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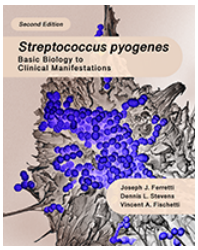

7. *Streptococcus pyogenes* Metabolism

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Introduction

Streptococcus pyogenes (Group A *Streptococcus*/GAS *or S. pyogenes*) is endowed with a formidable arsenal of virulence factors that allow it to evade 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). Core metabolism and its associated regulatory networks are the lifeline of the organism, which confer upon it the ability to efficiently exploit the resources of diverse host niches for the manufacture of the cellular building blocks it requires to maximize fitness. Hence, as much as it may seem dry and dull, the subject of metabolism provides a framework for understanding the pathogenesis of the multiple diseases caused by *S. pyogenes*. In this chapter, we integrate historical analyses with numerous recent studies on gene regulation, post-translational regulation, and biofilm formation to provide a unified understanding for how metabolism and its regulation impact *S. pyogenes* virulence.

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

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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 way 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, 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, 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. To understand how specific metabolic pathways uniquely present in a lactic acid bacterium adapted to these complex nutritional environments, a genome scale *in silico* model of essential metabolism of an M49 strain was constructed (Levering, et al., 2016). The model was further constrained through analysis of growth in a minimal medium to compare a WT M49 strain to a mutant that lacked *arcA* (encoding the aerobic respiration control protein/arginine deaminase) for calculation of nutrient uptake and production fluxes. This resulted in the identification of 480 genes associated with 575 reactions and 558 metabolites that are predicted to be required for growth (Levering, et al., 2016). Of these genes, 58% were subsequently identified in a genome-wide transposon mutagenesis analysis using M1T15488 and M49 strains to identify genes essential for fitness in several *in vivo* animal models of *S. pyogenes* infection (Le Breton, et al., 2015). By identifying metabolic pathways critical for growth, these studies may help define new targets for the development of novel drugs to counteract *S. pyogenes* infection.

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. The 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. Consequently, 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) promoting sustained asymptomatic colonization. Subsequently, *S. pyogenes* can invade and proliferate in the equally nutritionally challenging intracellular environment of the host and deeper tissues. In certain hosts, *S. pyogenes* can reemerge after an extended period of quiescence (Osterlund, Popa, Nikkilä, Scheynius, & Engstrand, 1997) and overcome the 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 (Henningham, Döhrmann, Nizet, and Cole, 2015).

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 how 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 (National Library of Medicine, 2022).

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 this chapter, these uncharacterized pathways are described in context with published *S. pyogenes* genome sequences and transcriptome analyses, or the corresponding pathways described in other Gram-positive pathogens, to emphasize their pathophysiologically relevant contribution to *S. pyogenes* virulence and the mechanism of pathogenesis. This discussion will include pathways affected during biofilm formation and those affected by post-translational modifications by serine/threonine/tyrosine kinases and phosphatases.

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 (the conversion of glucose to glucose-6-P and fructose-6-P to fructose 1,6–bis-P) and second half (the conversion of 1,3-diphosphoglycerate to 3-phosphoglycerate and phosphoenolpyruvate to pyruvate) 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, as has been described for other lactic acid bacteria (Carlsson, Kujala, & Edlund, 1985) (Figure 1).

In many bacteria, methylglyoxal (MGO) is produced as a by-product of glycolysis. MGO is a dicarbonyl α-oxoaldehyde metabolite that is a highly reactive electrophilic compound with a molecular weight of 72Da, and is highly toxic. It is generated from the spontaneous degradation of the triose phosphate intermediates, glyceraldehyde-3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP) (Silva, Gomes, Ferreira, Freire, & Cordeiro, 2013). In most bacteria, MGO exists only transiently, as it is rapidly detoxified by converting it to lactate in a two-step process mediated by methylglyoxal synthase (MGS) and glyoxalase (Glo). Intriguingly, while MGS is not present in *S. pyogenes*, it does contain an orphan Glo (GloA, Spy_1787/VOC family protein or SP5448_09040 of M1T1 strain 5448). Analysis of a GloA mutant revealed that it was more sensitive to methylglyoxal produced by human neutrophils through the activity of neutrophil myeloperoxidase (Zhang, Ong, Walker, & McEwan, 2016). These data implicate GloA in *S. pyogenes*' defense against innate immunity and suggests that GloA may serve as an important target to develop a novel therapeutic agent (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; 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, &

