a C2611U mutation in the domain V of all six 23S rRNA copies, and was phenotypically resistant to azithromycin and clindamycin, but remained susceptible to erythromycin and spiramycin [Figures 2 and 6, Table 2] (Malbruny, et al., 2002). The latter strain harbored an insertion of six nucleotides in the *rplD* gene sequence, which resulted in a KG insertion after position 69 in the L4 ribosomal protein, and was phenotypically resistant to azithromycin and spiramycin, but remained susceptible to erythromycin (borderline) and clindamycin [Figures 2 and 6, Table 2] (Malbruny, et al., 2002). Since then, additional ribosomal mutations in the L4 ribosomal protein have been reported in a few clinical isolates [Figure 6]: a two-amino-acid deletion (65RW66), an RA insertion after position 73, and a TG deletion at positions 70 to 71 (Bingen, et al., 2002; Bozdogan, Appelbaum, Ednie, Grivea, & Syrogiannopoulos, 2003). Other mutations in 23S rRNA have also been identified in clinical isolates [Figure 6]: a A2058G mutation (5/6 mutated *rrn* copies) with resistance to all macrolides, ketolides, and lincosamides (Jalava, Vaara, & Huovinen, 2004); A2058G (n=4) and A2059G (n=2) mutations in clinical isolates that present a $cMLS_B$ phenotype (Tanz, et al., 2004; Richter, et al., 2005); dual mutations (A2058G and U2166C) in seven clonally-related strains resistant to macrolides and ketolides (Farrell, Shackcloth, Barbadora, & Green, 2006); and in two isolates with a A2058G substitution with a $cMLS_B$ phenotype (Montes, Tamayo, Mojica, García-Arenzana, Esnal, & Pérez-Trallero, 2014).

Active efflux due to *mef* genes is responsible for resistance only to 14- and 15-membered macrolides (the so-called M phenotype), while there is no resistance to 16-membered macrolides, lincosamides, and streptogramins (Sutcliffe, Tait-Kamradt, & Wondrack, 1996). Mef proteins are proton-dependent efflux pumps that belong to the major facilitator superfamily (MFS) with 12 transmembrane segments (TMS) (Poole, 2005). Several allelic variants or subclasses of the *mef* gene have been described, mainly *mef*(A) originally reported in *S. pyogenes* (Clancy, et al., 1996) and *mef*(E) later identified in *S. pneumoniae* (Tait-Kamradt, et al., 1997). Because these two *mef* genes show 90% nucleotide identity, it has been proposed to consider them as a single class, designated *mef*(A) (Roberts, et al., 1999). However, some authors argued for the need to distinguish these determinants, since there are major differences between them (Klaassen & Mouton, 2005). First, erythromycin MICs associated with the *mef*(A) gene (MIC_{50}, 16 μg/ml) are higher than those associated with *mef*(E) (MIC_{50}, 8 μg/ml) (Amezaga, Carter, Cash, & McKenzie, 2002; Blackman Northwood, et al., 2009). Second, and most importantly, these determinants are carried by completely different genetic elements (Del Grosso, et al., 2002). Note that the *mef*(A) subtype is by far the most prevalent *mef* allele among *S. pyogenes* clinical isolates (Blackman Northwood, et al., 2009; Sangvik, Littauer, Simonsen, Sundsfjord, & Dahl, 2005; Ardanuy, et al., 2005).

In *S. pneumoniae*, the *mef*(A) gene is part of Tn*1207.1* (ca. 7 kb), a 7.2-kb defective transposon integrated into the chromosome, while the *mef*(E) is borne by a transferable macrolide efflux genetic assembly (mega) element (ca. 5 kb) that is integrated into composite transposons Tn*2009* (ca. 23 kb) or Tn*2010* (ca. 26 kb) (Varaldo, Montanari, & Giovanetti, 2009). In *S. pyogenes*, the *mef*(A) gene is carried by larger and mobile composite

elements (all chimeric in nature since they result from an insertion of a transposon into a prophage) that are different, depending on whether the isolates are susceptible or resistant to tetracyclines [Figure 7] (Varaldo, Montanari, & Giovanetti, 2009; Giovanetti, Brenciani, Lupidi, Roberts, & Varaldo, 2003; Banks, Porcella, Barbian, Martin, & Musser, 2003; Brenciani, et al., 2004; Giovanetti, Brenciani, Vecchi, Manzin, & Varaldo, 2005; Iannelli, Santagati, Oggioni, Stefani, & Pozzi, 2014). In tetracycline-susceptible isolates, a regular Tn*1207.1* forms the left end of the 52-kb Φ1207.3 (formerly Tn*1207.3*) (Iannelli, Santagati, Oggioni, Stefani, & Pozzi, 2014; Santagati, et al., 2003), or is part of Φ10394.4 (ca. 59 kb) [Figure 7] (Banks, Porcella, Barbian, Martin, & Musser, 2003; Banks, et al., 2004), with both integrated into the same chromosomal gene (*comEC*) and inserted into the same prophage (Varaldo, Montanari, & Giovanetti, 2009; Brenciani, et al., 2004). It appears that Φ1207.3 is more common than Φ10394.4 in *mef*(A)-positive, tetracycline-susceptible *S. pyogenes* isolates, and it has been shown that it was transferable both intra- and interspecifically (Varaldo, Montanari, & Giovanetti, 2009; Santagati, et al., 2003). In tetracycline-resistant isolates, evidence has emerged of a genetic linkage between *mef*(A) and *tet*(O) in a mobile phage-like element (Giovanetti, Brenciani, Lupidi, Roberts, & Varaldo, 2003; Giovanetti, Brenciani, Vecchi, Manzin, & Varaldo, 2005). Actually, this linkage corresponds to a variety of related *tet*(O)-*mef*(A) elements in which *mef*(A) is contained in a range of changeable and defective variants of Tn*1207.1* (Brenciani, et al., 2004; Giovanetti, Brenciani, Vecchi, Manzin, & Varaldo, 2005). The most common representative is the transferable Φm46.1 element (ca. 60 kb) that is integrated into the chromosome within the 23S rRNA uracil methyltransferase gene (Giovanetti, Brenciani, Lupidi, Roberts, & Varaldo, 2003; Giovanetti, et al., 2014; Brenciani, Bacciaglia, Vignaroli, Pugnaloni, Varaldo, & Giovanetti, 2010). As described in *S. pneumoniae*, *mef*(E) is part of a typical mega element and is sometimes physically associated with *tet*(M) in the composite transposon Tn*2009* [Figure 8] (Del Grosso, et al., 2011). Note that both *mef*(A) and *mef*(E) alleles are always adjacent to the *msr*(D) gene originally named *mel* (Varaldo, Montanari, & Giovanetti, 2009; Ambrose, Nisbet, & Stephens, 2005). Similar to *msr*(A) in staphylococci, this gene also codes for an ABC family protein that contains the two prototypical ATP-binding domains, but lacks any obvious TMS. Consequently, it has not been clearly proven if this class 2 ABC protein functions as a drug exporter (Davidson, Dassa, Orelle, & Chen, 2008). Nonetheless, *msr*(D) alone is sufficient to confer a 64-, 128-, and 16-fold increase in MICs of erythromycin, clarithromycin, and telithromycin, respectively, but not to streptogramins, which distinguishes it from Msr(A) (Daly, Doktor, Flamm, & Shortridge, 2004).

Additional *mef* alleles have been detected in *S. pyogenes*, such as *mef*(I) and *mef*(O), as well as diverse mosaic variants (Sangvik, Littauer, Simonsen, Sundsfjord, & Dahl, 2005; Mingoia, et al., 2007). The subclass *mef*(I), 91% and 94% identical to *mef*(A) and *mef*(E), respectively, was first identified in *S. pneumoniae* and then among *S. pyogenes* clinical isolates (Blackman Northwood, et al., 2009; Mingoia, et al., 2007). In *S. pneumoniae*, *mef*(I) is embedded in a genetic element that also contains *tet*(M) and *catQ*. This element, designated 5216IQ complex (ca. 30 kb), consists of two portions, one derived from Tn*5252* and Tn*916* (which harbors a copy of *tet*(M) not expressed due to the lack of the promoter, the ribosome-binding site, and a part of the leader peptide) and another called IQ module, which encloses *mef*(I) and *catQ*, a gene that encodes a chloramphenicol acetyltransferase found in *Clostridium perfringens* [Figure 8] (Mingoia, et al., 2007). In *S. pyogenes*, *mef*(I) and *catQ* are also linked through a partial fragment of the 5216IQ complex, designated the 5216IQ-like complex, with a defective IQ module and a partial or absent Tn*916* (Del Grosso, et al., 2011). Note that all these IQ elements are ICEs that belong to the Tn*5253* family, and are named ICE*Spy*029IQ (ca. 55 kb) and ICE*Spy*005IQ (ca. 50 kb) in *S. pyogenes* [Figure 8] (Mingoia, et al., 2014; Mingoia, Morici, Brenciani, Giovanetti, & Varaldo, 2014). The *mef*(O) allele was first described in *S. pyogenes*, and exhibits 88% and 89% nucleotide identity with *mef*(A) and *mef*(E), respectively (Sangvik, Littauer, Simonsen, Sundsfjord, & Dahl, 2005).

Even if there are significant differences in the distribution of MLS resistance genes that depend on the country or year of isolation, the most common gene present in an international study appeared to be *mef*(A) (ca. 45%), followed by *erm*(B) (30%), and *erm*(TR) (ca. 25%) (Farrell, Morrissey, Bakker, & Felmingham, 2002).

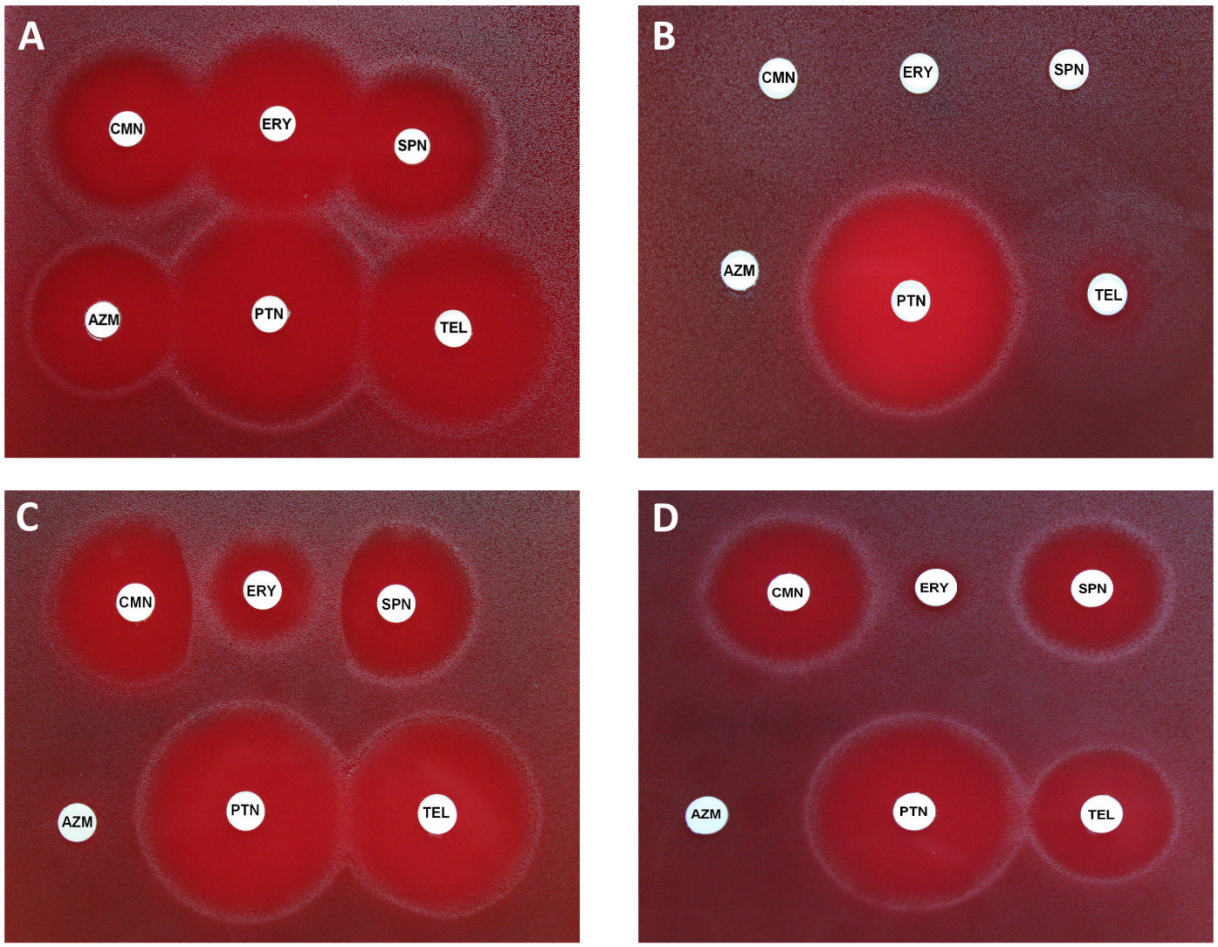

Figure 1. Common phenotypes of MLS resistance in *S. pyogenes*. (A) Wild-type susceptible strain (*S. pyogenes* ATCC 19615); (B) *S. pyogenes* containing an *erm*(B) gene constitutively expressed; (C) *S. pyogenes* containing an *erm*(TR) gene inducibly expressed (a D-shaped zone can be observed between ERY and CMN/SPN); (D) *S. pyogenes* resistant to ERY and AZM by *mef*(A)-mediated efflux (note the absence of the D-shaped zone). AZM, azithromycin; CMN, clindamycin; ERY, erythromycin; PTN, pristinamycin; SPN, spiramycin; TEL, telithromycin. L, lincomycin (Photo credits: Michel Auzou).

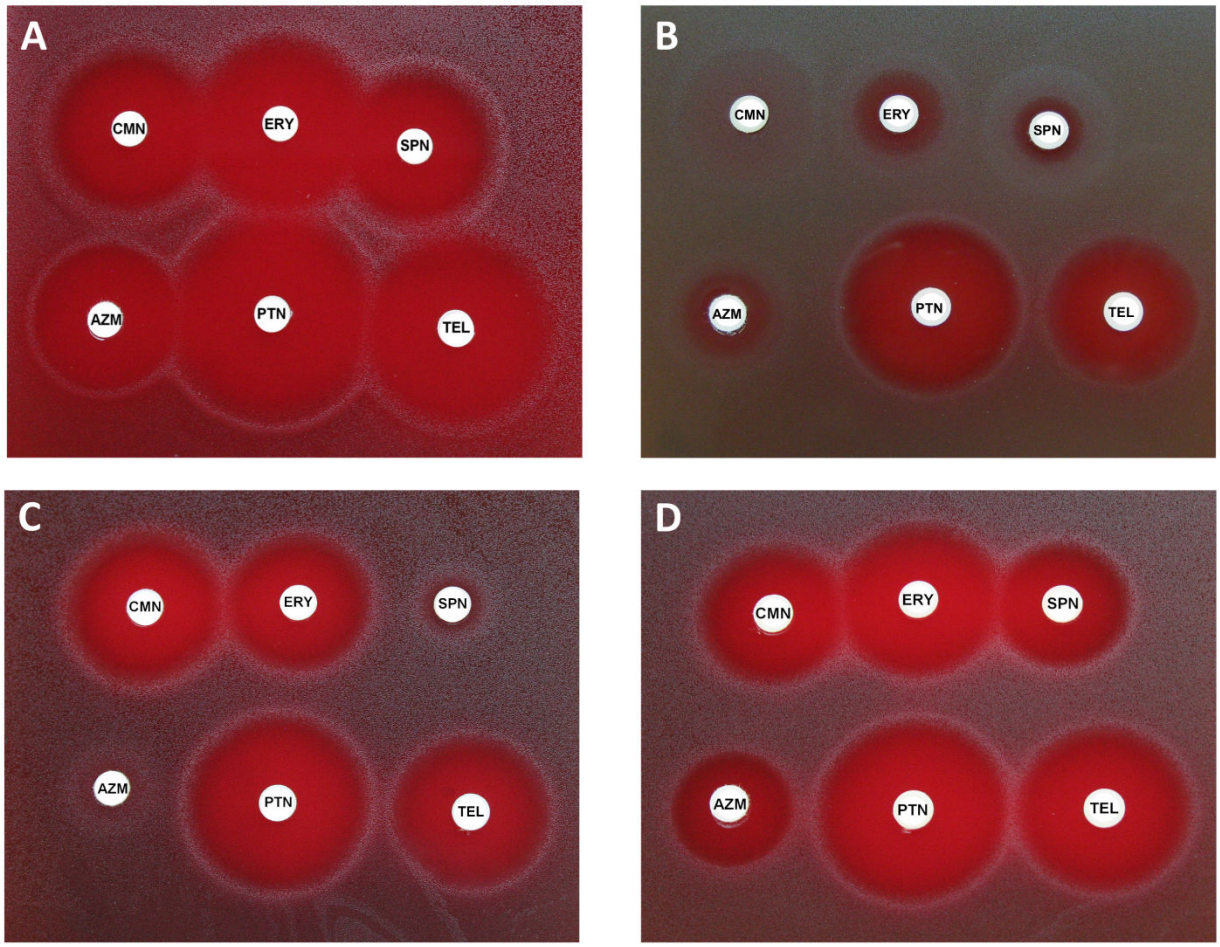

Figure 2. Unusual phenotypes of MLS resistance in *S. pyogenes*. (A) Wild-type susceptible strain (*S. pyogenes* ATCC 19615); (B) *S. pyogenes* containing an *erm*(TR) gene constitutively expressed due to attenuator alterations (Fines, Gueudin, Ramon, & Leclercq, 2001); (C) *S. pyogenes* 237 with L4 ribosomal protein mutation (KG insertion after position 69) (Malbruny, et al., 2002); (D) *S. pyogenes* 544 with 23S rRNA mutation (C2611U) (Malbruny, et al., 2002). AZM, azithromycin; CMN, clindamycin; ERY, erythromycin; PTN, pristinamycin; SPN, spiramycin; TEL, telithromycin. L, lincomycin (Photo credits: Michel Auzou).