Fig.1

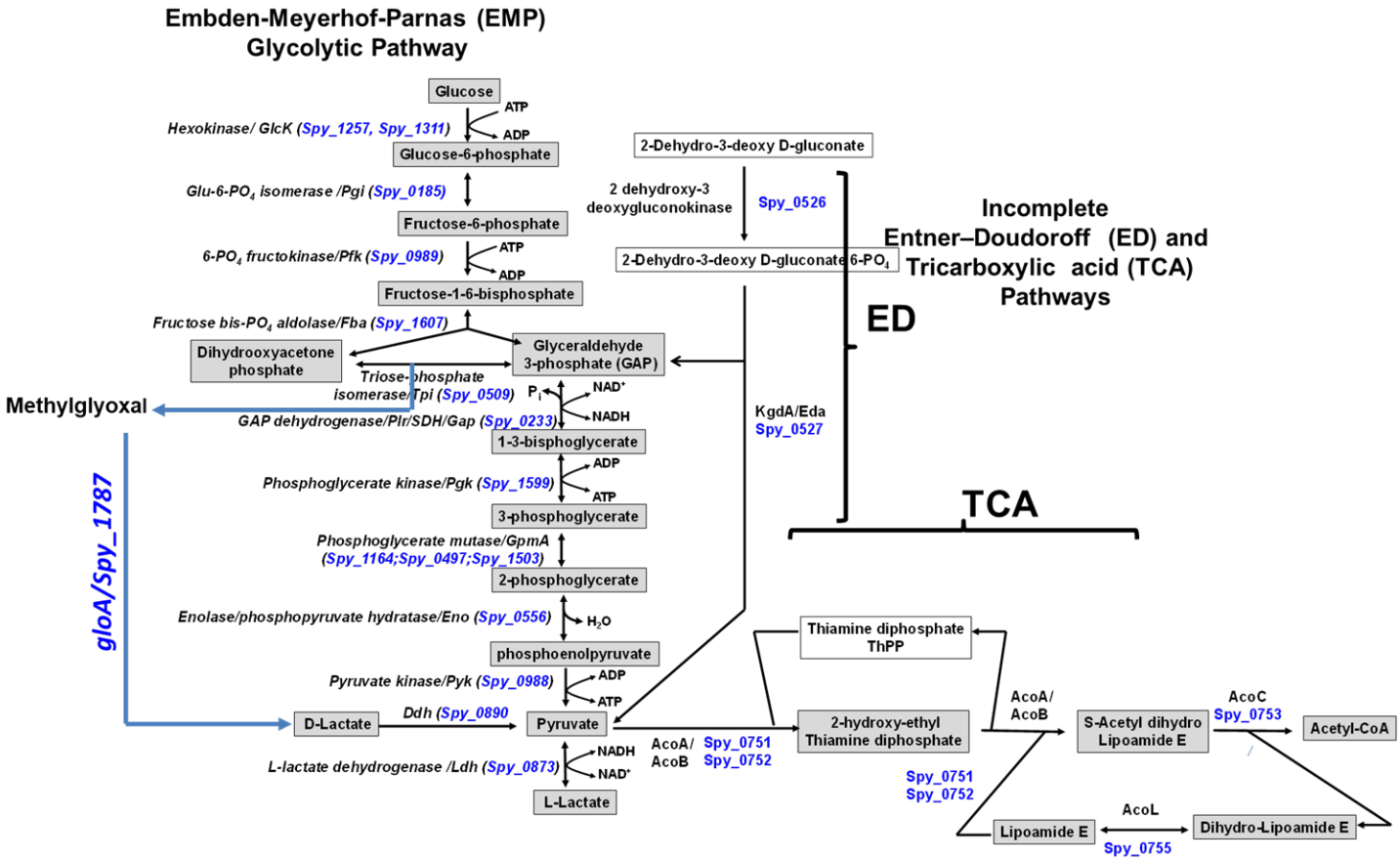

Figure 1: The Embden-Meyerhof-Parnas (EMP) pathway and its association with the incomplete Entner-Doudoroff (ED) and tricarboxylic acid (TCA) pathways present in *Streptococcus pyogenes*. *S. pyogenes* uses glucose as its sole carbon source for energy. The significance of the presence of incomplete ED and TCA pathways is currently unknown. The EMP pathway is responsible for the net gain of two ATP molecules and two $NADH_2$ molecules. GAS lacks a methylglyoxal synthase enzyme, which catalyzes the formation of methylglyoxal from triosephosphate. Methylglyoxal is then converted to D-lactate by Glo1 and subsequently tp pyruvate by LDH. The function of this orphan glyoxalase Glo1 is explained in the text. 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.

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 and a putative PTS β-glucoside transporter system (SPy_0475/bglP/ transporter, SPy_0476/bglB/phospho-β-glucosidase). These PTS systems couple the import of one or more carbohydrates with their phosphorylation prior to metabolism, which can influence GAS pathogenesis. In a recent global analysis of 14 annotated PTS permeases (EIIC), the *bglP – bglB* operon was found to be crucial for GAS growth and survival in human blood in in two different M1T1 strains (Braza, et al., 2020). Transcription of the operon is under the control of a licT anti-terminator that is repressed by glucose, but induced by the β-glucoside salicin. Mutants that lack BglP and BglB had defects in regulation of virulence-associated genes that control biofilm formation and streptolysin S (SLS)-mediated hemolysis. Together, these data show that salicin can influence GAS pathophysiology and support the idea that the carbohydrate content of the microenvironment plays a major role in dynamically managing GAS growth, virulence factor expression, and tissue damage (Paluscio, Watson, Jr., & Caparon, 2018).

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).

In certain *Streptococcus* species and other Gram-positive bacteria, the Cre-mediated CcpA regulation at some genes can be independent of HPr (Willenborg, de Greef, Jarek, Valentin-Wiegand, & Goethe, 2014). DebRoy et al. (DebRoy, et al., 2021) investigated whether this is also true for *S. pyogenes*. Using a RNAseq/ CHIP- seq approach, they found that abrogation of CcpA- HPr interaction impacted ~40% of 514 CcpA regulon genes, while CcpA directly bound to ~20% of the CcpA regulon, including the genes that encode the virulence factors Streptolysin S, PrtS (IL-8 degrading proteinase), and SpeB. They also found that CcpA-mediated regulation of

certain genes is likely controlled by an unknown mechanism that does not involve phosphorylation of Hpr at the canonical residues Ser46 and Ser15. A detailed discussion of *S. pyogenes* transcriptional regulation is described in another chapter in this book and in a recent review (Rom, Hart, & McIver, 2021).

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, & 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), the 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).

As described above, CcpA-repression of maltose and maltodextrin transport genes is semi-dependent on its interaction with HPr. An exception is that CcpA repression of maltose transport via Spy_1692 (EIIc, Figure 2) is independent of HPr (DebRoy, et al., 2021). Dual regulation by HPr-dependent and independent CcpA and MalR ensures that the latter contributes to *S. pyogenes* virulence in a site-specific manner at the oropharynx. An additional example is the 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, Sumby, Sitkiewicz, Granville, DeLeo, & Musser, 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, Sumby, Sitkiewicz, Granville, DeLeo, & Musser, 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 mechanisms that underlie SptR-regulated carbohydrate metabolism and virulence are currently unknown.

Fig. 2

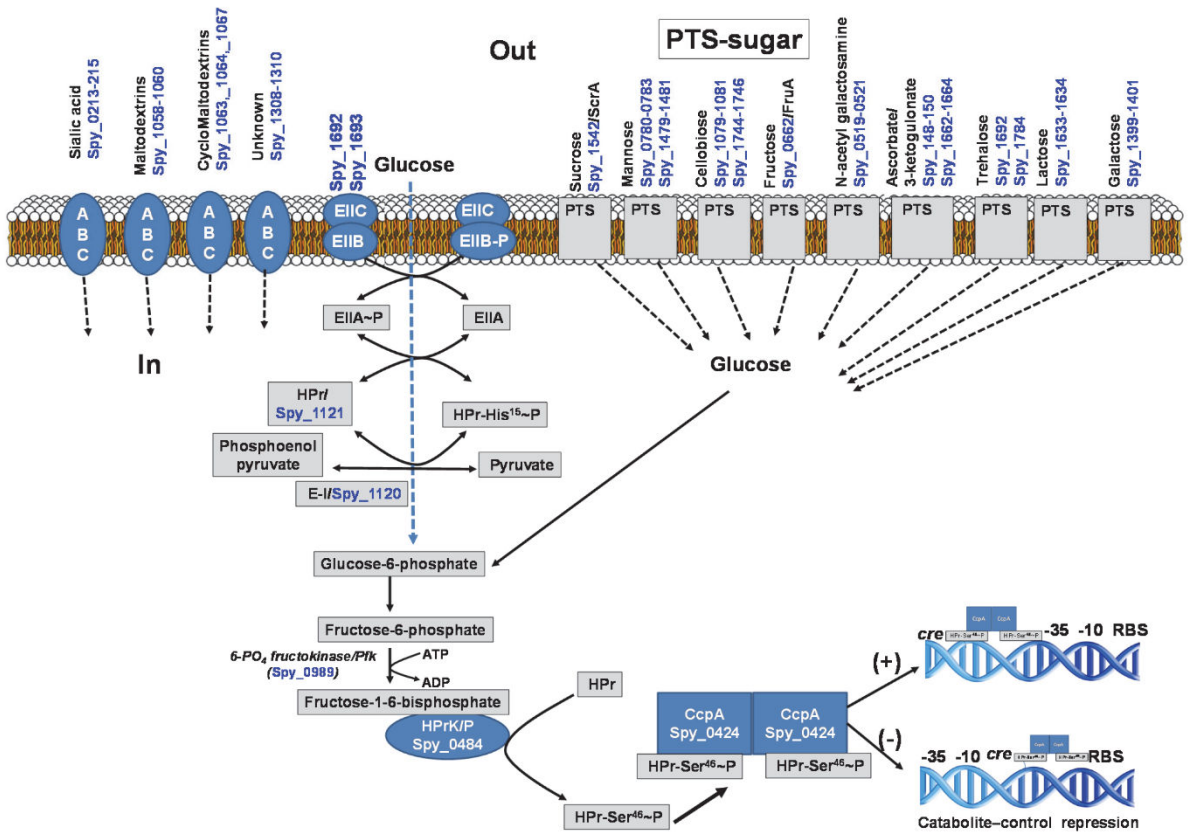

Figure 2: Phosphoenol pyruvate (PEP)-dependent phosphotransferase (PTS) pathway and catabolite repression. The PEP-PTS pathway in *S. pyogenes* couples the phosphorylation and import of glucose and non-glucose sugars. *S. pyogenes* possesses 14 PTS and four ABS transport systems to transport glucose 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.

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

A

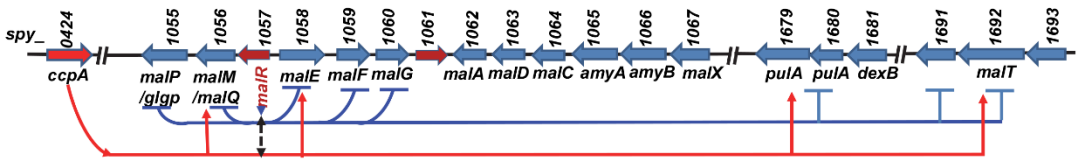

Fig. 3

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.

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). Although lactose and galactose transport in *S. pyogenes* are regulated by CcpA by both HPr-independent and semi-dependent mechanisms (DebRoy, et al., 2021), their regulation includes additional unique features. 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 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). This pathway is also influenced by the concentration of Zn^{2+} when *S. pyogenes* comes in contact with neutrophils (Ong, Walker, & McEwan, 2015), which release Zn^{2+} to inhibit the growth of phagocytosed pathogens (Botella, Stadthagen, Lugo-Villarino, de Chastellier, & Neyrolles, 2012). In response, *S. pyogenes* switches its carbon metabolism away from glucose to favoring galactose as revealed by increased *lacC.2* expression and LacC.2 enzyme activity. Thus, Zn^{2+} toxicity is less pronounced when *S. pyogenes* is grown in the presence of galactose (Ong, Walker, & McEwan, 2015). The mechanisms of metal toxicity are described in more detail below (see section on ion metabolism).

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 many 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-