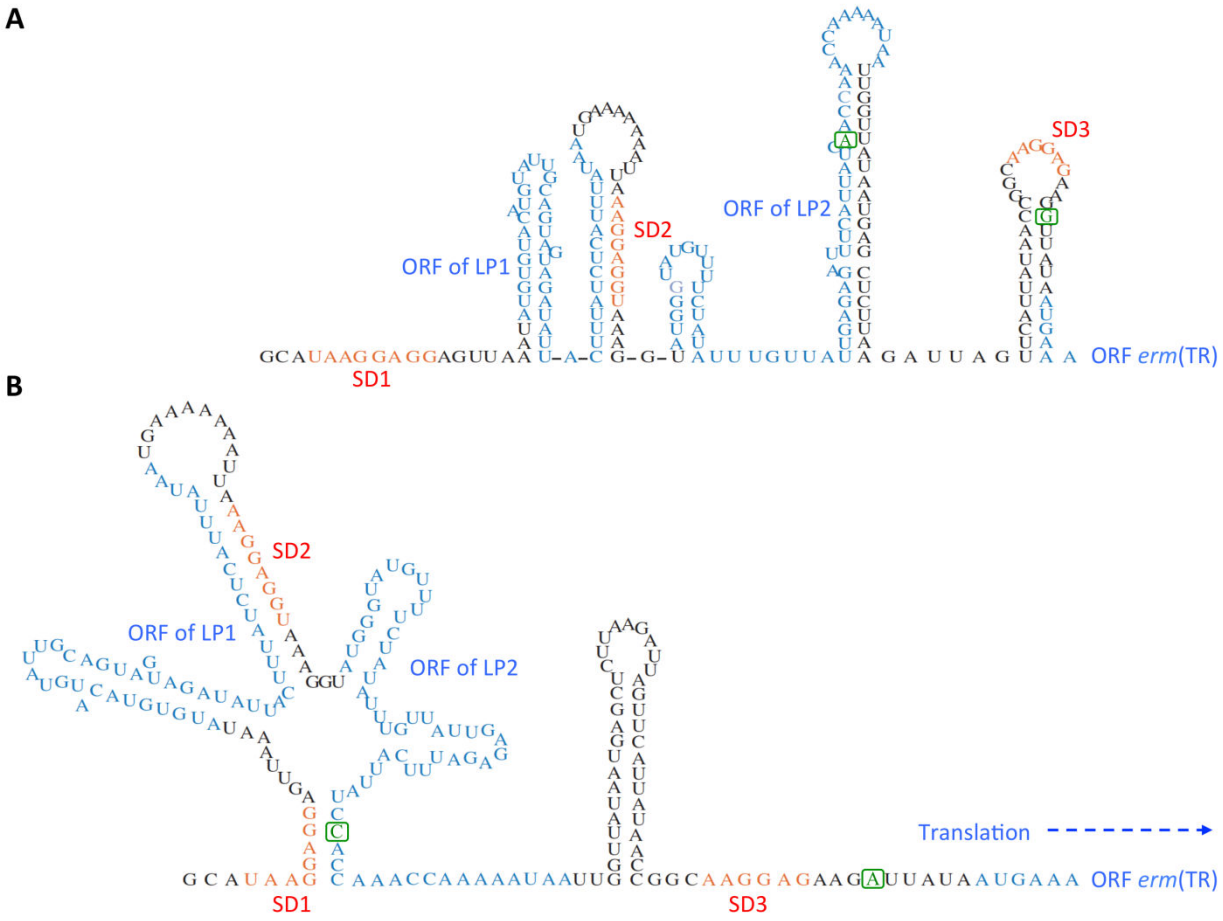

Figure 3. Predicted mRNA secondary structures of the *erm*(TR) regulatory region (called the attenuator). (A) Conformational isomer of the wild-type *erm*(TR) mRNA (Malhotra-Kumar, et al., 2009). Open reading frames (ORFs) are indicated in blue: LP1, leader peptide 1 (15 amino acids); LP2, leader peptide 2 (19 amino acids). Proposed Shine-Dalgarno (SD) sequences are indicated in red: SD1, SD2, and SD3 correspond to ribosome-binding sites for LP1, LP2, and *erm*(TR), respectively. (B) Proposed structure of the *erm*(TR) mRNA in presence of A137C and G205A mutations (boxed and colored in green), responsible for the releasing of the SD3 and the initiation codon of *erm*(TR) and then leading to translation (Malhotra-Kumar, et al., 2009).

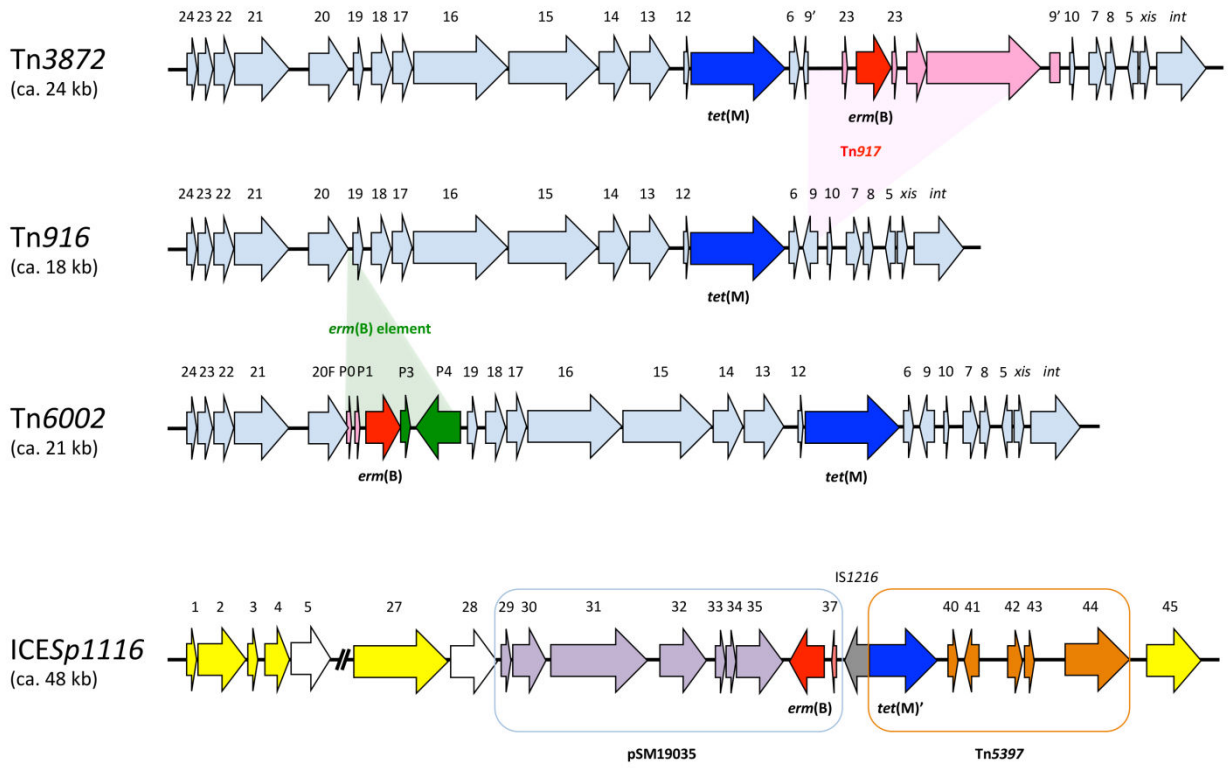

Figure 4. *erm*(B)-carrying genetic elements (Varaldo, Montanari, & Giovanetti, 2009; Brenciani A. , et al., 2007; Brenciani A. , et al., 2012; Brenciani A. , et al., 2014). Open reading frames (ORFs) are indicated by horizontal arrows; *erm*(B) and *tet*(M) genes are indicated in red and blue, respectively. Light blue arrows indicate Tn*916* and Tn*916*-related ORFs other than *tet*(M). Pink, green, yellow, light purple, and orange arrows indicate ORFs from Tn*917*, the *erm*(B) element, Tn*Gallo*1, pSM19035, and Tn*5397* elements, respectively.

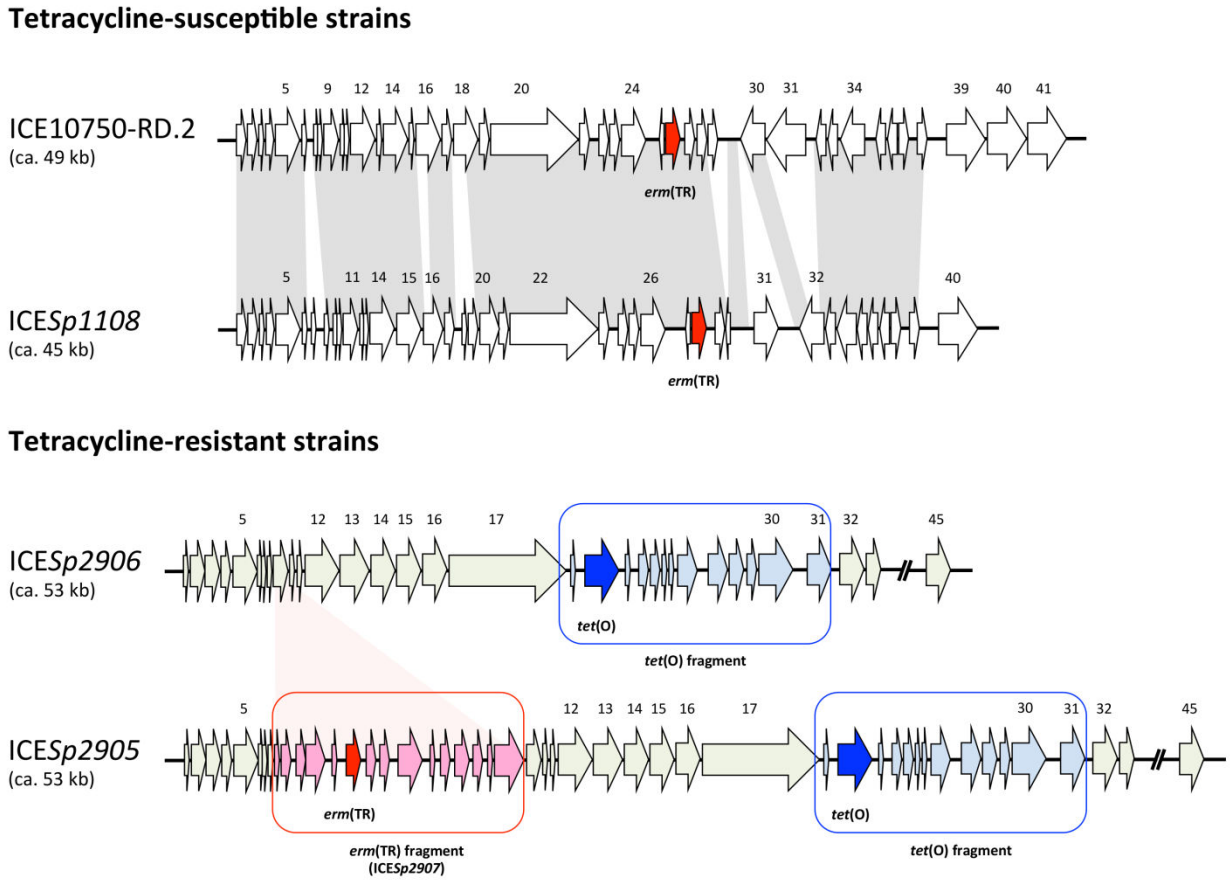

Figure 5. *erm*(TR)-carrying genetic elements that depend on tetracycline susceptibility/resistance (Varaldo, Montanari, & Giovanetti, 2009; Brenciani A. , et al., 2011; Giovanetti E. , Brenciani, Tiberi, Bacciaglia, & Varaldo, 2012). Open reading frames (ORFs) are indicated by horizontal arrows. *erm*(TR) and *tet*(O) genes are indicated in red and blue, respectively. Gray areas between ORF maps indicate areas with >90% homology. Light blue and pink arrows indicate ORFs from *tet*(O) and *erm*(TR) fragments (both boxed), respectively.

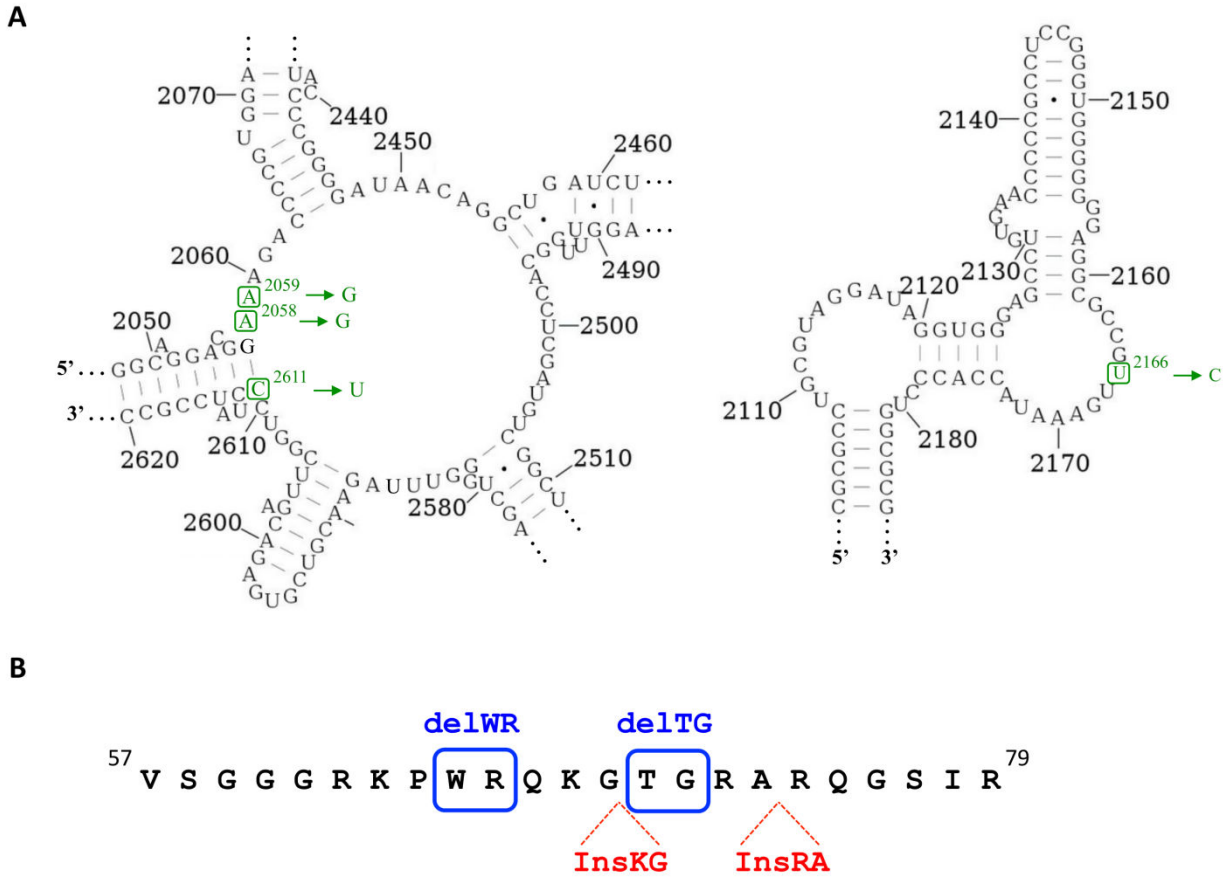

Figure 6. Ribosomal mutations responsible for MLS resistance in *S. pyogenes*. (A) Secondary structure of the domain V of 23S rRNA. Positions (according to *E. coli* numbering) and mutations described in *S. pyogenes* are indicated in green (Tanz, et al., 2004; Richter, et al., 2005; Montes M. , et al., 2014; Malbruny, et al., 2002; Jalava, Vaara, & Huovinen, 2004; Farrell, Shackcloth, Barbadora, & Green, 2006). (B) Partial amino acid sequence (from position 57 to 79, *S. pyogenes* numbering) of the L4 ribosomal protein (*rplD* gene). Deletions and insertions reported in *S. pyogenes* are indicated in blue and red, respectively (Malbruny, et al., 2002; Bingen, et al., 2002; Bozdogan, Appelbaum, Ednie, Grivea, & Syrogiannopoulos, 2003).

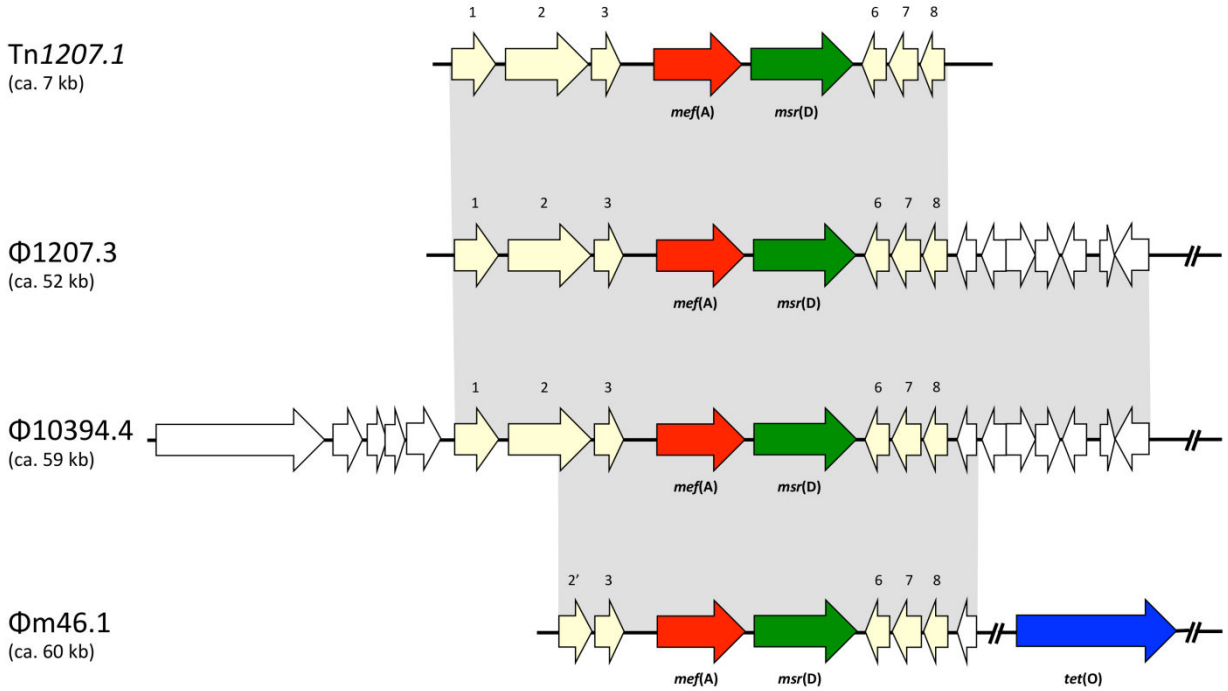

Figure 7. *mef*(A)-carrying genetic elements (Varaldo, Montanari, & Giovanetti, 2009; Giovanetti E. , Brenciani, Lupidi, Roberts, & Varaldo, 2003; Brenciani, et al., 2004; Giovanetti E. , Brenciani, Vecchi, Manzin, & Varaldo, 2005; Iannelli, Santagati, Oggioni, Stefani, & Pozzi, 2014; Iannelli, Santagati, Oggioni, Stefani, & Pozzi, 2014). Open reading frames (ORFs) are indicated by horizontal arrows. *erm*(TR), *msr*(D), and *tet*(O) genes are indicated in red, green, and blue, respectively. Gray areas between ORF maps indicate areas with >90% homology. Light yellow arrows indicate ORFs from Tn*1207.1*.

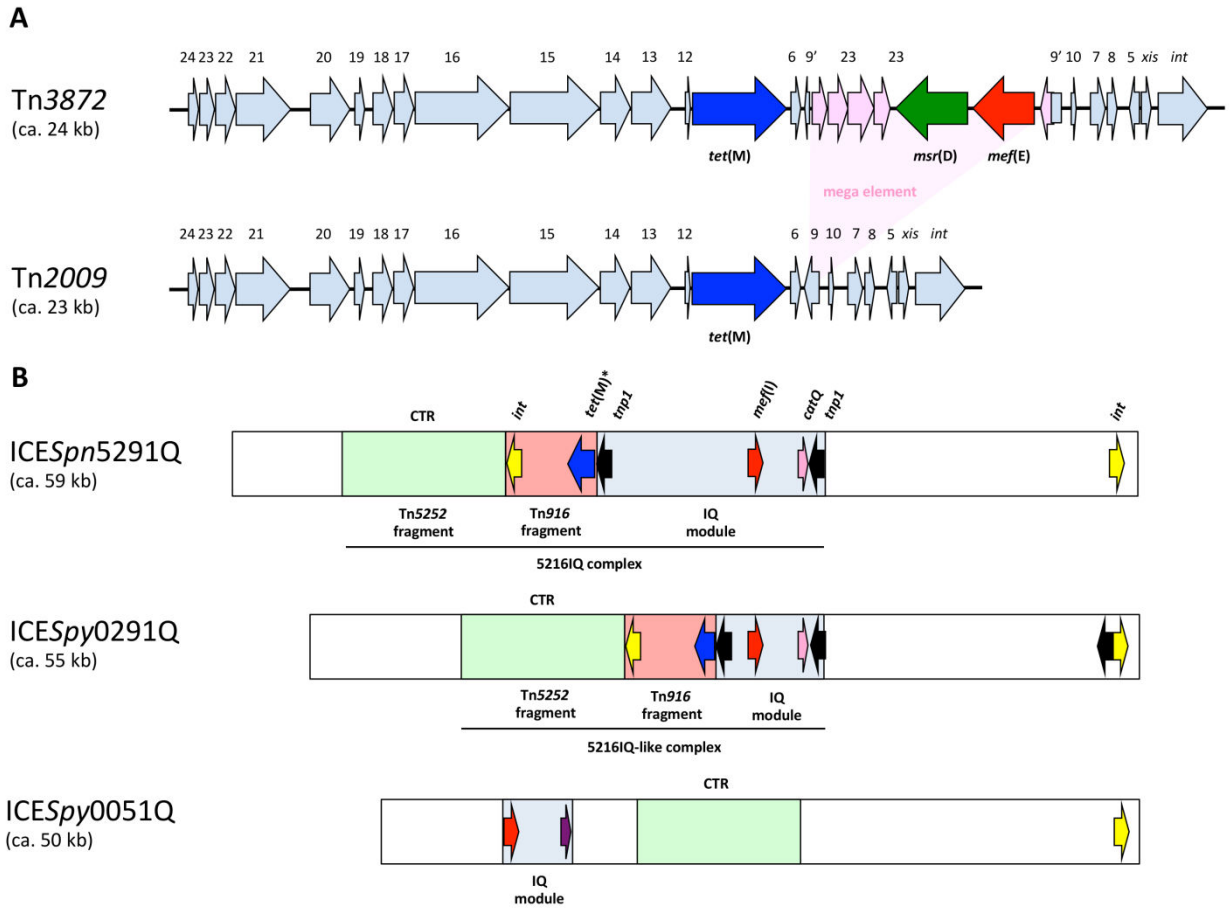

Figure 8. Genetic elements carrying *mef* genes other than *mef*(A) (Varaldo, Montanari, & Giovanetti, 2009; Del Grosso, et al., 2011; Mingoia M. , et al., 2014; Mingoia M. , Morici, Brenciani, Giovanetti, & Varaldo, 2014). Open reading frames (ORFs) are indicated by horizontal arrows. (A) *mef*(E)-carrying elements. *mef*(E), *msr*(D), and *tet*(M) genes are indicated in red, green, and blue, respectively. Light blue arrows indicate Tn*3872* ORFs other than *tet*(M). Pink arrows indicate ORFs from the mega element (macrolide efflux genetic assembly). (B) *mef*(I)-carrying elements. *mef*(I), *tet*(M)* (silent copy of *tet*(M) due to the lack of the promoter, the ribosome-binding site, and a part of the leader peptide), *catQ* genes are indicated in red, blue, and pink, respectively. The Tn*5252* fragment (which corresponds to the conjugal transfer-related [CTR] functional module), the Tn*916* fragment, and the IQ module are indicated in boxes colored in green, red, and blue, respectively. ORFs coding for transposase (*tnp1*) and integrase (*int*) genes are also indicated in black and yellow, respectively.

Table 2. Profiles of susceptibility to MLS antibiotics in *S. pyogenes* according to phenotypes/ genotypes of resistance (Arpin, Canron, Noury, & Quentin, 1999; Giovanetti, Montanari, Mingoia, & Varaldo, 1999; Giovanetti, Montanari, Marchetti, & Varaldo, 2000; Betriu, et al., 2000; Malbruny, et al., 2002; Bingen, et al., 2002).

Phenotype[a]	MIC (μg/ml)								
	WT	$cMLS_B$	$iMLS_B$-A	$iMLS_B$-B	$iMLS_B$-C	M	Other		
Genotype	-	*erm*(B)	*erm*(B)	*erm*(A)	*erm*(A)	*mef*(A)	23S rRNA (C2611U)	23S rRNA (A2058G)	L4
14-membered macrolides									
Erythromycin	0.03-0.06	≥128	≥128	≥128	2-8	8-16	0.12	≥256	0.5-2
Clarithromycin	≤0.01-0.06	≥128	≥128	≥128	1-2	8	-	-	1

Table 2. continued from previous page.

Phenotype[a]	MIC (µg/ml)								
	WT	$cMLS_B$	$iMLS_B$-A	$iMLS_B$-B	$iMLS_B$-C	M	Other		
15-membered macrolides									
Azithromycin	0.12-0.25	≥128	≥128	≥128	8-16	4-8	2	≥256	1-8
16-membered macrolides									
Spiramycin	0.25-0.5	≥128	≥128	0.25-0.5	0.25-1	0.25-0.5	0.5	64	64
Josamycin	0.25-0.5	≥128	≥128	0.03-0.12	0.03-0.12	0.03-0.12	-	64	1-2
Ketolides									
Telithromycin	≤0.01-0.06	2-8	4-8	≤0.01-0.06	≤0.01	0.12-0.25	0.06	1-16	0.06-0.12
Lincosamides									
Clindamycin	0.03-0.12	≥128	0.12-0.25	0.06-0.12	0.03-0.06	0.03-0.12	2	16	0.06-0.12
Streptogramins									
Quinupristin-dalfopristin	0.12-0.25	0.12-0.5	0.12-0.5	0.12-0.5	0.12-0.5	0.12-0.5	0.25	-	0.25

[a] WT, wild-type; $cMLS_B$, constitutive MLS_B resistance; $iMLS_B$, inducible MLS_B resistance (existence of 3 types: A, B, and C).

Fluoroquinolones

Fluoroquinolones (FQs) are bactericidal agents that are widely used in both human and veterinary medicine. The targets of FQ molecules are the type II topoisomerases (namely, DNA gyrase and DNA topoismerase IV) that are both heterotetramers that consist of two subunits, $GyrA_2B_2$ and $ParC_2E_2$ (Hawkey, 2003). Due to the greater clinical use of FQ, bacterial resistance to these compounds has been increasingly reported during the last three decades. In Gram-positive cocci, FQ resistance results from target alterations due to point mutations that occur primarily in 120-bp conserved fragments, the so-called quinolone resistance-determining regions (QRDRs), of both *parC* and *gyrA* genes (Hooper, 2002). Active efflux of hydrophilic FQ molecules is also possible (Poole, 2005). Note that plasmid-mediated resistance has been recently described in *Enterobacteriaceae*, but has not yet been found in Gram-positive cocci (Cattoir & Nordmann, 2009).

In streptococci, high-level FQ resistance is only mediated by target modifications, whereas active efflux may confer low-level resistance in *S. pneumoniae* and viridans group streptococci (Guerin, Varon, Hoï, Gutmann, & Podglajen, 2000). However, the latter mechanism has not yet been detected in *S. pyogenes* (Malhotra-Kumar, et al., 2005). Importantly, the emergence and spread of FQ resistance may also be due to interspecies recombination within the *parC* QRDR and the acquisition of resistance via horizontal gene transfer from *S. dysgalactiae* subsp. *equisimilis*, which shares a global gene pool with *S. pyogenes* (Pletz, et al., 2006; Duesberg, et al., 2008; Pinho, Melo-Cristino, & Ramirez, 2010). Due to the lack of natural transformability of *S. pyogenes*, the genetic information is likely to be transferred by transduction through bacteriophages (Pinho, Melo-Cristino, & Ramirez, 2010). Different substitutions have been reported in clinical isolates of *S. pyogenes*: Ser81Ala/Tyr/Phe, Glu85Ala and Met99Leu in GyrA; Ser79Ala/Tyr/Phe, Asp83Asn, Asp91Asn, Ala121Val, Gly128Val, and Ser140Pro [Figure 9] (Yan, et al., 2000; Alonso, Galimand, & Courvalin, 2002; Richter, et al., 2003; Reinert, Lütticken, & Al-Lahham, 2004; Albertí, et al., 2005; Orscheln, et al., 2005) Note that highly-resistant strains (levofloxacin MIC ≥16 mg/L) generally possess several mutations in the QRDRs of both *gyrA* and *parC* genes (Yan, Fox, Holland, Stock, Gill, & Fedorko, 2000; Richter, et al., 2003; Reinert, Lütticken, & Al-Lahham, 2004; Rivera, et al., 2005; Alonso, Mateo, Ezpeleta, & Cisterna, 2007; Wajima, et al., 2013), while low-level resistance (levofloxacin MIC, 2-4 mg/L) are commonly due to a single substitution in ParC (Alonso, Galimand, & Courvalin, 2002; Orscheln, et al., 2005; Rivera, et al., 2005; Wajima, et al., 2013; Yan, et al., 2008).

While high-level resistance remains exceptional among clinical isolates, FQ-non-susceptible strains that exhibit a low-level resistance (usually defined as ciprofloxacin MIC from 2 to 8 mg/L) may be common in some countries, such as Belgium (ca. 5% in 1999–2002, ca. 7% in 2003–2006), the USA (ca. 11% in 2002–2003), Spain (ca. 3% in 1999–2004, ca. 13% in 2005–2007), Portugal (ca. 5% in 1999–2006), Japan (ca. 16% in 2010–2012), and Italy (ca. 9% in 2012) (Malhotra-Kumar, et al., 2009; Malhotra-Kumar, et al., 2005; Orscheln, et al., 2005; Wajima, et al., 2013; Petrelli, et al., 2014). Notably, a vast majority of these isolates belong to *emm*-type 6, while some other genotypes (such as *emm*75 and *emm*89) have also been recorded (Malhotra-Kumar, et al., 2009; Malhotra-Kumar, Lammens, Chapelle, Mallentjer, Weyler, & Goossens, 2005; Montes, Tamayo, Orden, Larruskain, & Perez-Trallero, 2010; Yan, et al., 2008; Petrelli, Di Luca, Prenna, Bernaschi, Repetto, & Vitali, 2014; Alonso R. , Mateo, Galimand, Garaizar, Courvalin, & Cisterna, 2005). Indeed, *emm*-type 6 *S. pyogenes* seems to have an intrinsic reduced susceptibility to FQs, as a result of a polymorphism at position 79 of the *parC* gene (Orscheln, et al., 2005; Montes, Tamayo, Orden, Larruskain, & Perez-Trallero, 2010). However, despite this polymorphism, these strains are not more likely to develop high-level FQ resistance when compared to other *emm* types (Billal, et al., 2007).

Tetracyclines

Tetracyclines are broad-spectrum antibiotics with bacteriostatic activity. They inhibit protein synthesis by binding to the bacterial 30S ribosomal subunit and blocking entry of the amino-acyl tRNA into the A site of the ribosome (Chopra & Roberts, 2001). The prevalence of tetracycline resistance varies greatly (approximately from 10 to 40%), depending on the countries or regions reporting (Brown & Rybak, 2004; Al-Lahham, De Souza, Patel, & Reinert, 2005; Jones, Sader, & Flamm, 2013; Jasir, et al., 2000; Hammerum, Nielsen, Agersø, Ekelund, & Frimodt-Moller, 2004; Ayer, et al., 2007).

Three mechanisms of tetracycline resistance have been described: drug inactivation, active efflux, and ribosomal protection (Chopra & Roberts, 2001). Tetracycline/minocycline resistance is often encoded by the *tet*(M) gene in Gram-positive bacteria, and more rarely by *tet*(O), *tet*(Q), *tet*(S), *tet*(T), and *tet*(W) genes, which all encode ribosomal protection proteins (Chopra & Roberts, 2001). Tetracycline resistance alone is usually due to the efflux genes *tet*(K) and *tet*(L).

In *S. pyogenes*, *tet*(M) is also the major resistance determinant, while *tet*(O), *tet*(S), and *tet*(T) have all been reported (Hammerum, Nielsen, Agersø, Ekelund, & Frimodt-Moller, 2004; Clermont, Chesneau, De Cespédès, & Horaud, 1997; Betriu, et al., 2002; Betriu, Culebras, Rodríguez-Avial, Gómez, Sánchez, & Picazo, 2004; Nielsen, Hammerum, Ekelund, Bang, Pallesen, & Frimodt-Møller, 2004; Dundar, Sayan, & Tamer, 2010). The predominance of *tet*(M) may be explained by the fact that this gene is carried by conjugative transposons (such as Tn*916*) or by composite structures (such as Tn*3701*), which can easily translocate from chromosome to chromosome (Le Bouguénec, de Cespédès, & Horaud, 1988; Burdett, 1990). While a highly significant association between *tet*(M) and *erm*(B) has been shown, there is also evidence of a genetic linkage between *tet*(O) and *erm*(TR)/*mef*(A) [Figures 4, 5, and 7] (Giovanetti, Brenciani, Lupidi, Roberts, & Varaldo, 2003; Brenciani, et al., 2004; Ayer, Tewodros, Manoharan, Skariah, Luo, & Bessen, 2007).

Tigecycline is a novel glycylcycline that has potent activity against a wide spectrum of both Gram-positive and -negative bacteria, including those that are resistant to classical tetracyclines. Indeed, it is not affected by the two major mechanisms of tetracycline resistance, i.e. active efflux and ribosomal protection (Borbone, et al., 2008). For instance, MICs of tetracycline, minocycline, and tigecycline against *tet*(M)- and *tet*(O)-positive *S. pyogenes* strains are 32, 2-4, and 0.03-0.06 µg/ml and 16-32, 2, and 0.03 µg/ml, respectively (Borbone, et al., 2008).

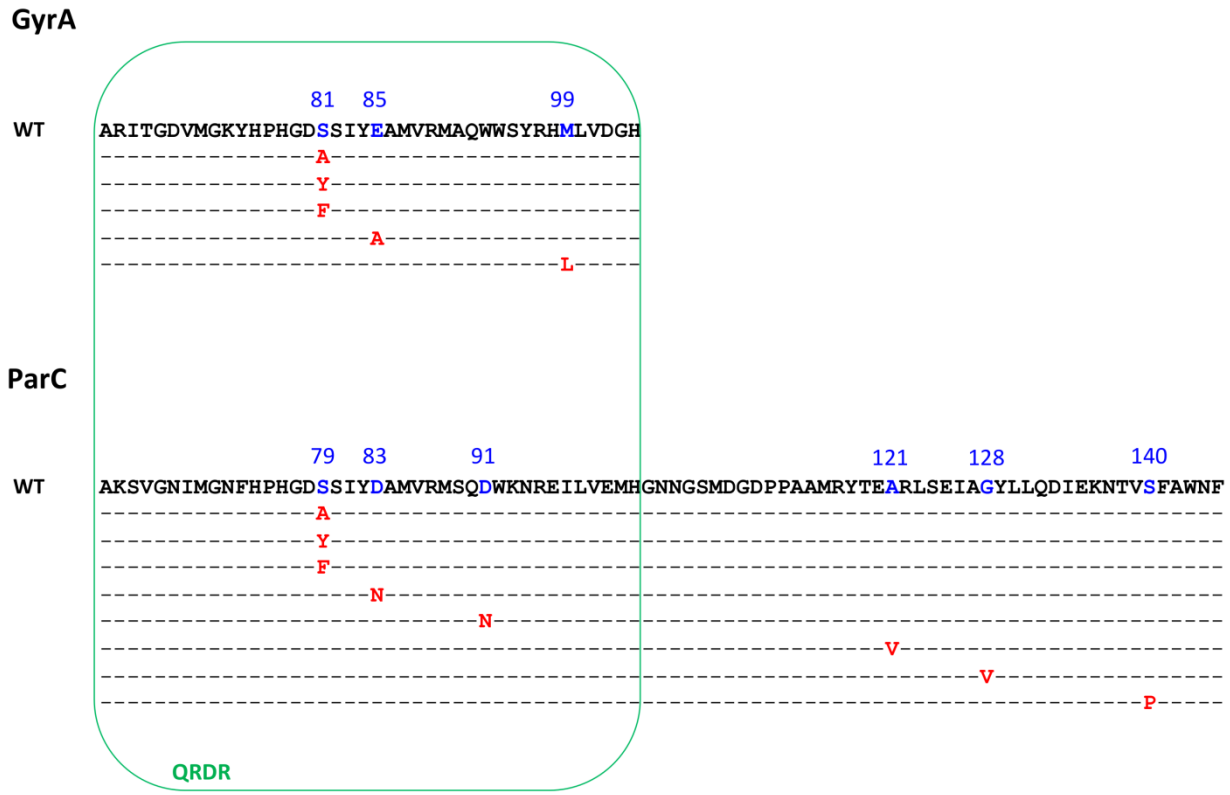

Figure 9. Amino acid sequences in *S. pyogenes* of quinolone resistance-determining regions (QRDRs) of GyrA (amino acids from 65 to 104, which correspond to positions 67-106 in *E. coli* numbering) and ParC (amino acids from 63 to 102, which correspond to positions 64-103 in *E. coli* numbering). Classical QRDRs (40 amino acids) are boxed in green. Mutations described in clinical isolates are indicated in red, as well as their occurring positions (blue) (Yan, Fox, Holland, Stock, Gill, & Fedorko, 2000; Alonso, Galimand, & Courvalin, 2002; Richter, et al., 2003; Reinert, Lütticken, & Al-Lahham, 2004; Albertí, et al., 2005; Orscheln, et al., 2005; Rivera, et al., 2005; Biedenbach, Toleman, Walsh, & Jones, 2006; Alonso, Mateo, Ezpeleta, & Cisterna, 2007; Wajima, Murayama, Sunaoshi, Nakayama, Sunakawa, & Ubukata, 2008; Montes, Tamayo, Orden, Larruskain, & Perez-Trallero, 2010; Pires, et al., 2010; Arai, et al., 2011; Wajima, et al., 2013).

Others

Glycopeptides

Glycopeptides (vancomycin and teicoplanin) are high-molecular weight molecules that interact with the D-Alanine-D-Alanine (D-Ala-D-Ala) termini of pentapeptide peptidoglycan precursors (Gold, 2001). They actually form a stable complex that involves five hydrogen bonds, which prevent the transglycosylation and transpeptidation reactions of the peptidoglycan synthesis.

In enterococci, resistance to glycopeptides is based on the presence of operons encoding enzymes i) that synthesize new precursors with low affinity where the last D-Ala residue is changed by a D-Lactate (D-Lac) or a D-Serine (D-Ser) residue; and ii) that eliminate or prevent the formation of a native precursor with a high affinity. Precursors with the D-Ala-D-Lac terminus have a 1,000-fold lower affinity to vancomycin than those ending in D-Ala-D-Ala, which results in a high-level resistance (MICs >16 µg/ml). On the other hand, the precursors that end in D-Ala-D-Ser have a 7-fold less affinity for vancomycin, which leads to a low-level resistance (MICs from 8 to 16 µg/ml) (Courvalin, 2006). Eight acquired operons (*vanA, vanB, vanD, vanE,*

vanG, vanL, vanM, and *vanN*) and one intrinsic operon (*vanC1-4*) have been characterized to date (Watanabe, et al., 2009; Hegstad, Mikalsen, Coque, Werner, & Sundsfjord, 2010; Cattoir & Leclercq, 2013). The *vanA*, *vanB*, *vanD*, and *vanM* operons confer resistance through the synthesis of peptidoglycan precursors with C-terminal D-Ala-D-Lac residues, whereas isolates that harbor the *vanC*, *vanE*, *vanG*, *vanL*, and *vanN* operons contain precursors that end in D-Ala-D-Ser (Cattoir & Leclercq, 2013). Only *vanA*, *vanB*, and *vanG* have been identified in rare strains of *Streptococcus* spp. but never among *S. pyogenes* clinical isolates (Poyart, et al., 1997; Mevius, et al., 1998; Park, Nichols, & Schrag, 2014). The *vanB* gene cluster (*vanB2* subtype) has only been identified in two *Streptococcus lutetiensis* isolates (MICs of vancomycin and teicoplanin at ≥256 and 3-4 µg/ml, respectively) as well as in two *vanA*-positive *Streptococcus gallolyticus* isolates (MICs of vancomycin and teicoplanin at ≥256 and 32-64 µg/ml, respectively) in France and the Netherlands (Poyart, et al., 1997; Mevius, et al., 1998; Dahl & Sundsfjord, 2003; Bjørkeng, Hjerde, Pedersen, Sundsfjord, & Hegstad, 2013). Two isolates of *Streptococcus agalactiae* and one of *Streptococcus anginosus* have been reported with low-level resistance to vancomycin (MIC of 4 µg/ml) in the US (Park, Nichols, & Schrag, 2014; Srinivasan, et al., 2014). Recently, a third strain of *vanA*-positive *S. gallolyticus* (MICs of vancomycin and teicoplanin both at ≥256 µg/ml) was described in Spain (Romero-Hernández, et al., 2015).