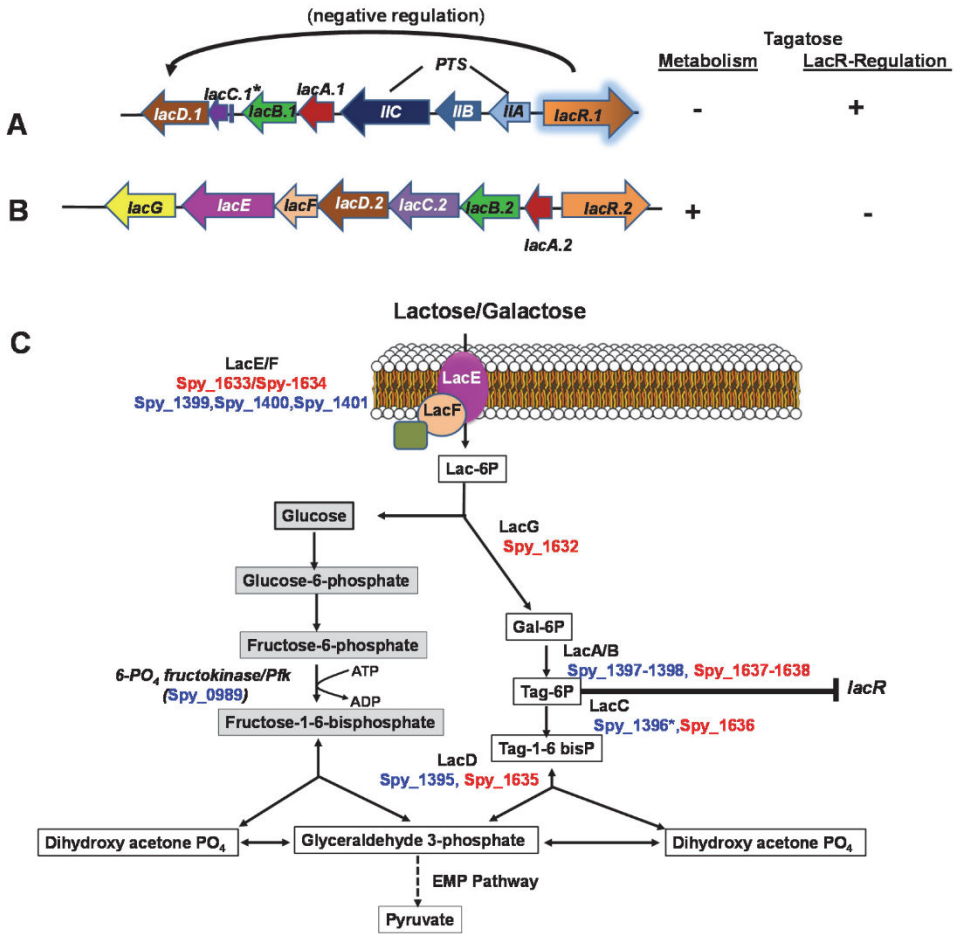

Fig. 4

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.

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 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).

Fig. 5

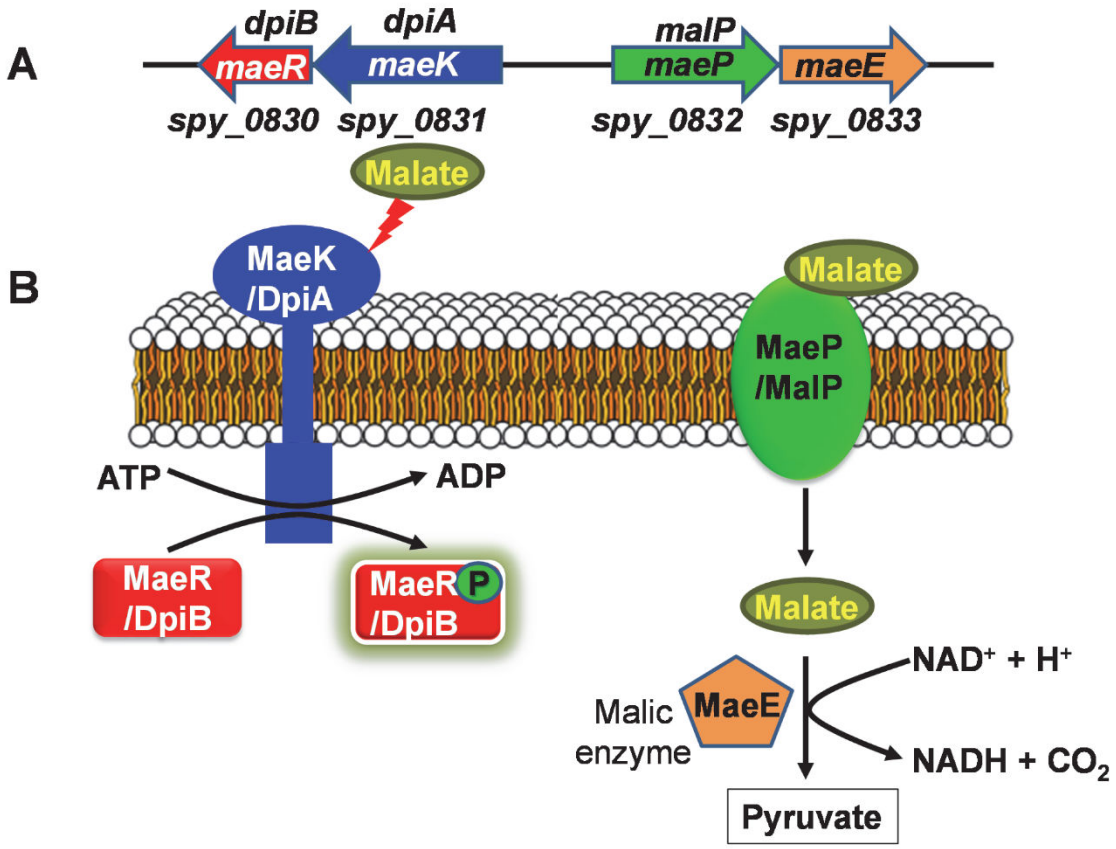

Figure 5: The malic enzyme (ME) pathway in *S. pyogenes*. (A) The arrangement of the open-reading frame that constitutes the ME locus of *S. pyogenes*. (B) A schematic presentation of the ME pathway depicts malate utilization via malate permease (MaeP/MalP). Once transported to the cytoplasm, the intracellular 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.

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 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 remains 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 the 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 in 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.