Sulfonamides and trimethoprim

Both sulfonamides and 2,4-diaminopyrimidines are synthetic agents and act as bacteriostatic inhibitors of bacterial biosynthesis of tetrahydrofolic acid, which is an essential cofactor for nucleic acid and protein syntheses (Masters, O'Bryan, Zurlo, Miller, & Joshi, 2003). They are generally given together due to a synergistic effect in vitro, and the most commonly used combination is sulfamethoxazole-trimethoprim, also called cotrimoxazole (or SXT) (Masters, O'Bryan, Zurlo, Miller, & Joshi, 2003). The sulfonamides are structural analogs of para-aminobenzoic acid that interfere with the formation of dihydropteroic acid, which is the first step in dihydrofolic acid synthesis, by blocking the enzymatic activity of the dihydropteroate synthase (DHPS). Trimethoprim inhibits a later step of the metabolic pathway (namely, the formation of tetrahydrofolic acid from dihydrofolic acid) by binding to the dihydrofolate reductase (DHFR) enzyme.

Bacterial resistance to sulfonamides results from chromosomal point mutations in the *dhps* (*fol*P) gene coding for the natural DHPS, or the acquisition of plasmid-borne *sul* genes coding for resistant DHPS enzymes (Huovinen, Sundström, Swedberg, & Sköld, 1995). Resistance to trimethoprim in bacteria may be due to one or more of the following mechanisms: auxotrophy in thymine/thymidine; reduced intracellular antibiotic concentration (impaired permeability, active efflux); production of a naturally-insensitive DHFR; alterations and/or overexpression of the intrinsic DHFR encoded by the *fol*A gene; or the presence of an additional plasmid-mediated *dfr* gene that codes for a resistant DHFR (Huovinen, Sundström, Swedberg, & Sköld, 1995).

Because of early inappropriate antimicrobial susceptibility testing (AST) approaches, *S. pyogenes* has been considered to be universally resistant to SXT for a long time. However, recent studies using standardized conditions (particularly AST media with low thymidine content) have confirmed the in vitro susceptibility of *S. pyogenes* to SXT (Bowen, et al., 2012). When appropriate methods are used, MICs of SXT are typically low [Table 1] and the prevalence of resistance among clinical isolates has ranged from 0% to 3% (Bowen, et al., 2012; Yourassowsky, Vanderlinden, & Schoutens, 1974; Eliopoulos & Wennersten, 1997). High-level sulfonamide resistance (MIC ≥128 µg/ml) in *S. pyogenes* is due to alterations within the chromosomally encoded DHPS (Swedberg, Ringertz, & Sköld, 1998; Jönsson, Ström, & Swedberg, 2003). Acquired resistance to trimethoprim in *S. pyogenes* may be due to the acquisition of transferable *dfrF* or *dfrG* genes (MICs from 32 to >512 µg/ml) or an amino acid substitution (Ile100Leu) in the intrinsic DHFR (MIC from 8 to 16 µg/ml) (Bergmann, Sagar, Nitsche-Schmitz, & Chhatwal, 2012; Bergmann, van der Linden, Chhatwal, & Nitsche-Schmitz, 2014).

Rifampin

Rifampin is a broad-spectrum antibiotic with bactericidal activity and is widely used for the treatment of tuberculosis. Like other members of the rifamycin family, it inhibits bacterial transcription through high-affinity binding to the DNA-dependent RNA polymerase (Lester, 1972). More precisely, rifampin interacts with the RNA polymerase β subunit encoded by the *rpoB* gene.

Rifampin resistance mainly results from chromosomal *rpoB* mutations that are responsible for target alterations, which occur at a frequency of ca. 10^{-6}-10^{-8}. These mutations are generally clustered in an 81-bp conserved fragment (the so-called rifampin resistance-determining region [RRDR]), which corresponds to codons 507-533 (according to *Escherichia coli* numbering) (Herrera, Jiménez, Valverde, García-Aranda, & Sáez-Nieto, 2003).

In *S. pyogenes*, rifampin resistance appears to be rare among clinical isolates (<0.5%) while MICs for wild-type strains are very low [Table 1] (Perez-Trallero, Urbieta, Montes, Ayestaran, & Marimon, 1998; Aubry-Damon, Galimand, Gerbaud, & Courvalin, 2002). High-level resistance to rifampin (>128 µg/ml) is due to RpoB changes at position 522 (Ser522Leu) (Aubry-Damon, Galimand, Gerbaud, & Courvalin, 2002; Herrera, et al., 2002).

Bacitracin

Like β-lactams and glycopeptides, bacitracin disrupts bacterial cell wall synthesis. It actually acts by preventing dephosphorylation and recycling of the lipid carrier (undecaprenol pyrophosphate) that allows the translocation of *N*-acetylmuramyl pentapeptide intermediates onto the surface of the cytoplasmic membrane (Butaye, Devriese, & Haesebrouck, 2003). Bacitracin is a narrow-spectrum antibiotic that is only active against Gram-positive bacteria.

Bacitracin is used in some topical preparations in human and veterinary medicine and has been employed in clinical laboratories as a presumptive marker for *S. pyogenes* identification. Indeed, among β-hemolytic streptococci, only *S. pyogenes* is susceptible to bacitracin. However, some bacitracin-resistant clones (MICs ≥16 µg/ml) have been uncommonly reported (York, Gibbs, Perdreau-Remington, & Brooks, 1999; Malhotra-Kumar, Wang, Lammens, Chapelle, & Goossens, 2003; Perez-Trallero, Garcia, Orden, Marimon, & Montes, 2004; Pires, et al., 2009). Notably, bacitracin resistance is commonly associated with macrolide resistance ($cMLS_B$ phenotype) mediated by the *erm*(B) gene with most of the isolates belonging to the *emm*28 genotype (Malhotra-Kumar, Wang, Lammens, Chapelle, & Goossens, 2003; Perez-Trallero, Garcia, Orden, Marimon, & Montes, 2004; Perez-Trallero, Garcia, Orden, Marimon, & Montes, 2004; Pires, et al., 2009; Mihaila-Amrouche, Bouvet, & Loubinoux, 2004). Even though the molecular mechanism of resistance is not well defined in this species, it might be associated with an overproduction of undecaprenol kinase encoded by the *bacA* gene, as shown in *E. coli* (Cain, Norton, Eubanks, Nick, & Allen, 1993; Chalker, et al., 2000). Notably, an ABC transporter (encoded by the *bcrABD* operon) responsible for bacitracin resistance in *E. faecalis* has not been detected among bacitracin-resistant *S. pyogenes* clinical isolates (Pires, et al., 2009; Manson, Keis, Smith, & Cook, 2004).

Chloramphenicol

The phenicols are inhibitors of bacterial protein synthesis and prevent peptide chain elongation after reversible binding to 23S rRNA (Schlünzen, et al., 2001). Chloramphenicol resistance is primarily due to the acquisition of chloramphenicol *O*-acetyltransferase (CAT) enzymes or to active efflux mediated by specific or multidrug transporters (Schwarz, Kehrenberg, Doublet, & Cloeckaert, 2004). Target modifications are also possible through point mutations or Cfr-mediated methylation in 23S rRNA (Schwarz, Kehrenberg, Doublet, & Cloeckaert, 2004; Kehrenberg, Schwarz, Jacobsen, Hansen, & Vester, 2005). CATs inactivate the antibiotic by acetylation in the three-step reaction, and there are two types of enzymes, according to their structure: type A CATs and type B CATs (also known as xenobiotic acetyltransferases or XATs) (Schwarz, Kehrenberg, Doublet, & Cloeckaert,

2004). Notably, chloramphenicol resistance is inducibly expressed through a translational attenuation regulation (Lovett, 1996).

Chloramphenicol resistance is rarely encountered in clinical isolates of β-hemolytic streptococci (ca. 0.5%), while it appears to be much more common among enterococcal species (ca. 40-50%) (Trieu-Cuot, et al., 1993). In *S. pyogenes*, only a few type-A CATs have been detected, such as *cat(pC221)* (group A-7), *cat(pC194)* (group A-9), *catS* (group A-12), and *catQ* (group A-16) (Del Grosso, et al., 2011; Schwarz, Kehrenberg, Doublet, & Cloeckaert, 2004; Trieu-Cuot, et al., 1993). Notably, *mef*(I) and *catQ* were demonstrated to be co-located in Tn*5253*-like ICEs (see above) [Figure 8] and were cotransferred by transformation (Mingoia, et al., 2014).

Fusidic acid

Fusidic acid is a bacteriostatic antibiotic that inhibits bacterial protein synthesis by interfering with elongation factor G (EF-G) (Collignon & Turnidge, 1999). While it is very active against staphylococci (MIC_{50} and MIC_{90} at 0.12 and 0.25 µg/ml, respectively), β-hemolytic streptococci, including *S. pyogenes*, are less susceptible (MIC_{50} and MIC_{90} both at 4 µg/ml) [Table 1] (Jones, Mendes, Sader, & Castanheira, 2011). Acquired resistance has only been characterized in *S. aureus*, and is caused by mutations in the EF-G that encodes the *fusA* gene, or results from horizontal transfer of a plasmid-mediated determinant (*fusB-E*) (Turnidge & Collignon, 1999; O'Neill, McLaws, Kahlmeter, Henriksen, & Chopra, 2007). No *S. pyogenes* isolate highly resistant to fusidic acid has been reported to date.

Fosfomycin

Fosfomycin interferes with the cell wall synthesis at an early intra-cytoplasmic stage by specifically and irreversibly inhibiting the enzymatic activity of UDP-*N*-acetylglucosamine enolpyruvyltransferase (named MurA) (Falagas, Giannopoulou, Kokolakis, & Rafailidis, 2008). It exerts a slow bactericidal activity against a large panel of both Gram-positive and -negative bacteria, but is mainly used for staphylococcal infections and uncomplicated acute cystitis in young women (Patel, Balfour, & Bryson, 1997). Fosfomycin is moderately active against *S. pyogenes* with MICs from 2 to 64 µg/ml, while no high-level resistance has been reported in this species to date (Falagas, et al., 2010).

New Antibiotics

Linezolid

Linezolid is a member of the family of oxazolidinones and targets the initiation phase of protein synthesis by direct interaction with the PTC in 23S rRNA (Hancock, 2005). It is only active against Gram-positive bacteria, including *S. pyogenes* (MIC_{50} and MIC_{90} at 1 µg/ml) [Table 1] (Gemmell, 2001; Brauers, Kresken, Hafner, & Shah, 2005). In staphylococci and enterococci, linezolid resistance is due to the emergence of ribosomal point mutations (23S rRNA, L4, or L22) or acquisition of the Cfr protein (Cattoir & Giard, 2014), but none of these resistance mechanisms has been identified in *S. pyogenes* to date.

Daptomycin

Daptomycin is a cyclic lipopeptide antibiotic that exhibits a potent and rapid bactericidal activity against Gram-positive bacteria (Kanafani & Corey, 2007). Daptomycin irreversibly binds to the bacterial cell membrane in a calcium-dependent manner, which causes depolarization and thus results in cell death (Hancock, 2005). It is highly active against β-hemolytic streptococci with MICs from 0.01 to 0.06 µg/ml [Table 1] (King & Phillips, 2001). No daptomycin-resistant *S. pyogenes* isolate has been reported to date.

Conclusion

Except for MLS and tetracyclines, *S. pyogenes* has remained highly susceptible to antimicrobial agents in vitro since the 1940s, particularly to penicillins, which are usually the first-line treatment. Indeed, even if therapy failures are quite common with β-lactams in clinical practice, no acquired mechanism of resistance has been reported to date. Note that there have not been any minor changes in the MICs of penicillin. In cases of allergy or therapy failure, MLS antibiotics are considered to be alternate options. However, macrolide resistance may become a problem, since it has emerged in numerous countries, and as a result, in vitro antimicrobial susceptibility testing should be performed. Such testing will not only allow researchers to distinguish susceptible phenotypes from resistant phenotypes, but also to differentiate between the different resistant phenotypes (such as $cMLS_B$, $iMLS_B$, and M phenotypes) since they unravel the potential activity of the different MLS members. Besides MLS antibiotics, *S. pyogenes* can also acquire resistance to the tetracycline family. Notably, numerous clinical isolates are co-resistant to MLS and tetracyclines, since both resistance determinants are borne by the same mobile genetic elements. High-level resistance to aminoglycosides or fluoroquinolones remains very uncommon, while there is no (or exceptional) resistance to other antibiotics. More specifically, no resistance has been described to date for newer molecules (such as linezolid, tigecycline, and daptomycin).

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Current Approaches to Group A Streptococcal Vaccine Development

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Introduction and Historical Perspectives

The search for safe and effective vaccines to prevent *Streptococcus pyogenes* infections has been ongoing for decades. The fact that there is not a licensed vaccine is remarkable, considering that *S. pyogenes* is one of the most studied human bacterial pathogens. Considerable detailed information is available on the molecular pathogenesis of infection (Walker, et al., 2014), the structure and function of multiple virulence determinants, and on protective immune responses in animals (Lancefield, 1962) and humans (Wannamaker, Denny, Perry, Siegel, & Rammelkamp, Jr., 1953). The global burden of *S. pyogenes* disease is substantial, and excess mortality from acute rheumatic fever (ARF), rheumatic heart disease (RHD), and invasive infections is significant (see the chapter on the world disease burden of *S. pyogenes*). The world needs a safe, effective, and affordable *S. pyogenes* vaccine.

Evidence indicates that natural infection with *S. pyogenes* leads to a protective immunity, which could be mimicked by appropriately constructed vaccines. The peak incidence of *S. pyogenes* infections occurs in schoolchildren and declines in adulthood. The relative resistance of adults has been ascribed to an accumulation of protective antibodies against type-specific regions of the M protein, conserved M epitopes, or other conserved antigens that follow multiple *S. pyogenes* infections during childhood. Lancefield first demonstrated type-specific protective immunity in mice and subsequently showed that in humans, bactericidal M protein antibodies persisted for years after infection (Lancefield, 1959). The most direct evidence of vaccine prevention of infection was a series of studies by Fox et al. who immunized volunteers with purified M protein preparations through

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either the parenteral or mucosal routes, and showed the protection that followed these "challenge" infections (Fox, Pachman, Wittner, & Dorfman, 1970; Fox, Waldman, Wittner, Mauceri, & Dorfman, 1973; Polly, Waldman, High, Wittner, & Dorfman, 1975). Wannamaker and his colleagues performed a study on 131 military recruits that demonstrated that infections with homologous serotypes occurred six times more frequently in individuals without type-specific antibodies, as compared to individuals with antibodies to that particular type (Wannamaker, Denny, Perry, Siegel, & Rammelkamp, Jr., 1953). However, the same study demonstrated that type-specific serum antibodies had little effect on "transitory acquisitions" of *S. pyogenes* infections in the pharynx (Wannamaker, Denny, Perry, Siegel, & Rammelkamp, Jr., 1953), an observation that was confirmed in a subsequent study in families in Egypt (Guirguis, Fraser, Facklam, El Kholy, & Wannamaker, 1982). Finally, Beachey showed that serum from volunteers immunized with a purified pepsin extract of M24 contained antibodies that were bactericidal against type 24 streptococci (Beachey, Stollerman, Johnson, Ofek, & Bisno, 1979). Vaccine development has been hampered by the fact that there is not a well-defined human immune correlate of protection against *S. pyogenes* infection. The studies of Lancefield and Fox allude to a type-specific immunity that is mediated by antibodies against the M protein, and that bactericidal antibodies are associated with protection against symptomatic infection. However, others argue that repeated *S. pyogenes* infections evoke antibodies against conserved antigens (shared by most or all serotypes) that may explain the immunity acquired by adults.

Aside from the biological and technical hurdles, it has become increasingly evident that the clinical development of *S. pyogenes* vaccines is somehow "impeded." There have been multiple impediments, both historical and contemporary. The major concern has been that *S. pyogenes* vaccine antigens may contain autoimmune epitopes that could potentially trigger ARF—one of the very diseases that vaccines are designed to prevent. Another impediment is the complexity of the epidemiology of the *S. pyogenes* infections, including the number of *emm* types, anatomic sites of infection (throat and skin), and geographic differences in the prevalence and burden of the epidemiology and diseases (Steer, Law, Matatolu, Beall, & Carapetis, 2009). A major economic impediment is the fact that 95% of all serious *S. pyogenes* diseases occur in low- and middle-income countries (Carapetis, Steer, Mulholland, & Weber, 2005) where the return on investment by vaccine manufacturers is predicted to be insufficient to match development costs. Additionally, there is a perception that there would be a lower demand for vaccines in high-income countries, where prevention of pharyngitis would be the major outcome, given the cost-benefit analysis.

The history of *S. pyogenes* vaccine development dates back more than 90 years. Clinical trials of group A streptococcal vaccines were performed as early as 1923 (Bloomfield & Felty, 1923). Children and adults have been vaccinated with everything from intravenous injections of whole, heat-killed streptococci to intramuscular injections of highly purified fragments of M proteins. The majority of the clinical trials since 1960 were performed with relatively crude preparations of M proteins or cell walls of group A streptococci (Table 1). The major problem associated with these vaccines was reactogenicity, which limited the total amount of vaccine that could be delivered, in many cases. From more recent studies, we presume that the intense inflammation associated with these crude preparations was due to contaminating antigens, which may have included known toxins, such as streptolysin S or 0, pyrogenic exotoxins (erythrogenic toxins), or unknown toxins. At least some of the reactogenicity may have been due to the presence of superantigens, which is a property associated with pyrogenic exotoxins (Kotb, 1995).

A study by Massell et al. (Massell, Michael, Amezcua, & Siner, 1968) was highly controversial because of a subsequent report that linked the vaccine to at least two (and possibly three) cases of acute rheumatic fever (Massell, Honikman, & Amezcua, 1969). The relatively crude M protein vaccine was derived from hot acid extracts of type 3 streptococci. Subjects from this trial were siblings of patients that had had documented acute rheumatic fever. The basis for their concern was the apparent increase in the "attack rate" of ARF in the 21 subjects, as compared to historical data in their clinic population. The authors admit that "final conclusions are not justified from this limited experience with only 21 vaccinated children" (Massell, Honikman, & Amezcua,

1969). Nonetheless, following the Massell publication, a US federal ban was essentially imposed on *S. pyogenes* vaccine testing in humans that remained in effect for over 30 years, until its reversal in 2006.

The studies by Fox (Fox, Pachman, Wittner, & Dorfman, 1970; Fox, Waldman, Wittner, Mauceri, & Dorfman, 1973; Polly, Waldman, High, Wittner, & Dorfman, 1975) and Beachey (Beachey, Stollerman, Johnson, Ofek, & Bisno, 1979) (Table 1) heralded a new age of vaccine development that employed highly purified, well-characterized M antigens. With effective methods of extracting M proteins from intact streptococci and removing potentially reactogenic contaminants, it was anticipated that multivalent vaccines could be developed in ways that paralleled those used for multivalent pneumococcal polysaccharide vaccines. Subsequent structure/function studies revealed that some M proteins contained epitopes that evoked antibodies that cross-reacted with human tissues (Cunningham, 2000). This observation resulted in a reevaluation of the use of large fragments of M proteins and led to the current approach of including only N-terminal M peptides devoid of potential autoepitopes in multivalent vaccines (Dale, 2008). Simultaneously, many investigators began to explore alternatives to M protein-based vaccines, with the goal of identifying common protective antigens that would circumvent the possibility of inducing tissue cross-reactive antibodies and also provide broad coverage against most (if not all) group A streptococci, independent of their serotype (Table 2).