Fig. 6

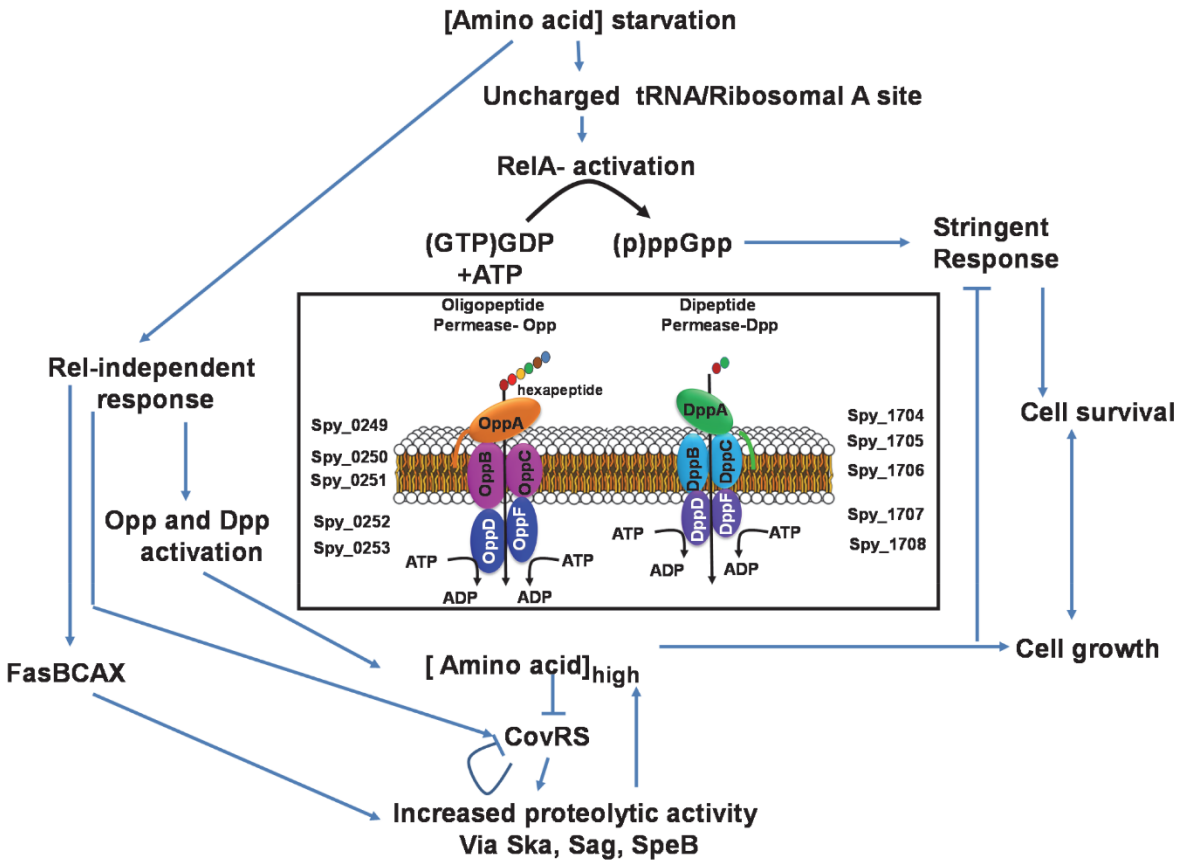

Figure 6: RelA-dependent and -independent amino acid starvation response networks of S. pyogenes. Arrows indicate positive regulation, while blunt-ended arrows indicate negative regulation or repression. The schematically presented network highlights the up-regulation of several genes including those 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.

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, the excretion of acetate creates an acidic environment, which can induce acid stress and may accumulate to toxic levels.

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

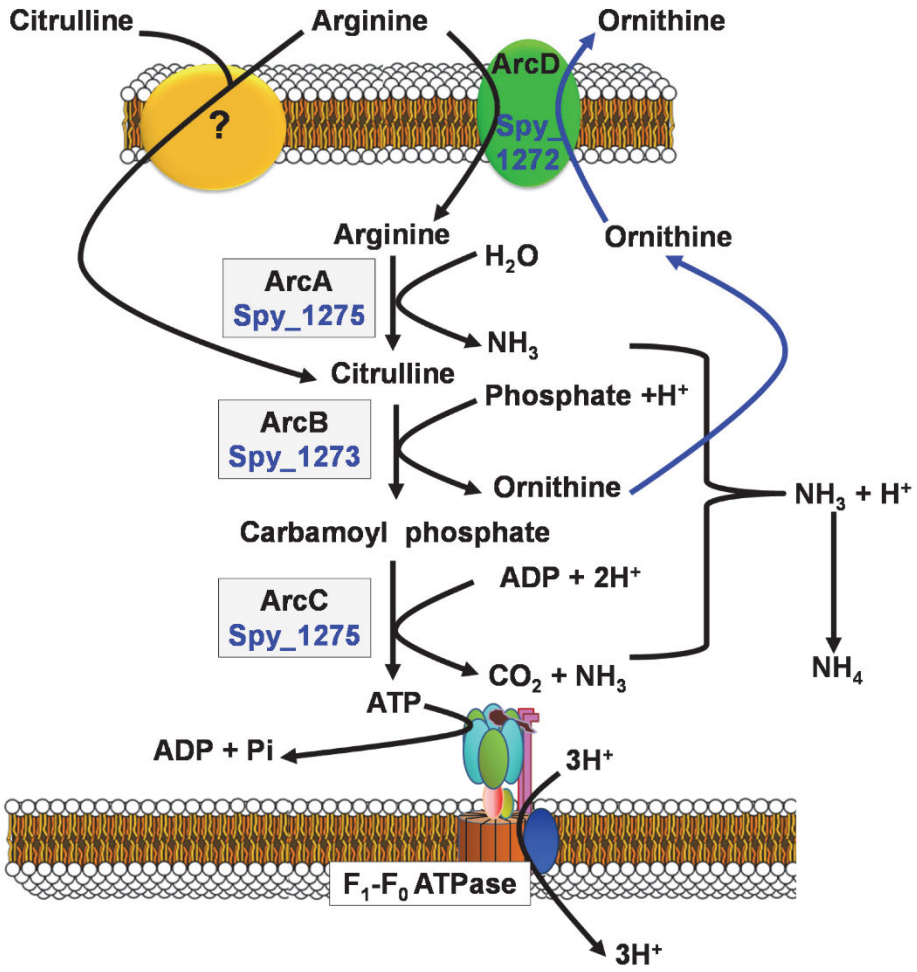

Fig. 7

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.

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.

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

Fig. 8

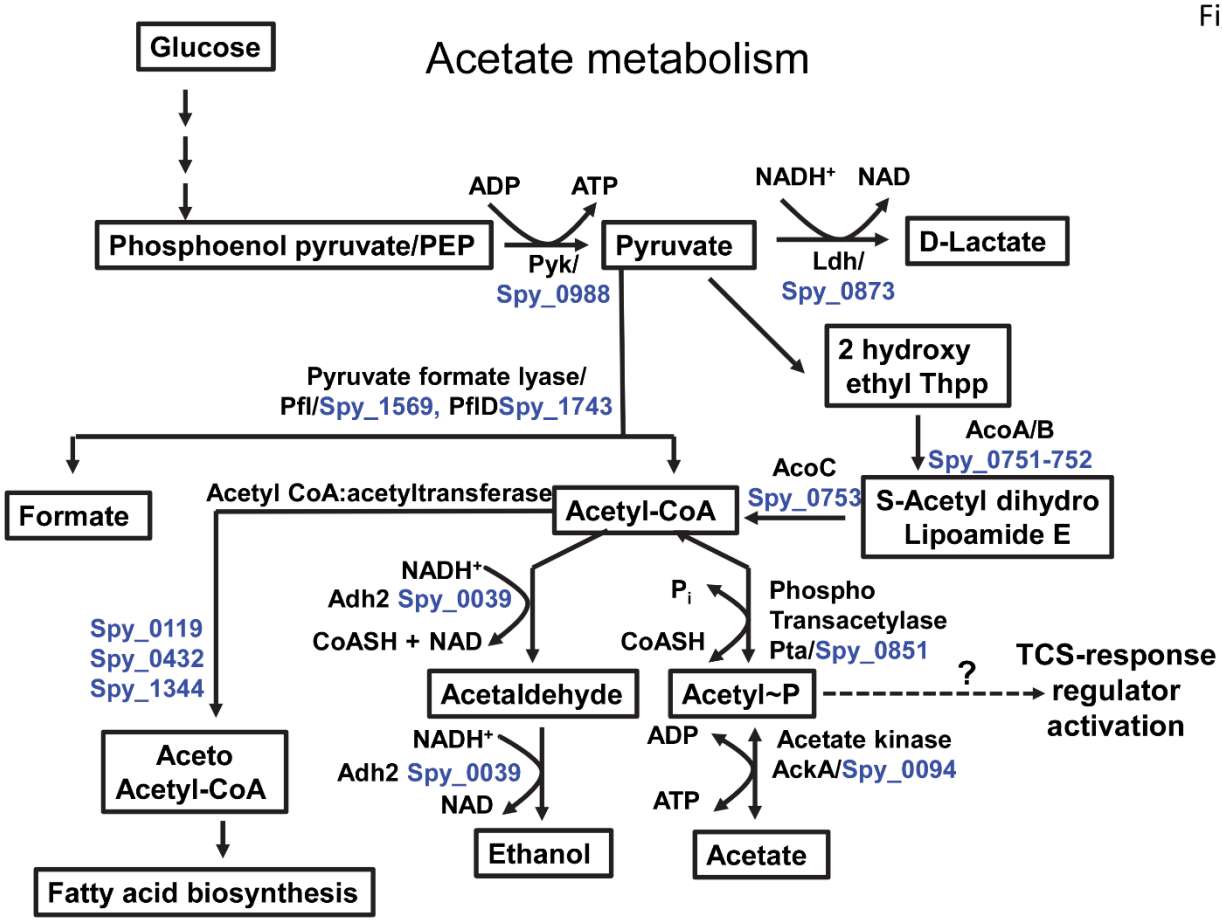

Figure 8: Acetate metabolism pathways for partially oxidized metabolites. Acetate activation pathways involve two key enzymes: Ack (acetate kinase) and Pta (phospho-transacetylase). These enzymes catalyze the formation of energy rich-acetyl-phosphate (Acetyl~P, ΔG^{o} = -43.3 kJ/mol) from acetate and 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.

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. 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, Zhang, Grimes, Qi, Lee, & Rock, 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, Lamberet, Staels, Trieu-Cuot, Gruss, & Poyart, 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.

Fig. 9

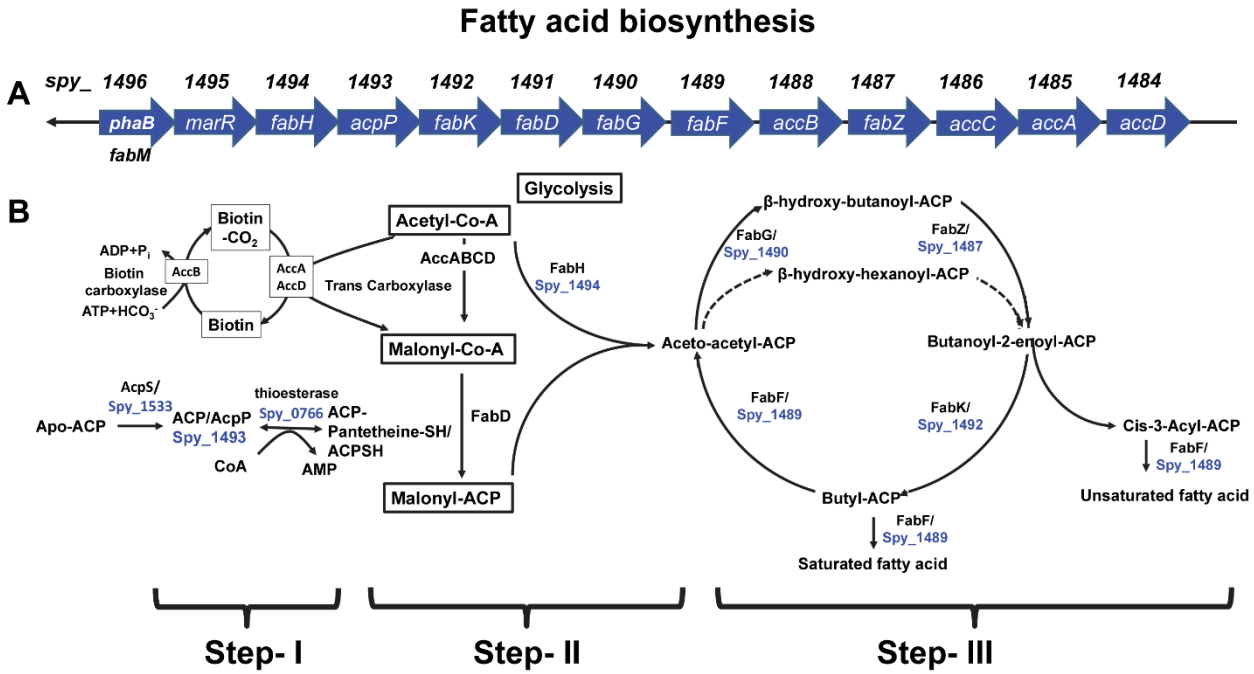

Figure 9: Fatty acid biosynthesis pathway in *S. pyogenes*. (A). The genomic organization of genes involved in fatty acid biosynthesis in *S. pyogenes*. (B). Fatty acid biosynthesis is divided in to three major steps involving several enzymes.
Step I. In this step, the acetyl-CoA carboxylase reaction 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.