This chapter describes current efforts to develop safe and effective vaccines to prevent *S. pyogenes* infections. Although clinical development has been slow, there are a number of available approaches, based on a detailed understanding of the molecular pathogenesis of infection and protective immune responses in animals and humans. The development of M protein-based vaccines has taken full advantage of molecular techniques, rapid and reproducible *emm* typing methods, and modern molecular engineering that involves gene synthesis and scalable production. Common M epitopes have been engineered into a vaccine that contains a minimal B cell epitope to optimize functional antibody responses. Through genome-based reverse vaccinology, several common antigens have been identified as potential vaccine components. Although the global epidemiology of *S. pyogenes* infections is still not well defined, a growing amount of information is being used to inform vaccine design. As more vaccines enter clinical trials, there is a need to define common denominators in protocol design, particularly as they relate to safety assessments and efficacy.

Table 1. Summary of previously published Group A streptococcal vaccine trials in humans.

Study (Ref.)	# Subjects	Antigen	Dose (max.)	Dose (total)
Young, 1946 (Young, 1946)	>1000	Whole, killed types 17 and 19	$1x10^9$ CFU (i.v.)	$3x10^9$ CFU (i.v.)
Schmidt, 1960 (Schmidt, 1960)	7	M protein acid extracts	100 ug	700 ug
Potter, 1962 (Potter, Stollerman, & Siegel, 1962)	34	Cell walls Types 12 and 5	44 ug	44 ug
Wolfe, 1963 (Wolfe, Jr., Hayashi, Walsh, & Barkulis, 1963)	71	Cell walls Type 14	1.6 m	43.2 mg
Fox, 1966 (Fox, Wittner, & Dorfman, 1966)	33	M protein Types 12 and 24	20 ug	20 ug
Massell, 1968 (Massell, Michael, Amezcua, & Siner, 1968)	21	M3 protein Crude acid extracts	1 mg	4.5 mg
Fox, 1969 (Fox, Pachman, Wittner, & Dorfman, 1970)	80	M12 protein	40 ug	120 ug
	Infants, children			
Fox, 1973 (Fox, Waldman, Wittner, Mauceri, & Dorfman, 1973)	19	M1 protein	90 ug	270 ug
Challenge infections of control and immunized subjects				

Table 1. continued from previous page.

Study (Ref.)	# Subjects	Antigen	Dose (max.)	Dose (total)
Polly, 1975 (Polly, Waldman, High, Wittner, & Dorfman, 1975)	21	M1 protein	300 ug	900 ug
Delivered as an upper airway aerosol, subjects challenged				
Beachey, 1979 (Beachey, Stollerman, Johnson, Ofek, & Bisno, 1979)	12	M24 protein highly purified "pep M"	200 ug	1.1 mg

Table 2. Candidate *S. pyogenes* vaccine antigens.

Vaccine class	Vaccine antigen	Development stage	Reference
M protein: Type-specific region	6- and 26-valent N-terminal 30-valent N-terminal	Completed phase I and II clinical trials Preclinical	(McNeil, et al., 2005) (Dale, Penfound, Chiang, & Walton, 2011)
M protein: Conserved region	Whole C-repeat conserved region	Antigen – animal studies Vector - *Streptococcus gordonii* completed clinical trial	(Bessen & Fischetti, 1988a; Bronze, Courtney, & Dale, 1992) (Kotloff, et al., 2005)
	Minimal epitope J8/J14/p145	Animal studies and natural infection studies, phase I trial of J8	(Batzloff M. , Yan, Davies, Hartas, & Good, 2004)
	StreptInCor-B and T epitopes from conserved region	Animal studies	(Wannamaker, Denny, Perry, Siegel, & Rammelkamp, Jr., 1953; Lancefield, Persistence of type-specific antibodies in man following infection with group A streptococci, 1959)
GAS carbohydrate	GAS carbohydrate	Animal studies and natural infection studies	(Sabharwal, et al., 2006)
Non M protein vaccine candidates	GAS C5a peptidase	Animal studies and natural infection studies	(Shet, Kaplan, Johnson, & Cleary, 2003)
	Fibronectin-Binding Protein	Animal studies	(Kawabata, et al., 2001)
	Streptococcal protective antigen	In clinical trials as component of multivalent M	(Dale J. B., Chiang, Liu, Courtney, & Hasty, 1999)
	Serum Opacity Factor	Animal studies	(Courtney, Hasty, & Dale, 2003; Schulze, Medina, & Guzmán, 2006)
	Streptococcal pyrogenic exotoxin B (extracellular cysteine protease)	Animal studies	(Kapur, et al., 1994)
	Streptococcal Pyrogenic Exotoxin C	Animal studies	(McCormick, et al., 2000)
	Pili (T antigen)	Animal studies	(Köller, et al., 2010)
	Serine protease (SpyCEP)	Animal studies	(Zingaretti, et al., 2010)
	Serine esterase (Sse)	Animal studies	(Liu, Zhu, Zhang, & Lei, 2007)
	GAS 40	Animal studies	(United States of America Patent No. WO2005032582 A8, 2005)
	Nine common antigens	Animal studies	(Fritzer, et al., 2010)

Table 2. continued from previous page.

Vaccine class	Vaccine antigen	Development stage	Reference
	G-related alpha2-macroglobulin binding protein (GRAB)	Identified only	(McMillan, et al., 2004a)
	Metal transporter of streptococcus (MtsA)	Identified only	(McMillan, et al., 2004a)
	Superoxide dismutase	Identified only	(McMillan, Davies, Browning, Good, & Sriprakash, 2004b)
	Lipoproteins	Identified only	(Lei, Liu, Chesney, & Musser, 2004)

Multivalent M protein-based vaccines

The surface M protein of *S. pyogenes* is a major virulence determinant and also a protective antigen (as discussed in the chapters on ultrastructure). Antibodies against M protein opsonize the organism and promote C3-mediated phagocytosis, which is associated with protection against infection in animals (Lancefield, 1962). These observations have served for many years as the basis for the development of M protein-based *S. pyogenes* vaccines. Intact M proteins not only contain protective (opsonic) epitopes, but also, contain human tissue cross-reactive epitopes in some cases (Cunningham, 2000). Because of the theoretical possibility of inducing autoantibodies, it has been challenging to separate the protective epitopes from the autoimmune epitopes so that vaccine preparations would contain only protective M protein peptides. Multiple studies from several laboratories have shown that the epitopes contained in the hypervariable, type-specific N-terminus of the M proteins evoke antibodies with the greatest bactericidal activity and that they are the least likely to cross-react with host tissues (Dale, 1999). In addition, the majority of the autoimmune epitopes of M proteins that have been identified are located in the middle of the mature M proteins and are distinct from the type-specific, protective epitopes (Dale, 1999). These observations have led some investigators to focus on the N-terminal type-specific peptides of M proteins for inclusion in multivalent vaccines (Dale, Chiang, & Lederer, 1993; Dale, Simmons, Chiang, & Chiang, 1996; Dale, 1999). Synthetic and recombinant peptides as small as 10 amino acids have been shown to protect animals against subsequent challenge infections with homologous serotypes of *S. pyogenes* (Dale & Chiang, 1995).

The finding that small peptides from the M proteins could evoke bactericidal antibodies that were not cross-reactive with human tissue prompted investigators to identify methods of designing and formulating vaccines that contained protective epitopes from multiple M serotypes. One approach has been to design fusion proteins that contain N-terminal M peptides linked in tandem, and the first of these was a trivalent synthetic peptide that was linked to an unrelated carrier (Beachey, Seyer, & Dale, 1987). Subsequent vaccines were produced using recombinant techniques, in which specific 5' regions of the *emm* genes were amplified by PCR and linked together in-frame using unique restriction sites. Vaccines containing four (Dale, Chiang, & Lederer, 1993), six (Dale, 1999), eight (Dale, Simmons, Chiang, & Chiang, 1996), twenty-six (Hu, et al., 2002), and thirty (Dale, Penfound, Chiang, & Walton, 2011) peptides from different M serotypes have been shown to evoke broadly opsonic antibodies in animals without evoking tissue cross-reactive antibodies. Clinical trials designed to assess the safety and immunogenicity of the hexavalent (Kotloff, et al., 2004) and 26-valent (McNeil, et al., 2005) vaccines have previously been performed. Both vaccines were safe, well-tolerated, and evoked bactericidal antibodies against the vaccine serotypes of *S. pyogenes*.

The availability of more extensive epidemiologic data from a North American pharyngitis study (Shulman, et al., 2004; Shulman, et al., 2009), the CDC's ongoing ABC surveillance in the US (O'Loughlin, et al., 2007), and the StrepEuro study of invasive *S. pyogenes* strains (Luca-Harari, et al., 2009) has permitted the formulation of a 30-valent vaccine (Figure 1) with greater potential efficacy (Dale, Penfound, Chiang, & Walton, 2011). The serotypes

represented in the 30-valent vaccine account for 98% of all cases of pharyngitis in the US and Canada, 90% of invasive disease in the US and 78% of invasive disease in Europe. The vaccine was highly immunogenic in rabbits when delivered intramuscularly on alum. The 30-valent vaccine evoked bactericidal antibodies against all of the vaccine serotypes of *S. pyogenes*, which is comparable to or greater than that observed with the 26-valent vaccine (Hu, et al., 2002).

An unexpected observation was that the 30-valent vaccine evoked bactericidal antibodies against a number of non-vaccine serotypes of *S. pyogenes*. Altogether, 83 different *emm* types have been tested in bactericidal assays. Significant killing (>50%) was observed with 73/83 (88%) of the isolates. Of the non-vaccine types tested, 43/53 (81%) were killed, with an average killing rate of 80%. Extrapolating these results to epidemiologic studies in populations at high risk for ARF/RHD suggests a high potential coverage rate for the 30-valent vaccine. For example, the potential efficacy in preventing pharyngitis in school children in Bamako, Mali could be as high as 84% (Dale, et al., 2013) and in the Vanguard Community of Cape Town, as high as 90% (Engel, et al., 2014) when cross-opsonic antibody activity was factored into the analysis (Table 3).

The cross-opsonization of multiple non-vaccine types of *S. pyogenes* promoted by the 30-valent vaccine was largely unexplained, until the global M protein study group provided a collection of *S. pyogenes* isolates for structural analyses of M proteins. The combined results of functional antibody activity and the new cluster system (Sanderson-Smith, et al., 2014) has resulted in a revised hypothesis of cluster-specific immunity, rather than type-specific immunity (see below). These observations may form the foundation for additional computational design studies that may be able to formulate optimal M protein-based vaccines with broad efficacy throughout the world.

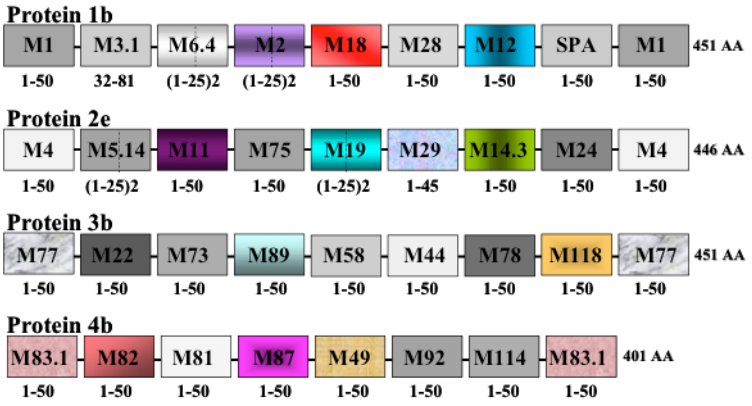

Figure 1. Schematic diagram of the four proteins that comprise the 30-valent M protein-based *S. pyogenes* vaccine.

Table 3. Potential efficacy of the 30-valent vaccine considering vaccine types (VT) and non-vaccine types (NVT) opsonized by the 30-valent antisera.

	% Total isolates	
	VT only	VT + NVT (cross-opsonized)
Pharyngitis-NA	98	98
Invasive Disease-US	90	93
Invasive Disease-Europe	78	97
Pharyngitis-Bamako	37	**84**
Pharyngitis-Cape Town	59	**90**

Molecular typing and protection against Group A *Streptococcus*

Traditional serotyping and *emm* typing of *S. pyogenes*

Since Lancefield's first publication in 1919, immunity against *S. pyogenes* infections has been believed to be "type specific" (Dochez, Avery, & Lancefield, 1919). Subsequent pioneering work in the 1950s showed that the presence of type-specific antibodies was responsible for immunity against the homologous serotype of *S. pyogenes* (Denny, Jr., Perry, & Wannamaker, 1957; Wannamaker, Denny, Perry, Siegel, & Rammelkamp, Jr., 1953; Watson, Rothbard, Swift, & de Mello, 1946; Lancefield, 1959), which established the basis for "type-specific immunity." These studies led to the development of serotyping as a method to distinguish between strains of *S. pyogenes*, based upon antibodies raised against heterologous strains in rabbits, also known as "M typing." With the advent of molecular technologies and relatively easy access to sequencing facilities, time-consuming serotyping has been progressively replaced by "*emm* typing," a molecular typing method based on PCR and sequencing. Molecular typing of *S. pyogenes* relies on sequence analysis of the *emm* gene, which encodes the N-terminus of the M protein. This portion of the M protein consists of a highly variable amino acid sequence that results in antigenic diversity (Whatmore, Kapur, Sullivan, Musser, & Kehoe, 1994; Beall, Facklam, & Thompson, 1996; O'Brien, et al., 2002). To date, 223 different *emm* types have been globally reported (McMillan, et al., 2013). When the overall architecture of the M protein is considered, all *emm* types fall into three main groups with distinct molecular structures that correspond to the previously described *emm* pattern-typing. *emm* patterns distinguish three distinct groupings (patterns A–C, D, and E), based on the presence and arrangement of *emm* and *emm*-like genes within the *S. pyogenes* genome (McMillan, et al., 2013; Bessen & Lizano, 2010). Specific *emm* types share the same *emm* pattern grouping (McMillan, et al., 2013; McGregor, et al., 2004) and the

emm pattern correlates well with tissue tropism (patterns A–C for pharyngitis, pattern D for impetigo, and pattern E for both) (Bessen & Lizano, 2010). Patterns A–C and D also correspond to the previously called class I / serum opacity factor (*sof*) negative M proteins, whereas pattern E corresponds to the class II/*sof* positive (Smeesters, McMillan, & Sriprakash, 2010b). Approximately 75% of *emm* types belong to the pattern D and E groups (McMillan, et al., 2013). Despite their epidemiologic relevance, these *emm* types have not been as extensively characterized as those of the pattern A–C group.

emm-cluster typing

Standard *emm*-typing only considers a small region of the protein sequence to classify *S. pyogenes* isolates into *emm* types (around 15% of the complete protein) (McMillan, et al., 2013; Smeesters, McMillan, & Sriprakash, 2010b). As a consequence, *emm*-typing is not informative of the entire sequence, predicted conformational structure, or functional domains of the remainder of the M protein molecule. This limitation of *emm*-typing is relevant because M proteins are multi-functional and contain distinct domains that bind many host proteins across their entire length (Figure 2) (Smeesters, McMillan, & Sriprakash, 2010b). It is also likely that a number of subtle interactions are possible between the M protein and the immune system components, with the consequence of determining the virulence capacity of the M variants (Smeesters, McMillan, & Sriprakash, 2010b). These interactions also have the potential to interfere with the immunity induced by an *S. pyogenes* infection.

A novel *emm*-cluster typing system has recently been proposed for *S. pyogenes* (Sanderson-Smith, et al., 2014). This system classifies the 223 *emm* types (McMillan, et al., 2013) into 48 functional *emm* clusters containing closely related M proteins that share structural and functional properties. *emm* clusters help to predict the virulence potential of any *S. pyogenes* isolate by ascribing M protein binding attributes to *emm* types that belong to the same *emm* cluster (Smeesters, McMillan, & Sriprakash, 2010b; Sanderson-Smith, et al., 2014). This system also correlates to the antigen content of the M protein vaccine and serves as a framework to investigate immunologic cross-protection between *emm* types (Sanderson-Smith, et al., 2014; Smeesters, Mardulyn, Vergison, Leplae, & Van Melderen, 2008; Smeesters, Dramaix, & Van Melderen, 2010a).

Global epidemiology and vaccine design

Strain diversity and vaccine coverage based upon *emm* type

Molecular epidemiology studies have shown considerable variation in *emm* type distribution at both the country and global regional level (Steer, Law, Matatolu, Beall, & Carapetis, 2009; Smeesters, McMillan, Sriprakash, & Georgousakis, 2009). Systematic reviews have highlighted differences in the *emm* type distribution of *S. pyogenes*, especially between high-income countries and resource-poor, predominantly tropical regions (Steer, Law, Matatolu, Beall, & Carapetis, 2009; Smeesters, McMillan, Sriprakash, & Georgousakis, 2009). While only a relatively small number of predominant *emm* types circulate in high-income countries, the diversity of strains associated with disease in low-income settings is much greater, which results in the possibility for low coverage of type-specific vaccines. Moreover, the profiles of the many *emm* types recovered from low-income countries differ considerably from one country to another, a factor that hinders vaccine development (Dey, et al., 2005; Abdissa, et al., 2006; Smeesters, et al., 2006). In high-income countries, 25 *emm* types accounted for 90.3% of all disease-causing isolates with the three most frequent *emm* types (*emm*1, *emm*12 and *emm*28) accounting for 40% of isolates. In contrast, 26 *emm* types accounted for only 61.8% of all isolates in the Pacific region with no predominant *emm* type. A similar distribution was observed in Africa, with 26 *emm* types accounting for only 62.5% of all isolates. In Asia, the Middle East, and in Latin America, obvious similarities to the *emm* type distribution observed in high-income countries were noted, although major differences in *emm* type distribution were observed between these regions and even at the country level. For example, a study in Brazil found that *emm* type distribution was considerably different between the slums and the wealthy suburbs from the same city

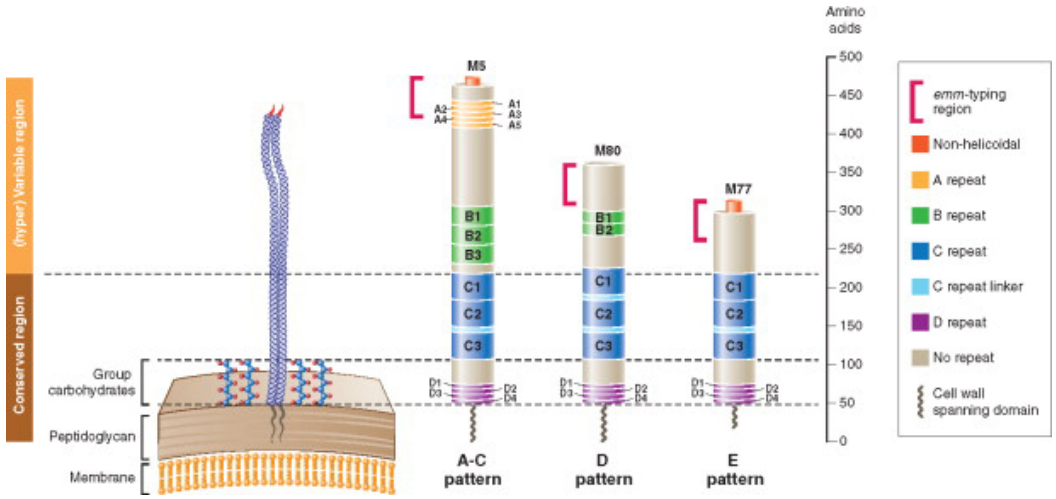

Figure 2. Three representative M proteins model. Three representative M proteins (M5, M80 and M77) were selected as prototypes for the structural characteristics within each *emm* pattern group. M protein length and the size of the repeat and non-repeat regions are drawn to scale. Pattern A-C *emm*-types represent the longest M proteins, with a (hyper)variable portion of about 230 residues. In comparison, pattern D and E proteins possess a (hyper)variable portion of ~ 150 and 100 residues, respectively. The 'A' repeats are absent from the vast majority of M proteins belonging to the pattern D and E groups. The 'B' repeats are present in most of the pattern A-C and D *emm*-types, but absent from most of the pattern E *emm*-types. Thirty-five conserved residues constitute the 'C' repeat unit. Consecutive 'C' repeat units are sometimes separated by a seven residue unit called 'C' repeat linker (See supplementary data S2). Twenty percent of the M proteins (such as M80) do not possess non-helicoidal amino terminus. This proportion is 10%, 19% and 25% amongst the pattern A-C, D and E *emm*-types respectively. The portion of the protein considered by the *emm*-typing method is represented.

of Salvador (Tartof, et al., 2010); the diversity of *emm* types in the suburbs had a similar profile to that of high-income countries, while the profile in the slums was more like that seen in resource-poor countries. This finding suggests that socio-economic factors have a considerable influence on the diversity of circulating *S. pyogenes*.

The Pacific region is characterized by a high *S. pyogenes* disease burden and a wide variety of circulating *emm* types (Steer, Law, Matatolu, Beall, & Carapetis, 2009; Smeesters, McMillan, Sriprakash, & Georgousakis, 2009). An analysis of two prospective surveillance studies of invasive *S. pyogenes* disease in New Caledonia in 2006 and 2012, respectively, found that 70% of strains recovered in New Caledonia in 2012 were different from those recovered six years earlier (Baroux, et al., 2014; Le Hello, et al., 2010). This observation is clearly different from those made in North America, where the overall *emm* type distribution has remained relatively stable over the past 10 years, with only a handful of strains responsible for the majority of infections (Shulman, et al., 2009; O'Loughlin, et al., 2007). The large diversity of strains detected in a number of Pacific countries as well as broad changes in the *emm* type distribution over time have implications for predicted vaccine coverage in each region, based on *emm*-type. Theoretical protection by the 26-valent type-specific M protein vaccine was estimated in 2009 to be 23.9% in the Pacific (Table 4). Similarly, a recent study in Hawaii showed that pharyngeal isolates of *S. pyogenes* represent a diverse collection of emm types, in which 50% were included in the 26-valent vaccine (Erdem, et al., 2009). In contrast, coverage of the vaccine in non-tropical, high income countries was 72.8%. Therefore, the high number of circulating *emm* types of *S. pyogenes* in *S. pyogenes*-endemic countries has presented a major hurdle to global vaccine development.

Table 4. Vaccine coverage of isolates by region and disease.

	Invasive	Pharyngeal	Skin	All
Africa	=	43.7% (33.2–54.1)	21.3%*	39.0% (27.2–50.7)
Asia	65.8% (47.4–84.1)	52.5% (39.3–65.7)	41.1% (37.2–44.9)	60.5% (50.8–70.1)

Table 4. continued from previous page.

	Invasive	Pharyngeal	Skin	All
Latin America	77.7% (62.6–92.8)	73.7% (49.6–97.8)	27.0% (19.6–34.4)	71.9% (46.8–97.0)
Middle East	67.5% (57.3–77.7)	54.7% (26.6–82.8)	=	63.2% (50.5–76.0)
Pacific	32.1% (14.4–49.8)	30.8% (26.9–34.6)	19.3% (10.4–28.2)	23.9% (17.8–29.9)
High-income countries	74.9% (71.1–78.7)	77.8% (68.1–87.5)	36.8% (14.5–59.0)	72.8% (66.4–79.2)
Combined	74.2% (70.5–77.9)	73.5% (61.4–85.5)	30.6% (18.3–42.8)	69.7% (63.1–76.2)

Data are vaccine coverage [% (95% Cl)]. * 95% CI not calculated because data are from one study only. = Data not available.

Strain diversity based on *emm*-cluster and the cross-protection hypothesis

The *emm*-cluster typing system has not yet been widely applied to global epidemiologic datasets. However, when applied to the data outlined above from New Caledonia the analysis demonstrated that *emm*-clusters associated with invasive infection did not vary greatly over the 6 year time course, in contrast to *emm*-types. Further, analysis of *emm*-cluster distribution in Australia, Fiji and New Caledonia combined found only a limited number of *emm* clusters were responsible for most of the disease burden in these three countries, while very few similarities could be found among the *emm* types (Baroux, et al., 2014; McDonald, Towers, Fagan, Carapetis, & Currie, 2007; Steer, et al., 2009).

While the concept of type-specific immunity against *S. pyogenes* is a broadly accepted paradigm, it has only been validated using a limited number of *emm* types; specifically those *emm* types that are common to high income countries, rather than those present in lower income settings. Previous studies suggest that the type-specific paradigm of immunity might not be directly applicable to the many strains of *S. pyogenes* currently circulating in low-incomes countries. First, preliminary analysis of the complete sequence of 51 M proteins has suggested that *emm* types found in low-income countries (Smeesters, et al., 2006) have complete M protein sequences that are highly related (Smeesters, Mardulyn, Vergison, Leplae, & Van Melderen, 2008; Smeesters, Dramaix, & Van Melderen, 2010a). This suggests that the immune response against the entire M protein may be similar between these *emm* types (Smeesters, Dramaix, & Van Melderen, 2010a; Smeesters P. R., 2014). Second, pre-clinical development of a 30-valent M protein vaccine has demonstrated *in vitro* cross-opsonization of *emm* types that are not included in the vaccine (Dale, Penfound, Chiang, & Walton, 2011; Dale, et al., 2013). Noteworthy, such *emm*-cluster typing appears to largely predict cross-opsonization of *emm* types within *emm* clusters, with some exceptions. As a result, the *emm* cluster system may serve as a framework to investigate this cross-protection phenomenon. Importantly, the *emm* cluster system does not contradict this concept of type specificity, because many of the "high-income *emm* types" occur in their own *emm*-cluster or with only a few other *emm*-types (Sanderson-Smith, et al., 2014). The concept of cross-protection across the numerous *S. pyogenes emm* types could lead to an M protein vaccine that could provide broad coverage in both high- and low-income settings.

Conserved region M-protein based vaccines

An alternate strategy to N-terminal M peptide vaccines that still use the extracellular domain of the M-protein has been to consider the conserved C-terminal region, and specifically, the C-repeat region. The C-repeat region is highly conserved between different *S. pyogenes* strains and therefore represents a possible vaccine candidate that may protect against multiple strains of *S. pyogenes*.

A number of research groups have explored the potential of the conserved region vaccine approach. To date, the main approaches that have been investigated include: (i) the use of the entire C-terminal region of the M6 strain as a recombinant protein (Bessen & Fischetti, 1990); (ii) the use of a 12 amino-acid minimal B-cell epitope from

the C-repeat region (J8) as a synthetic peptide (Batzloff, et al., 2003); and (iii) the use of B and T cell epitopes from the C-repeat region from an M5 strain as a synthetic peptide or recombinant protein (pepVac StreptInCor vaccine) (Guilherme, et al., 2006; Guilherme, et al., 2009). Although it is believed that vaccines incorporating the conserved region will protect against all *S. pyogenes* strains, there are no data from human trials as yet.

Vaccine based on C-Repeat Region of the M-protein

A primary route for *S. pyogenes* infection in humans is through the colonization of the mucosal epithelium of the pharynx where immunoglobulin A (IgA) provides a defense mechanism against bacterial infection. By focusing on the M-protein, Bessen and Fischetti demonstrated the ability of peptides that represent the conserved region of the M-protein of an M6 *S. pyogenes* isolate to induce IgA antibodies that passively protect mice when peptide antisera was mixed with *S. pyogenes* and intranasally administered (Bessen & Fischetti, 1988b). Expanding on this concept, these peptides were conjugated to cholera toxin B subunit (CTB). Mice vaccinated with these peptide-CTB conjugates had significantly reduced pharyngeal colonization following intranasal *S. pyogenes* challenges, as compared to cohorts of control mice (Bessen & Fischetti, 1988a).

Similarly, peptides corresponding to the C-repeat region of M6 were conjugated to cholera toxin B subunit (CTB) and administered to mice either orally or intranasally (Bessen & Fischetti, 1990). Cohorts of mice immunized with these conserved region peptide conjugates were significantly protected against intranasal colonization by homologous M6 or heterologous M14 streptococci, as compared to control cohorts that were only administered CTB (Bessen & Fischetti, 1990). When taken together, these data highlighted the role of conserved region peptide-specific Ig in controlling *S. pyogenes* colonization of the throat. Bronze et al. also highlighted the importance of a local mucosal immune response in protecting against streptococci infection (Bronze, McKinsey, Beachey, & Dale, 1988). Attenuated M24 streptococci when administered locally protected mice from subsequent intranasal infections with both homologous M24 and heterologous M6 streptococci (Bronze, McKinsey, Beachey, & Dale, 1988).

An advantage of targeting the C-repeat region as a vaccine candidate is the potential to induce host protection against all *S. pyogenes* strains; however, concerns about immunogenicity and the efficacy of conserved region epitopes have been raised. Jones and Fischetti investigated the potential of 19 monoclonal antibodies to opsonize M6 streptococci (Jones & Fischetti, 1988). Only one of the 19 monoclonal antibodies was capable of opsonizing the M6 strain, and this antibody was shown to target the amino-terminal region of the M-protein. Notably, the monoclonal antibodies targeting the C-repeat region, while not opsonic, were capable of fixing complement. In contrast, a peptide, SM5(164-197), towards the carboxyl terminal region of the M-protein of M5 GAS was capable of inducing antibodies that could opsonize M5, M6, M18, M19, and M49 streptococci (Sargent, Beachey, Corbett, & Dale, 1987). Antisera against this peptide, SM5(164-197), recognized sarcolemmal membranes and cardiac tissue, but not myosin (Sargent, Beachey, Corbett, & Dale, 1987). Rabbit antisera against four overlapping peptides from the C-repeat region of M6 streptococci were generated to determine if anti-C-repeat antibodies recognized myosin (Vashishtha & Fischetti, 1993). Low levels of antibodies to selected peptides in this study did bind to cleaved or denatured myosin (Vashishtha & Fischetti, 1993). This highlighted the importance of defining minimal epitopes for inclusion in an *S. pyogenes* vaccine.

Vaccine based on a minimal B-cell epitope

The search for *S. pyogenes* antigens that are conserved amongst the majority of *S. pyogenes* serotypes identified an epitope (referred to as P145) in the C-repeat region of the M-protein, which was recognized by individuals in a highly endemic community (Pruksakorn, Galbraith, Houghten, & Good, 1992; Pruksakorn, et al., 1994). It was found that these antibodies had opsonic potential in naturally infected individuals (Pruksakorn, Galbraith, Houghten, & Good, 1992; Brandt, et al., 1996). Interestingly, it was observed that opsonization with p145-immune sera only occurred when stationary-phase organisms were used (Brandt, et al., 1996; Hayman, Toth, Flinn, Scanlon, & Good, 2002), not log-phase organisms, as are used in the standard Lancefield assay. To

minimize/eliminate any chance of tissue cross-reactivity, a minimal B-cell epitope (capable of inducing protective antibodies) from this region was defined (Relf, et al., 1996; Hayman, et al., 1997). This 12-mer epitope within P145, referred to as J8i, was a B cell epitope that did not stimulate T cells in the different mouse strains examined (Hayman, et al., 1997). As a result, J8i was poorly immunogenic. Furthermore, J8i was too small to maintain its helical structure, which is required for its antigenicity. Therefore, a technology was developed to fold J8i as a helix to result in J8, a 28-mer synthetic peptide where only the central 12 amino acids (J8i) are derived from *S. pyogenes* sequences, with the flanking sequences derived from a non-streptococcal peptide (Relf, et al., 1996).

The short synthetic peptide, J8, was immunologically non-responsive in some outbred genetically diverse mouse populations. To overcome this limitation, the peptide was conjugated to the diphtheria toxoid (DT) (Batzloff, et al., 2003). When the peptide-DT conjugate, "J8-DT," was administered with CFA or with the human compatible adjuvant, alhydrogel, it was found to be highly immunogenic in inbred and outbred mice (Batzloff, et al., 2003). The induction of opsonic IgG following vaccination with J8-DT was demonstrated and the formulation was also able to significantly protect outbred mice from challenge with a *S. pyogenes* strain obtained from an Australian clinical isolate (Batzloff, et al., 2003). Both active and passive immunization using J8-DT induced significant protection following intraperitoneal *S. pyogenes* challenge (Batzloff, et al., 2003; Pandey, Batzloff, & Good, 2009; Sheel, Pandey, Good, & Batzloff, 2010). J8-DT vaccination induced vaccine-specific memory B cells (MBC) and long-lasting antibody responses, which then protected mice from systemic infection (Pandey, Wykes, Hartas, Good, & Batzloff, 2013). Most importantly, it was observed that exposure to *S. pyogenes* could boost the vaccine-induced antibody response and protect the immunized mice, and that the T-cell help for this boosting response could be provided by naïve T cells. Thus, even though the T-cell help for the primary response may come from the DT component of the vaccine, naïve T cells could work with the memory B cells to generate a protective response. This further highlights the uniqueness of the J8-DT vaccine, which contains a minimal GAS B cell epitope along with T-cell epitopes that do not belong to the pathogen, but to DT. Furthermore, IgG antibodies induced by J8-DT vaccination do not cross-react with human tissue (Hayman, et al., 1997). Recent data have demonstrated that J8-DT can also prevent pyoderma in an animal model that closely mimics human *S. pyogenes* skin infection (Pandey, 2015).

The protective potential of J8, and the closely related peptide J14, as an intranasal vaccine has also been demonstrated (Olive, Clair, Yarwood, & Good, 2002). Intranasal immunization with J14 using CTB, as well as the lipid amino terminal derivative Pam2Cys or a proteasome adjuvant, protected outbred mice from a lethal *S. pyogenes* challenge (Batzloff, Hartas, Zeng, Jackson, & Good, 2006; Batzloff, et al., 2005). In both cases, J14-specific mucosal IgA was generated, which resulted in reduced throat colonization following intranasal *S. pyogenes* challenge (Batzloff, Hartas, Zeng, Jackson, & Good, 2006; Batzloff, et al., 2005). Using a different delivery system, the J8-lipid core peptide (Toth, Danton, Flinn, & Gibbons, 1993) either with or without adjuvant was shown to induce opsonic serum IgG, which was protective against a GAS challenge (Olive, et al., 2005).

In addition to the experimental data described here, there is indirect evidence from studies of natural immunity in humans that supports the role of these epitopes in providing broad-based immunity. An earlier study in the Northern Territory of Australia found that p145 was a cryptic epitope, being poorly immunogenic as a result of natural exposure to *S. pyogenes*. However, after many years of *S. pyogenes* exposure, opsonic antibodies to p145 (from which J8 and J14 are derived), did develop, and increased with increasing age, which parallels the acquisition of immunity (Brandt, et al., 1996). J8 is hypothesized to be highly conserved because it is cryptic and as a result, it is hidden from the immune system following natural exposure, which results in the need for extensive exposure for the development of antibodies; however, a critical observation is that antibodies induced by J8 peptide immunization do recognize and opsonize *S. pyogenes*.

Moving forward on the basis of these immunogenicity and other safety data, J8-DT has successfully completed a human double-blinded Phase I pilot trial with no adverse events reported to date and with volunteers developing an antibody response to J8 (unpublished data).

Vaccine comprising B and T-cell epitopes

Another *S. pyogenes* vaccine candidate, developed by Brazilian researchers, is in progress. "StreptInCor" is based on the amino acid sequences from the M5 protein conserved region (C2 and C3 regions). To define the vaccine epitope, a large panel of approximately 900 sera and peripheral blood mononuclear cells (PBMC) were used. This enabled the identification of both B and T immunodominant epitopes, which led to the construction of StreptInCor, which is composed of 55 amino acid residues (Guilherme, et al., 2006). It has been shown that the vaccine epitope has three dimensional structural features that make it recognizable to any HLA class II (DRB1*/DRB3*/DRB4*/DRB5*), and which results in T-cell activation and differentiation into effectors and memory cells (Guilherme, et al., 2009; Guilherme, et al., 2011).

Mice vaccinated subcutaneously with this peptide using CFA as an adjuvant developed high levels of antigen-specific antibodies. Mucosal immunization with this peptide, using AFCo as a mucosal adjuvant, induced mucosal (IgA) and systemic (IgG) responses (Guilherme, et al., 2009). The protective efficacy of StreptInCor was demonstrated in various mouse strains, including BALB/C, Swiss, and HLA class II transgenic mice (Postol, et al., 2013; Guerino, et al., 2011). The vaccine did not induce cross-reactivity with cardiac proteins (Postol, et al., 2013) and no autoimmune or pathological reactions were observed in histopathological evaluations (Guerino, et al., 2011). Recent data demonstrated that anti-StreptInCor antibodies were able to opsonize several *S. pyogenes* strains (De Amicis, et al., 2014).

Genome-based discovery of *S. pyogenes* vaccine candidates

Without a doubt, the completion of the genome sequence of *Haemophilus influenzae* in 1995 (Fleischmann, et al., 1995) opened a new era in biological sciences and their medical applications. Indeed, the possibility of simultaneously exploring each of the single genes of an organism offered completely new insights on how the synthesis of its proteome is coordinated in response to the environment and on the phylogenetic relationships both between and within different species.

The omics revolution had also a great impact in vaccinology. It became apparent that *in silico* data mining for bacterial secreted and surface proteins could successfully be exploited for the discovery of vaccine antigen candidates against relevant bacterial pathogens. The genes that encode potentially suitable vaccine targets could be expressed and purified by such high throughput methods as recombinant proteins in non-pathogenic hosts and tested in pre-clinical models for their immunogenicity and their ability to neutralize the original infectious agent. This genomic approach to vaccine discovery, which was termed reverse vaccinology (RV), was first applied with success to the newly available vaccine against the *N. meningitidis* serogroup B (Pizza, et al., 2000). In this section we will discuss how genomic based information has guided the discovery of protective antigen candidates against the group A streptococcus, one of the most elusive bacterial targets for which an effective vaccine is still not available, despite decades of intense research.

Discovery of *S. pyogenes* pili and their potential as vaccine candidates

The genome-based discovery of *S. pyogenes* pili occurred in 2005 (Mora, et al., 2005) soon after this type of structure was identified as an important vaccine target in the related species of group B streptococcus. Gram-positive pili appeared as long, flexible rods that protrude up to 3 μm from the bacterial surface (Telford, Barocchi, Margarit, Rappuoli, & Grandi, 2006). They are heteropolymeric structures that consist of a major protein subunit that constitutes the pilus backbone (BP), plus one or two minor subunits (AP1 and AP2) present at the tip and on its base respectively, all covalently assembled by a series of transpeptidase reactions catalyzed by

class B and class C sortases. The sortase A that is responsible for linking proteins that bear an LPXTG motif to the peptidoglycan cell wall also anchors the pilus structure.