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; 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

Fig. 14

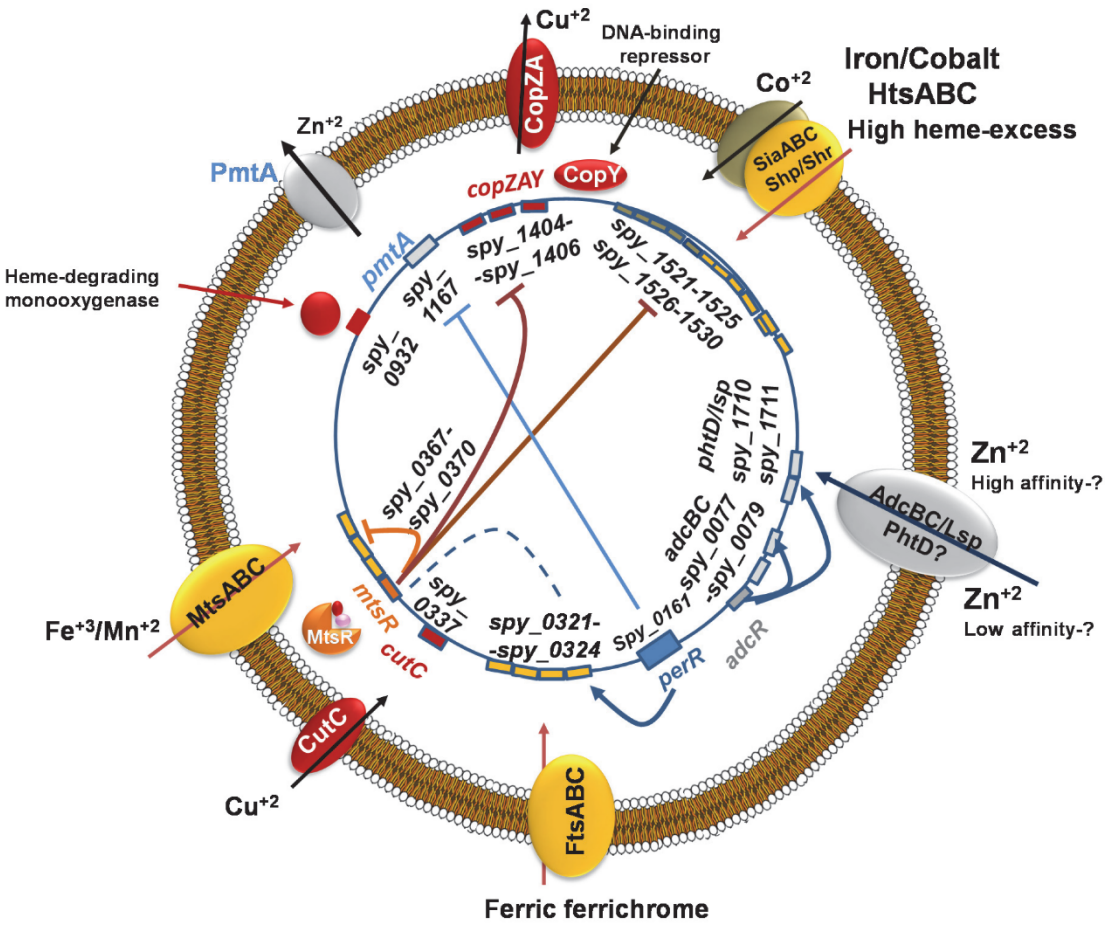

Figure 14: A schematic diagram of the involvement of genes and the corresponding proteins in the transport of various ions (Fe/Mn, heme, ferrichrome, Co, Cu, and Zn), and their regulation in *S. pyogenes*. Arrows indicate positive regulation. Blunt-ended arrows indicate repression/ negative control. 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.

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, 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-