The observation that the genes encoding the group B streptococci pilus proteins and sortase enzymes appeared clustered together in a pathogenicity island has prompted the search for a similar island in the genomes of *S. pyogenes* that belong to different M types. The main candidate appeared to be the highly variable FCT genomic island, previously known for encoding fibronectin-binding proteins, collagen-binding proteins, and the T antigens. Antibodies specific to three of the proteins encoded in this region were shown to react with high molecular weight polymers on *S. pyogenes* extracts and pilus-like structures on the bacterial surface (Figure 3). The major *S. pyogenes* pilus BP protein turned out to be the T antigen that was first described by Rebecca Lancefield, the variability of which constituted the basis for the classification of the species into different T types. Knockout mutants lacking this protein, or the sortase machinery encoded in the FCT region, were deprived of pilus polymers on their surface. The relevance of pili to *S. pyogenes* pathogenesis was highlighted by experiments demonstrating their involvement in the development of biofilms (Manetti, et al., 2007), in mediating cell adhesion to the human epithelia (Abbot, et al., 2007) and in the formation of microcolonies that helped protect the bacteria from phagocytic killing (Becherelli, et al., 2012).

Notably, immunization of mice with a combination of recombinant pilus proteins was shown to confer protection against *S. pyogenes* challenge. However, as anticipated above, the *S. pyogenes* FCT region displays considerable genetic diversity, with nine different FCT variants identified to date based on their gene composition and DNA sequence. As a consequence of this variability, protection by *S. pyogenes* pilus proteins is specific to strains that bear the variants used for immunization.

However, sequence analysis matched with epidemiological data indicated that 90% of strains currently circulating in the US and European Union belong to 12 T types. Therefore, since immunization with the pilus backbone confers protection against *S. pyogenes* challenge in mice, a vaccine that included the backbone proteins of the 12 T types was predicted to cover most *S. pyogenes* infections (Manetti, et al., 2007). More recently, we investigated cross-protection between strains that carry pili with homologous backbone proteins. To address this question, advantage was taken of the well-established opsonophagocytic assay in group B streptococci. Different *S. pyogenes* pili were systematically expressed in group B streptococci, and bacterial killing mediated by antibodies to the different pilus variants was assessed. This data showed that cross-protection could potentially be achieved between some of the T types that share sufficiently high homology levels, which potentially further restricts the number of backbone proteins that are required for wide coverage (Buccato, 2015).

Regardless the number of proteins needed to achieve broad coverage, the development of an *S. pyogenes* pilus-based vaccine could benefit from the application of "structural vaccinology." This approach has been successfully demonstrated in the case of group B streptococci, where a synthetic chimera that combines the protective domain of backbone proteins from different pilus variants was shown to induce functional antibodies that mediate cross-killing (Nuccitelli, et al., 2011).

Integrating genomics, proteomics and immunomics for *S. pyogenes* vaccine discovery

Reverse vaccinology is based on the straightforward consideration that if all annotated proteins from a given pathogen are available (for instance, by high throughput cloning and expression) and if all proteins can be screened against a robust and reliable surrogate-of-protection assay (for instance, by *in vitro* bactericidal assays or animal challenge models), then protective antigens will be identified. The meningococcal and group B streptococci examples demonstrate that the strategy works. However, since most of the assays available for protective antigen selection involve animal immunization, the number of antigens to be tested represents a severe bottleneck in the entire process. For this reason, more selective strategies have been applied over the last few years in order to quickly identify protective antigens. The ultimate goal, often referred to as the "Holy Grail

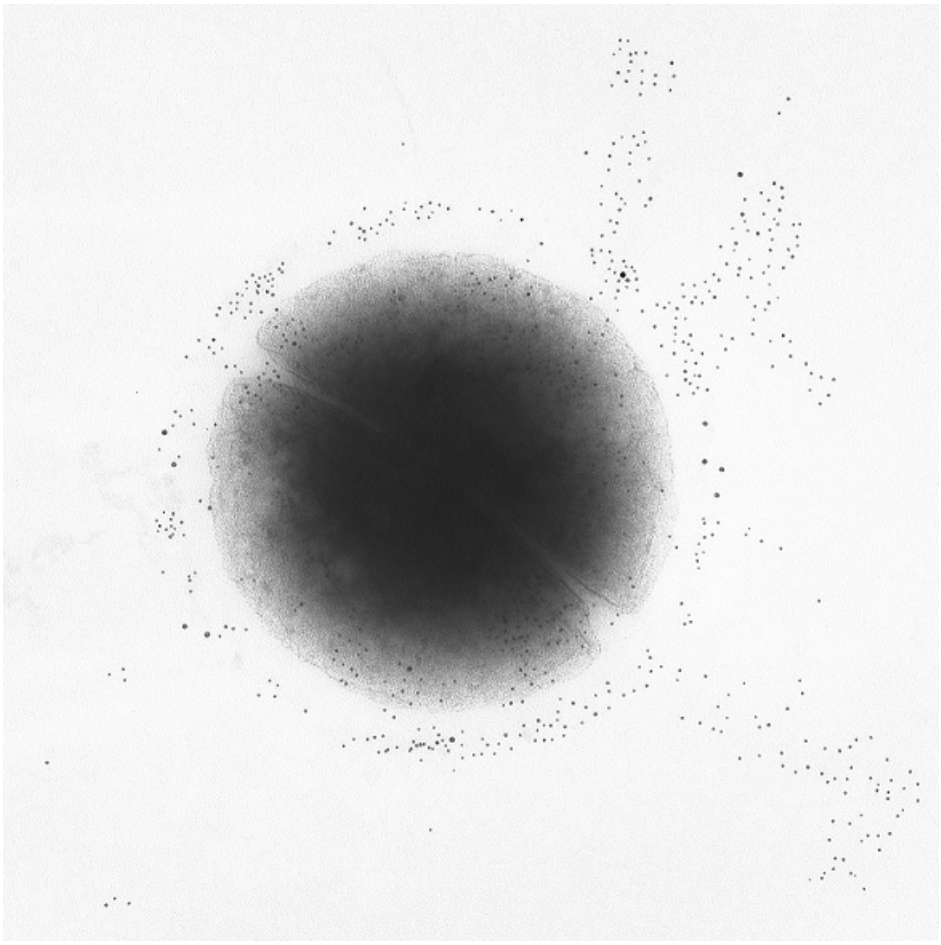

Figure 3. Streptococcal pili visualized by electron microscopy after labeling with subunit antibodies coupled to gold particles.

of Vaccinology," is to identify protective antigens by "simply" scanning the genome sequence of any given pathogen, which will let researchers avoid time-consuming "wet science" and "move straight from genome to the clinics."

With this objective in mind, a three-technology strategy that allows narrowing the number of antigens to be tested in the animal models down to less than ten was recently described (Bensi, et al., 2012). This approach has been successfully applied to *S. pyogenes,* and as a result, a three-antigen vaccine candidate is currently ready to enter Phase I clinical trials. The overall approach is based on the assumption that the antigens that induce broadly protective antibody responses are those that are conserved, well expressed, and either secreted or surface-associated. To identify this specific group of antigens, the genome sequences of all *S. pyogenes* isolates available in the public database were first analyzed to select conserved genes that potentially encode secreted and surface-associated proteins. These genes were then expressed in *E. coli* and the recombinant proteins were used in two ways: to produce mouse polyclonal antibodies and to build protein arrays. Polyclonal antibodies were subsequently exploited to establish which of the corresponding surface proteins were expressed at a high level in a battery of *S. pyogenes* isolates by using fluorescence-activated cell sorting (FACS) analysis (Technology 1), while protein arrays were used to select immunogenic proteins by screening a panel of sera from *S. pyogenes*-infected human patients (Technology 2). Finally, in Technology 3, secreted and surface-exposed proteins were identified by mass spectrometry (MS) by analyzing the supernatants (secretome) and the protease-derived peptides of "shaved" bacterial cells (surfome) from different isolates. Once available, the lists of antigens identified by MS, FACS, and protein array were merged in order to establish which proteins were identified by all three technologies. These "common" proteins are those that fulfill the assumption criteria, being: 1) well

expressed in a high number of strains; 2) immunogenic; and 3) are surface-exposed/secreted in multiple isolates. The three-technology strategy led to a global list of 40 antigens, with only six of these identified by all three experimental approaches. Remarkably, four of these six antigens were protective in three different mouse models (internasal, intraperitoneal and air pouch infection models) that used four *S. pyogenes* isolates belonging to different M types as challenge strains. The protective antigens include three particularly interesting proteins. One of them is SpyCEP, a serine protease that degrades IL-8 and other chemokines, which prevents neutrophil recruitment at the infection site (Edwards, et al., 2005). A second one is streptolysin O (SLO), a secreted toxin that kills eukaryotic cells through the formation of membrane pores. The third antigen is Spy0269, a previously uncharacterized hypothetical protein involved in bacterial cell division and probably also involved in adhesion to host cells (Gallotta, et al., 2014). The combination of SpyCEP, SLO and Spy0269 elicits robust protection in mice. Such protection is mediated by antibodies with different biological functions. They efficiently neutralize the hemolytic activity of SLO and the proteolytic activity of SpyCEP. In addition, the antibodies have bactericidal activity, as established by a whole blood bactericidal assay. Finally, the antibodies have the capacity to interfere with bacterial cell division and adhesion. Therefore, this multi-facet mechanism of protection makes this three-antigen COMBO vaccine particularly attractive for testing in human trials.

SOF/fibronectin-binding proteins and their potential as vaccine candidates

The binding of fibronectin (Fn) is an important function for *S. pyogenes*, as illustrated by the fact that *S. pyogenes* expresses at least 11 different Fn-binding proteins (Table 5). All of these Fn-binding proteins have been found to contribute to virulence and most are multifunctional. This section will focus on evidence concerning their potential as vaccine candidates, but reviews detailing their functions and contributions to virulence are also available (Walker, et al., 2014; Yamaguchi, Terao, & Kawabata, 2013). The Fn-binding proteins of *S. pyogenes* can be grouped into two basic categories; those that contain a common Fn-binding repeat region and those that contain a unique Fn-binding domain. Those with a common Fn-binding domain include SOF (SfbII), SfbX, protein F1 (SfbI), protein F2 and FbaB. Figure 4 shows a generic model of these proteins. Those with a unique Fn-binding domain include FbaA, Fbp54, GAPDH, M1/M3 proteins, Scl1, and Shr.

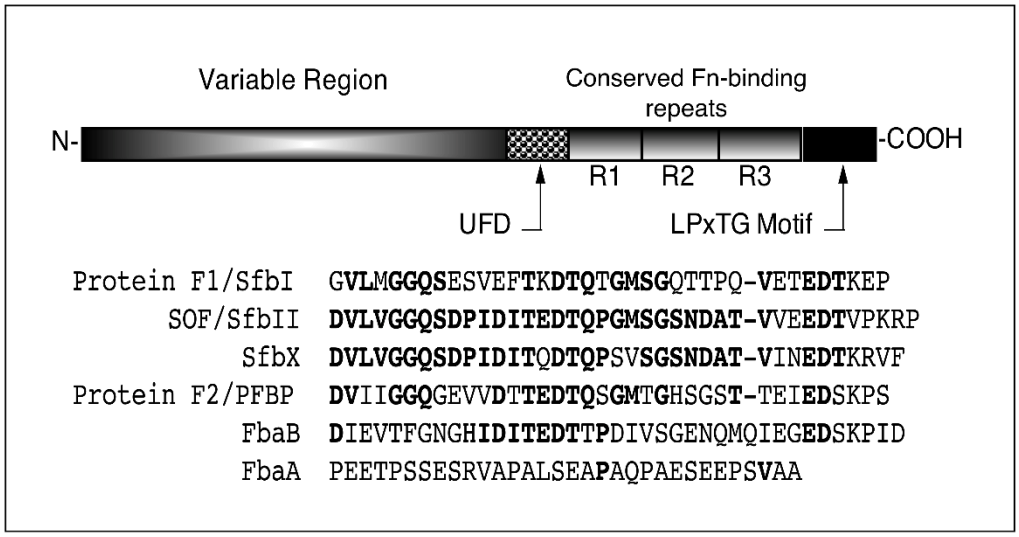

Figure 4. A schematic of *S. pyogenes* Fn-binding proteins. Some of the Fn-binding proteins contain an upper Fn-binding domain (UFD) that immediately precedes the repeat peptide domain (Schwarz-Linek, Höök, & Potts, 2006). A single repeat from each of the indicated Fn-binding proteins is shown and common amino acids are indicated in bold type. The number of repeats can vary. The repeat peptide of FbaA is shown but bears little homology to repeat peptides of other Fn-binding proteins, illustrating the uniqueness of its Fn-binding domain. The repeats are modified from those presented by Schwarz-Linek et al. (Schwarz-Linek, Höök, & Potts, 2004).

Table 5. Vaccine potential of Fn-binding proteins.

Fn-binding proteins	Antisera opsonizes	Protection in animal model	M types/% of clinical isolates	Reference
FbaA	yes	yes (IP)	1,2,4,13,22, 28,49,75,77	(Terao, Okamoto, Kataoka, Hamada, & Kawabata, 2005; Terao, et al., 2001)
FbaB	?	?	3,18	(Terao, Kawabata, Nakata, Nakagawa, & Hamada, 2002)
Fbp54	yes	yes (IP)	all	(Courtney, Li, Dale, & Hasty, 1994; Kawabata, et al., 2001)
GAPDH	yes	yes*	all	(Pancholi & Fischetti, 1992; Pancholi & Chhatwal, 2003; Boël, Jin, & Pancholi, 2005)
M1/M3 proteins	yes	yes	1,3	(Cue, Lam, & Cleary, 2001)
Protein F1/SfbI	no	yes (IN) no (SC)	~78%	(Hanski & Caparon, 1992; Schulze K. , et al., 2001; McArthur, et al., 2004; Olive, et al., 2007; Guzmán, Talay, Molinari, Medina, & Chhatwal, 1999; Sagar, et al., 2012)
Protein F2/PFBP	?	?	36-80%	(Jaffe, Natanson-Yaron, Caparon, & Hanski, 1996; Kreikemeyer, Oehmcke, Nakata, Hoffrogge, & Podbielski, 2004)
Scl1	?	?	all	(Fontán, Pancholi, Nociari, & Fischetti, 2000)
Shr	yes	yes	all	(Dahesh, Nizet, & Cole, 2012; Eichenbaum, 2012; Huang, Fisher, Nasrawi, & Eichenbaum, 2011)
SOF/SfbII	yes	yes (IP) no (IN)	~50%	(Courtney & Pownall, 2010; Rakonjac, Robbins, & Fischetti, 1995; Courtney, Hasty, & Dale, 2003; Gillen, et al., 2008; Schulze, Medina, & Guzmán, 2006)

Table 5. continued from previous page.

Fn-binding proteins	Antisera opsonizes	Protection in animal model	M types/% of clinical isolates	Reference
SfbX	?	?	~50% (all SOF + strains)	(Jeng, et al., 2003; Courtney, Nishimoto, Dale, Hasty, & Schmidt, 2004)

Abbreviations: Fn – fibronectin, IP- intraperitoneal challenge, IN – intranasal challenge, SC - subcutaneous challenge
? – no published report on whether the protein can induce opsonic or protective immune responses.
* - V. Pancholi, personal communication

SOF/SfbII

Serum opacity factor (SOF) opacifies human serum and has multiple functions (Courtney & Pownall, 2010). SOF and SfbII (streptococcal Fn-binding protein II) were cloned by different investigators and were provided with different names, based on the functions used to select clones that express the proteins (Rakonjac, Robbins, & Fischetti, 1995; Kreikemeyer, Talay, & Chhatwal, 1995). SfbII was subsequently found to be identical to SOF (Kreikemeyer, Martin, & Chhatwal, 1999).

Similar to M proteins, SOF varies structurally in its N-terminal domain, which results in over 50 different serotypes of SOF (Courtney & Pownall, 2010). Antisera to this variable N-terminal domain have been used to serotype strains of *S. pyogenes*, based on the inhibition of opacification of serum. The C-terminal domain of SOF contains an Fn-binding peptide repeat that is highly conserved.

The vaccine potential of SOF was first indicated from the findings that antisera against the N-terminal domain of SOF2 (SOF2ΔFN, SOF in which DNA encoding the Fn-binding domain was deleted) opsonized not only M type 2 *S. pyogenes*, but also M types 4 and 28 (Courtney, Hasty, & Dale, 2003). These findings suggested that SOF may contain shared protective epitopes. A combination of antiserum to SOF and M protein resulted in greater killing of *S. pyogenes* in human blood than either antiserum alone, which suggests that SOF may augment the killing efficiency of M protein-based vaccines. Antibodies to SOF that were affinity-purified from human serum were found to opsonize and kill *S. pyogenes,* which indicates that humans can respond to SOF and produce opsonic antibodies. Notably, an affinity matrix composed of the full-length SOF provided antibodies with a greater killing efficiency than a matrix composed of SOF2ΔFN (73% vs 43% killing), a finding that suggests that the Fn-binding domain of SOF may provide an extra degree of protection. In addition, IP and SC injections protected mice against challenges with *S. pyogenes*. These injections were well tolerated by mice, as no overt signs of toxicity were noted (Pancholi & Chhatwal, 2003).

Gillen et al. (Gillen, et al., 2008) compared mutants of recombinant SOF75 that did not opacify serum with wild-type SOF for their ability to stimulate a protective immune response in mice. The Fn-binding domain was also deleted in these constructs. SC immunization with SOF75ΔFN provided a significant degree of protection against a challenge from M49 *S. pyogenes* (100% survival rate vs 38% in controls), whereas the non-opacifying mutants of SOF75ΔFN provided a slight but insignificant degree of protection (50% survival). It is not clear why the non-opacifying mutants of SOF75 failed to provide adequate protection. However, these findings did indicate that SOF from one serotype can provide protection against a heterologous serotype of *S. pyogenes*.

In contrast to the above findings, Schulze et al. (Schulze, Medina, & Guzmán, 2006) found that intranasal immunization of mice with SOF failed to provide protection against an intranasal challenge with *S. pyogenes*. Although a significant immune response was generated against SOF, it was not determined if the antibodies were opsonic. Perhaps the intranasal route is not an optimal route for SOF to generate protection against a lethal challenge.

SOF is expressed in other streptococci. *S. suis* express SOF that does not contain an Fn-binding domain (Baums, et al., 2006) and *S. agalactiae* express FbpA, which is an opacity factor with a high degree of homology to SOF

from *S. pyogenes* (Courtney, et al., 1999). Thus, a vaccine against SOF may target other pathogenic streptococci, in addition to *S. pyogenes*.

Sfbx

Streptococcal Fn-binding protein x (Sfbx) is expressed by all SOF+ *S. pyogenes*, and its gene is found immediately downstream of *sof* and is cotranscribed with *sof* as a bicistronic message (Jeng, et al., 2003). Like SOF, *Sfbx* is expressed and its protein evokes an immune response during *S. pyogenes* infections in humans (Courtney, Nishimoto, Dale, Hasty, & Schmidt, 2004). Unlike SOF and M proteins, the N-terminal domain of SfbX does not vary (Courtney, Nishimoto, Dale, Hasty, & Schmidt, 2004). It is not known if Sfbx elicits a protective immune response, but antibodies against its Fn-binding domain should cross-react with those of SOF, because their Fn-binding repeats are almost identical. Rabbit antiserum to the N-terminal domain of Sfbx did not opsonize *S. pyogenes* that expresses this protein (Courtney, 2015), which suggests that this domain may not be an ideal candidate for a vaccine.

Protein F1/Sfb1

Protein F1 and SfbI (streptococcal Fn-binding protein I) are the same protein, but were discovered by different groups and were given different names (Hanski & Caparon, 1992; Talay, Valentin-Wiegand, Timmis, & Chhatwal, 1994). The N-terminal domains of SfbI from different serotypes have a 50-97% identity, whereas its Fn-binding domain is highly conserved (Towers, et al., 2003).

A number of studies have reported that IN vaccination of mice with SfbI conjugated to a variety of adjuvants provided protection against IN challenges with various serotypes of *S. pyogenes* (Table 5). Schulze et al. (Schulze, Medina, Talay, Towers, Chhatwal, & Guzmán, 2001) found that immunization with the Fn-binding repeats of SfbI afforded better protection than its N-terminal peptide. SfbI may also have adjuvant activity (Schulze, Medina, Chhatwal, & Guzmán, 2003; Schulze & Guzmán, 2003).

In contrast to the above studies, McArthur et al. (McArthur, et al., 2004) found that IN vaccination with Sfb1 failed to protect mice against death from an SC challenge of *S. pyogenes*. In addition, mouse and rabbit antisera against Sfb1 failed to opsonize *S. pyogenes*. The lack of protection in this study may be related to the failure to stimulate opsonic antibodies.

Protein F2, PFBP, and FbaB

To our knowledge, there has been no reported study on the stimulation of a protective immune response by protein F2, PFBP, or FbaB. Ramachandran et al. (Ramachandran, et al., 2004) proposed that PFBP (Rocha & Fischetti, 1999) and FbaB (Terao, Kawabata, Nakata, Nakagawa, & Hamada, 2002) are variants of protein F2 (Jaffe, Natanson-Yaron, Caparon, & Hanski, 1996). However, there is no significant homology between the central domains of protein F2 and FbaB, and it is unlikely that epitopes within these domains would stimulate a significant cross-protective immune response.

FbaA

FbaA is a Fn-binding protein expressed by a limited number of serotypes (Table 5). Immunization of mice with either the full-length FbA or with its Fn-binding domain provided significant protection against *S. pyogenes* (Terao, Okamoto, Kataoka, Hamada, & Kawabata, 2005). It is interesting to note that purified antibodies to FbaA also opsonized *S. pyogenes* (Terao, Okamoto, Kataoka, Hamada, & Kawabata, 2005). The highest level of opsonization was obtained with antibodies to the full length FbaA (68% killing) and intermediate levels obtained with antibodies to the N-terminal region of FbaA (57% killing) and Fn-binding repeats of FbaA (44% killing).

Fbp54

Fbp54 is an Fn-binding protein with a calculated molecular weight of 54 kDa (Courtney, Li, Dale, & Hasty, 1994), and its gene is found in all *S. pyogenes* strains (Kawabata, et al., 2001). The name Fbp54 is actually a misnomer and was based on the calculated mass of the cloned protein, which was thought to contain the entire gene at the time of cloning. Subsequent work (Courtney, 2015) and its sequence in *S. pyogenes* genomes indicated that an N-terminal fragment of 76 amino acids was missing.

Fbp54 is antigenic in humans (Courtney, Dale, & Hasty, 1996) and rabbit serum against Fbp54 opsonized *S. pyogenes* (Kawabata, et al., 2001). Immmunization with Fbp54 by various routes in mouse models was found to evoke protection against challenges with multiple serotypes of *S. pyogenes* (Kawabata, et al., 2001). Fbp54 is conserved in many streptococcal species with high identity to pavA of *S. pneumoniae* (Holmes, et al., 2001), Sfba from group B streptococci (Mu, et al., 2014), and FbpA of *S. gordonii* (Christie, McNab, & Jenkinson, 2002), which suggests that vaccines that contain Fbp54 may, in theory, target other streptococci, as well as *S. pyogenes*.

GAPDH

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a multifunctional, intracellular enzyme that is also found on the surface of *S. pyogenes* where it binds fibronectin in addition to other host proteins (Pancholi & Fischetti, 1992; Pancholi & Chhatwal, 2003; Jin, Agarwal, Agarwal, & Pancholi, 2011). Antibodies to GAPDH both opsonized *S. pyogenes* (Boël, Jin, & Pancholi, 2005) and protected mice from a *S. pyogenes* challenge (V. Pancholi, personal communication). However, there are similarities between human and bacterial GAPDH, and full autoimmune responses to GAPDH have not been thoroughly investigated (Fontán, Pancholi, Nociari, & Fischetti, 2000). In addition, GAPDH is expressed by many bacteria, and a GAPDH vaccine may target both pathogens and commensals.

Scl1

Scl1 is a collagen-like protein expressed by *S. pyogenes* that binds cellular Fn, but not plasma Fn (Oliver-Kozup, et al., 2013). Antibodies against Scl1 have been found in human serum and in mice infected with *S. pyogenes* (Hoe, Lukomska, Musser, & Lukomski, 2007). However, none of these responses have been shown to provide protection against infections. It remains to be resolved whether purified Scl1 will induce antibodies that will cross-react with human collagen and/or provide overall protection against *S. pyogenes* infections.

Shr

Shr (streptococcal hemoprotein receptor) is a virulence factor that, as the name implies, binds heme containing proteins and also binds extracellular matrix proteins laminin and fibronectin (Dahesh, Nizet, & Cole, 2012). Shr is highly conserved and is found in all sequenced genomes of *S. pyogenes* and in *S. dysgalactiae* subspecies *equisimilis* (Eichenbaum, 2012). Both IP and IN immunizations with Shr provided protection against *S. pyogenes* challenge infections in mice (Huang, Fisher, Nasrawi, & Eichenbaum, 2011). Furthermore, mice passively immunized with rabbit antiserum to Shr were protected against challenges with M1 and M3 *S. pyogenes*, which suggests that Shr may provide protection against multiple serotypes.

M1/M3 proteins

The M1 and M3 proteins bind Fn through interactions with their A and B repeat regions (Cue, Lam, & Cleary, 2001). Other serotypes of M proteins that have been tested do not bind Fn. However, the binding of Fn to M proteins has not been extensively investigated, and as a result, it is not clear if other M types may also bind Fn. The vaccine potential of both the conserved and non-conserved regions of M proteins is discussed elsewhere in this chapter.

In summary, many of the Fn-binding proteins have been found to provide protection against *S. pyogenes* infections in mouse models. Some of these were expressed by all types of *S. pyogenes* proteins (including Shr, GAPDH, and FBP54) but other, non-pathogenic bacteria may also express these proteins, and it can be debated if it would be wise to use a vaccine that targets both pathogens and commensals. Some of the Fn-binding proteins are expressed only by a limited number of serotypes (FbaA, FbaB) and may not be the best candidates to include in a multi-component vaccine. The Fn-binding domain shared by protein F1/SfbI, protein F2, SOF, and SfbX should be considered for inclusion into a multivalent vaccine, because this domain not only provided a significant degree of protection in animal models, but also had adjuvant activity. Although there is a fair degree of homology between the Fn-binding repeat domain of these proteins (Figure 3), the degree of cross-reactive immune responses to these domains has not yet been investigated. Thus, it is not known if the Fn-binding domain of one Fn-binding protein will provide cross-protection against serotypes of GAS expressing a different Fn-binding protein. Finally, the question arises whether the addition of the Fn-binding domain and/or the N-terminal domain of those Fn-binding proteins expressed by a significant number of serotypes would enhance the coverage of serotypes and protective efficacy of a multicomponent vaccine.

ScpA as a potential vaccine component

The streptococcal C5a protease (SCP) is expressed on the surface of all serotypes of *S. pyogenes* and most human isolates of groups B, C, and G streptococci, where it specifically destroys C5a. The enzyme also binds fibronectin and functions as a low level invasin for *S. pyogenes* (Courtney, Nishimoto, Dale, Hasty, & Schmidt, 2004), group B streptococci (Tenenbaum, et al., 2007), and group G streptococci (Wei, et al., 2013; Severin, et al., 2007). The genetic diversity of *S. pyogenes*, and the fact that other species of β hemolytic streptococci cause pharyngitis and may induce similar complications, suggest that a vaccine must include multiple, antigenically conserved proteins in order to significantly reduce the incidences of pharyngitis, skin infections, and various sequelae. The specific inhibition of complement C5a that mediates phagocyte recruitment was first discovered in *S. pyogenes* (Wexler, Chenoweth, & Cleary, 1985), but has since been shown to be a universal mechanism of pathogenesis. Other streptococcal species and unrelated bacteria, including staphylococci and borrelia, also interfere with C5a recruitment of phagocytes to infectious foci. Some *S. pyogenes* serotypes also produce proteases that destroy IL-8, another phagocyte chemoattractant (Hidalgo-Grass, et al., 2006; Sjölinder, et al., 2008). The high degree of sequence similarity of SCP from different serotypes of *S. pyogenes* and other β-hemolytic species, and its pivotal role in the pathogenesis of these streptococci, argue strongly for its inclusion in vaccines. Increases in SCP-specific antibodies were documented after episodes of pharyngitis in children and adults (Shet, Kaplan, Johnson, & Cleary, 2003) and human sera with high titers of anti-SCP neutralize cleavage of C5a by purified enzyme (Cleary, Matsuka, Huynh, Lam, & Olmsted, 2004). The primary goal of vaccine development is to prevent colonization of oral and vaginal mucosae and associated lymphoid tissue by all β-hemolytic streptococci that are commonly associated with human disease. Protection studies used truncated forms of recombinant SCP with mutations in the active site and employed subcutaneous, intranasal, or intravaginal rodent infection models. Intranasal and subcutaneous immunization with recombinant SCPA and adjuvants induces robust anti-SCP IgG and IgA responses that speed the clearance of streptococci from pharyngeal- and nasal-associated lymphoid tissue (NALT) after challenge with one of several serotypes tested (Cleary, Matsuka, Huynh, Lam, & Olmsted, 2004; Ji, Carlson, Kondagunta, & Cleary, 1997; Ji, McLandsborough, Kondagunta, & Cleary, 1996; Park & Cleary, 2005; Suvorov, et al., 2010). Antibody directed against SCPA inhibits cleavage of C5a and is also opsonic for both *S. pyogenes* and group B streptococci (Suvorov, et al., 2010). As expected, the intranasal administration of anti-SCPA prevented colonization of NALT, the mouse homologue of human tonsils (Park & Cleary, 2005).

Although GBS infections are less common, neonatal infections are associated with significant mortality. SCP is also an important virulence determinant for groups B and C streptococci, and vaccination with SCP prevented lethal infections of mice when challenged by either species (Wei, et al., 2013; Suvorov, et al., 2010; Cheng, et al., 2001; Cheng, et al., 2002). Vaccination of mice with SCP also prevented vaginal colonization of dams and

protected their pups against challenge with Type III group B streptococci. Lessons learned from peptide vaccines for prevention of pertussis and other mucosal infections suggests that an efficacious vaccine will likely require multiple surface antigens. Toward this end, Severin et al. used proteomic screens to identify other surface proteins that could be combined with SCP in a multi-component vaccine-peptides that are uniformly expressed by most *S. pyogenes* M types and other β-hemolytic streptococcal pathogens (Severin, et al., 2007).

Group A Streptococcus: Clinical Trial Design

History of *S. pyogenes* vaccine trials

As described above, the slow progress toward developing a vaccine for *S. pyogenes* that began with clinical trials in 1923 (Steer, Batzloff, Mulholland, & Carapetis, 2009) was halted in the late 1970s, following a report in 1969 of two definite and one probable cases of rheumatic fever in recipients of a crude M protein vaccine (Massell, Honikman, & Amezcua, 1969). Although subsequent review of this study casts doubt on the role of the vaccine in the onset of rheumatic fever, in 1979, the United States Food and Drug Administration (FDA) prohibited the administration of *S. pyogenes* vaccines in clinical trials in the US, a prohibition that remained in place for nearly 30 years. Since the lifting of the ban in the last decade, only a few clinical trials of candidate *S. pyogenes* vaccines have been performed, although several vaccine candidates are approaching the clinical development stage.

General design issues

As with any vaccine, potential *S. pyogenes* vaccines will need to first undergo phase 1 clinical trials to demonstrate their initial safety in humans. Dose-ranging studies will explore the optimal dose of the vaccine antigens, based on findings from pre-clinical animal studies. The number of doses and dose intervals will also be determined, which are typically based on both animal studies and clinical trials of vaccines with similar antigen compositions. For example, a purified or recombinant protein-based vaccine may initially be studied using a three-dose schedule, similar to that used with hepatitis B vaccines (two closely spaced injections and a third injection after a longer interval). Inclusion of an adjuvant will require demonstration of improved immunogenicity compared to the antigen alone. Inclusion of a control (either placebo or a licensed, non- *S. pyogenes* vaccine) will permit blinding of the study and provide an unbiased assessment of adverse events. While smaller phase 1 studies often use a placebo, larger phase 2 studies and studies in children may benefit from use of a licensed, non-*S. pyogenes* vaccine as the comparator vaccine so that all participants can derive a potential benefit from participation in the study. Although the target age for an *S. pyogenes* vaccine is likely to be pre-school-aged children, prior to the peak of *S. pyogenes* pharyngitis and rheumatic fever, clinical trials of *S. pyogenes* vaccine candidates will, out of necessity, first be performed in adults. Although regulatory agencies frequently require vaccines targeting infants to first be studied in older children, it is not clear whether subsequent studies of an *S. pyogenes* vaccine could be immediately undertaken in pre-school-aged children after phase 1 and early phase 2 studies in adults. An argument could be made that following experience with vaccination of approximately 100 adults with a candidate *S. pyogenes* vaccine, a phase 2 study in 3–5 year olds could be initiated.

Immunogenicity outcomes

Immunogenicity outcomes will be an important component of any clinical trial of an *S. pyogenes* vaccine. Whatever the antigen employed, demonstrating induction of a specific immune response to the antigen will be required, using validated immunological assays. For M protein vaccine candidates, assessment of antibody response against each component M type will be required; for the 30-valent *S. pyogenes* vaccine currently under development, this will require 30 assays for each participant at each time point. In contrast, single-antigen *S. pyogenes* vaccine candidates, such as the J8 vaccine from the conserved C-repeat section of the M protein, require only a single serological assay (Batzloff, et al., 2003). Vaccine candidates, such as the StrepInCor vaccine

that uses B and T cell epitopes from the C-repeat section, will likely require assays that measure T cell response in addition to the antibody responses (Guilherme, et al., 2006).

The lack of a definitive correlate of protection for *S. pyogenes* vaccines has led to the development of a number of functional assays to measure the biological activity of the antibodies elicited. The clinical trials undertaken with the hexavalent and 26-valent M protein-based *S. pyogenes* vaccines measured antibody responses by both enzyme immunoassay and by a functional opsonophagocytosis assay (Kotloff, et al., 2004; McNeil, et al., 2005). Functional assays are tedious to perform and are not easily adaptable for high throughput methodologies. While functional assays may be required in the early development of the 30-valent M protein *S. pyogenes* vaccine candidate, limiting such assays in larger, later-phase studies may be necessary for reasons of feasibility. For example, 30 opsonophagocyosis assays for each participant at every blood collection would amount to tens of thousands of assays in a large phase 2 or 3 study. It is not yet known whether the development and validation of functional assays will be required for *S. pyogenes* vaccine candidates that use non-M protein antigens. The identification of a correlate of protection using an easily performed, high-throughput assay would greatly alleviate these issues. Alternately, performing functional assays on a subset of participants (and with a subset of M proteins in the case of a multivalent M protein vaccine) might be an acceptable option.

Safety outcomes

Because of the history of safety concerns, adverse event monitoring in clinical trials of *S. pyogenes* vaccines will be subjected to even greater scrutiny than in other vaccine clinical trials. Routine adverse event monitoring will be required, including injection site reactions (such as erythema, swelling, tenderness) and systemic adverse events (such as fever, headache, fatigue, anorexia). In addition to these common adverse events and reporting of all serious adverse events, surveillance for adverse events of special interest to *S. pyogenes* vaccines, including carditis and arthritis, will be required. In early phase trials, monitoring will require regular interval physical examinations, baseline and routine serum chemistry and hematology, and assays to measure complement (C3) and inflammatory markers (C reactive proteins). In the clinical trials of the 26-valent M protein vaccine, baseline and follow-up electrocardiograms and echocardiograms were performed, as well as baseline and follow up assays for tissue cross-reactive antibodies (heart, kidney, cartilage and brain) (Kotloff, et al., 2004; McNeil, et al., 2005). As with the functional serological assays, tissue cross-reactive antibodies are not routinely available, and these tests are burdensome to perform. Further discussions with regulatory authorities and scientific experts will be needed to determine the stage in the clinical development process at which these assays will no longer be required. The use of echocardiograms as a screening tool is problematic, as use during clinical trials of the 26-valent M protein vaccine demonstrated a wide range of normal variation of non-pathological findings in normal, healthy individuals. The standardized, reproducible interpretation of echocardiograms in healthy adults also proved to be a challenge; these challenges may be even greater in healthy pre-school-aged children. The necessity for echocardiograms and electrocardiograms through all phases of the clinical vaccine development program will also be an important topic for discussion with regulatory authorities.

Efficacy studies

In the absence of a definitive immunological correlate of protection, phase 3 studies of candidate *S. pyogenes* vaccines will require efficacy outcomes. Ideally, these efficacy outcomes will be easily measured and confirmed, and will be sufficiently common so that study sample sizes will be manageable (Steer, Dale, & Carapetis, 2013). Pharyngitis is the most readily measured efficacy outcome, because its clinical symptoms are readily observed, and laboratory confirmation through rapid antigen testing and culture is easily performed and is readily available, inexpensive, and reproducible. However, the prevalence of asymptomatic streptococcal carriage, and the fact that pharyngitis is most common in school-aged children, yet the target population for vaccination is the pre-school-aged child, are important factors that must be considered, as they may impact the assessment of efficacy when pharyngitis is the outcome of interest. The latter issue could be addressed by focusing enrollment

of older pre-school-aged children, prolonging the duration of follow-ups, and marginally increasing the sample size. Impetigo is another common manifestation of *S. pyogenes* infection that could be used as an efficacy outcome, but it is unclear whether vaccination will protect against skin infection and its presence is less commonly confirmed by laboratory testing. However, the J8 vaccine, when administered, subcutaneously does protect mice from pyoderma that is due to multiple strains of *S. pyogenes* (Pandey, et al., 2015).

Acute rheumatic fever and invasive streptococcal infections are the most severe forms of *S. pyogenes* infection and would be important targets for prevention by a candidate *S. pyogenes* vaccine. However, ARF and invasive infections are uncommon, even in the developing world, where the incidence of these complications far exceeds rates observed in industrialized countries. A phase 3 efficacy study with prevention of rheumatic fever as the outcome would be problematic as it would require follow-up for participants to identify cases of acute rheumatic fever. In the context of this follow-up, should episodes of pharyngitis be identified, treatment with antibiotics would be required and cases of rheumatic fever would be avoided. As a result, the ethical issues involved in the design of such a study would be challenging. The efficacy of *S. pyogenes* vaccines against ARF may best be assessed in post-licensure phase 4 studies.

Development of an *S. pyogenes* human challenge model might provide an opportunity to identify correlates of protection that could be used in the design of a phase 3 *S. pyogenes* vaccine field trial. Challenge studies with *S. pyogenes* were used frequently in the 1960s and 1970s, prior to the FDA's hold on *S. pyogenes* vaccine clinical trials (Polly, Waldman, High, Wittner, & Dorfman, 1975; D'Alessandri, et al., 1978). These studies could only be undertaken in adults and would be used to inform the design of the pivotal phase 3 study in children, rather than to directly support the licensure of a candidate vaccine.

Conclusion

The overall goal of *S. pyogenes* vaccine development is to introduce vaccines that will significantly impact the global burden of disease. As summarized in this chapter, there are a number of candidate vaccine antigens that have been proven to be efficacious in animal models of *S. pyogenes* challenge infections. The impediments to bringing an effective and affordable vaccine to market have proven to be significant—but are not insurmountable. The greatest challenge will in developing vaccines that address the global need and that are effective in preventing the infections that may trigger ARF and RHD, as well as serious invasive infections. This may require a systematic experimental approach to develop combination vaccines that contain multiple protective antigens to achieve the desired level of protective immunity. The successful global deployment of safe and affordable vaccines could have a significant, positive impact on the morbidity and mortality that is attributable to *S. pyogenes* infections.

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