Fig. 10

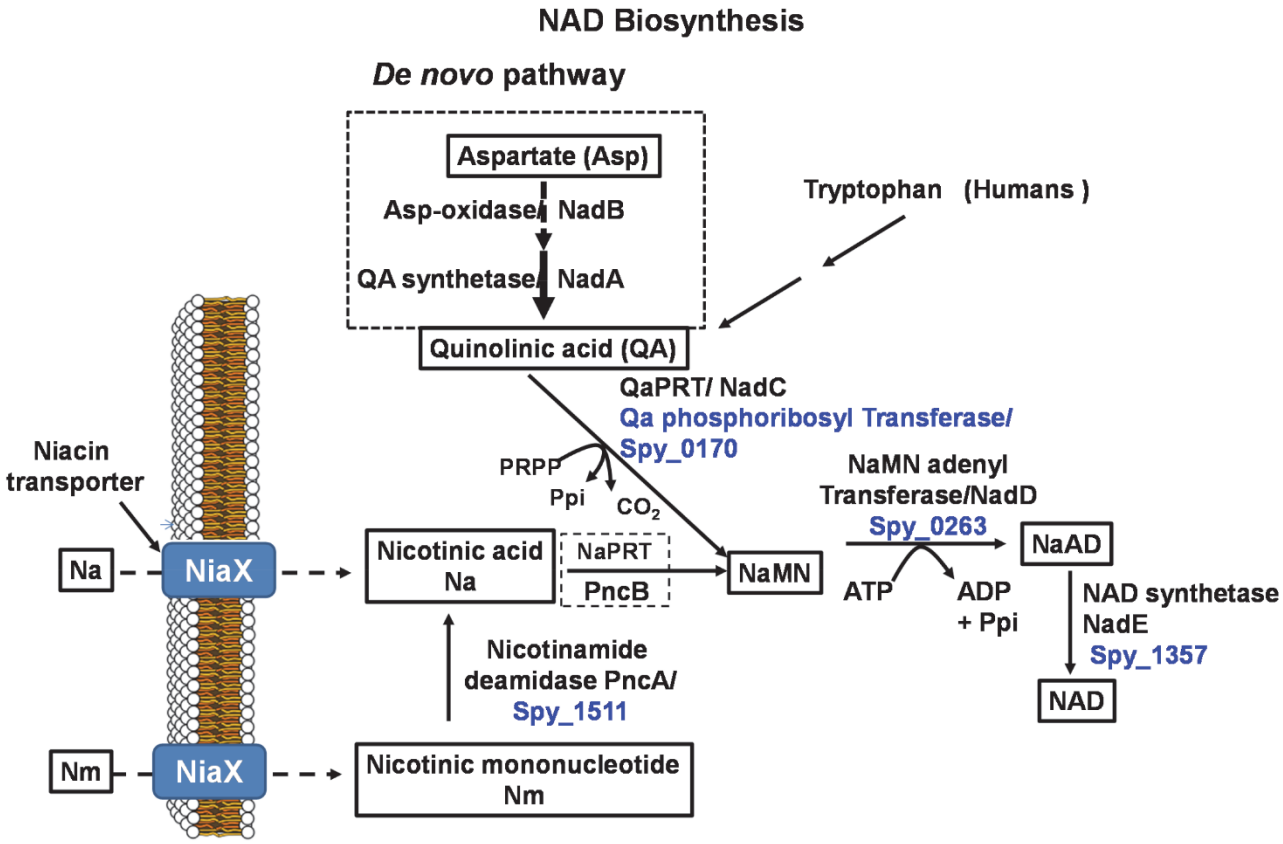

Figure 10: A schematic presentation of NAD biosynthesis in *S. pyogenes*. *S. pyogenes* lacks the NadA and NadB enzymes in the *de novo* NAD biosynthesis pathway (shown in the dotted rectangle). This organism also lacks the nicotinamide phosphoribosyl transferase (NaPRT/PncB). However, in the *de novo* 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.

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 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, Tsou, Kuo, Wang, Wu, & Liao, 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, Tsou, Kuo, Wang, Wu, & Liao, 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. A recent review provides detail analysis of the role of individual proteins involved in *S. pyogenes* oxidative stress resistance (Henningham, Döhrmann, Nizet, and Cole, 2015).

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/

Fig. 11

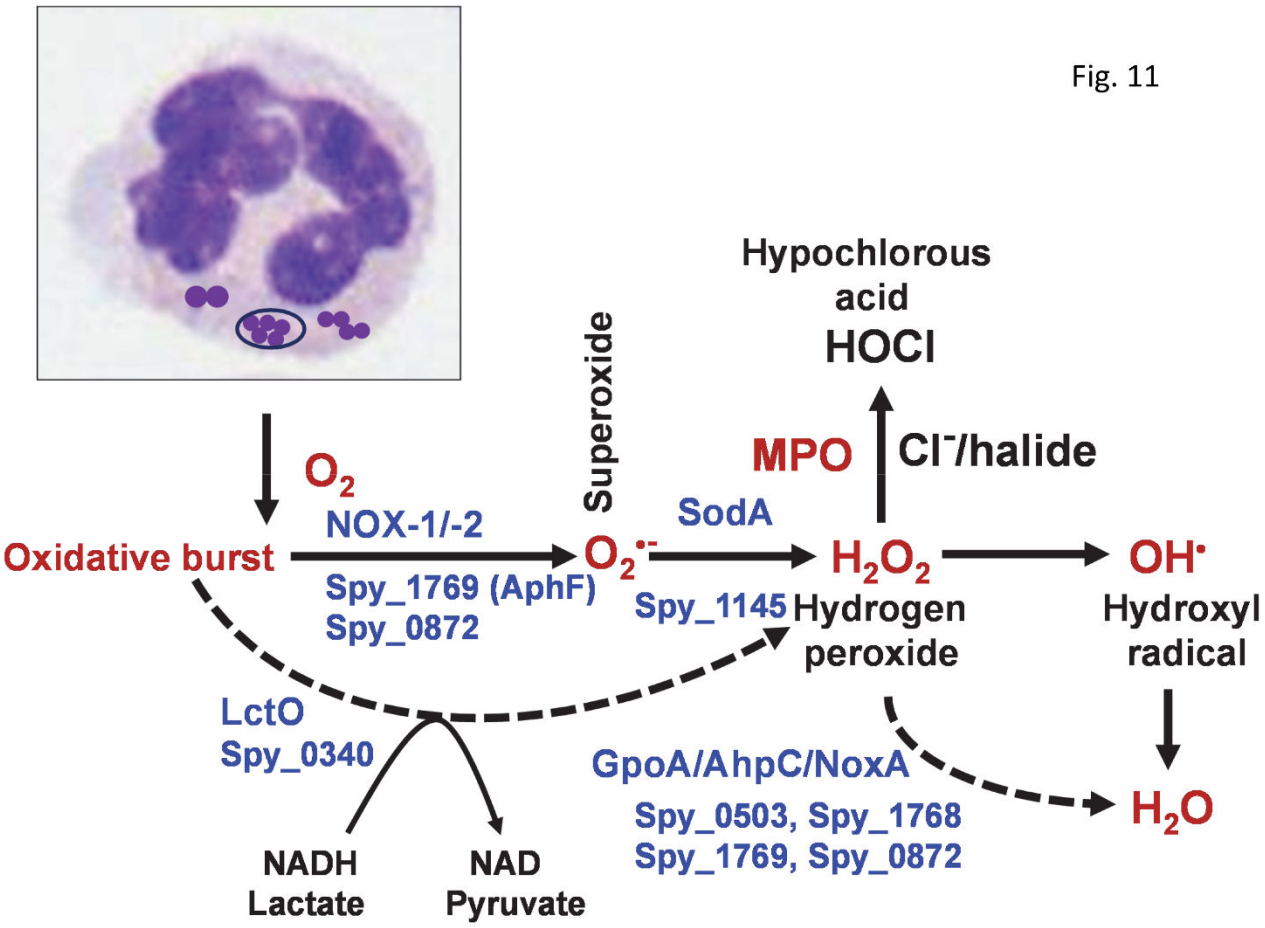

Figure 11: Aerobic metabolism of *S. pyogenes* and its role in thwarting deleterious effects of reactive oxygen intermediates (shown in red) generated by enzymes in human neutrophils during infection and phagocytosis. The schematic diagram shows the conversion of oxygen (O_2) to superoxide ion ($O_2^{\bullet -}$) 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.

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 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

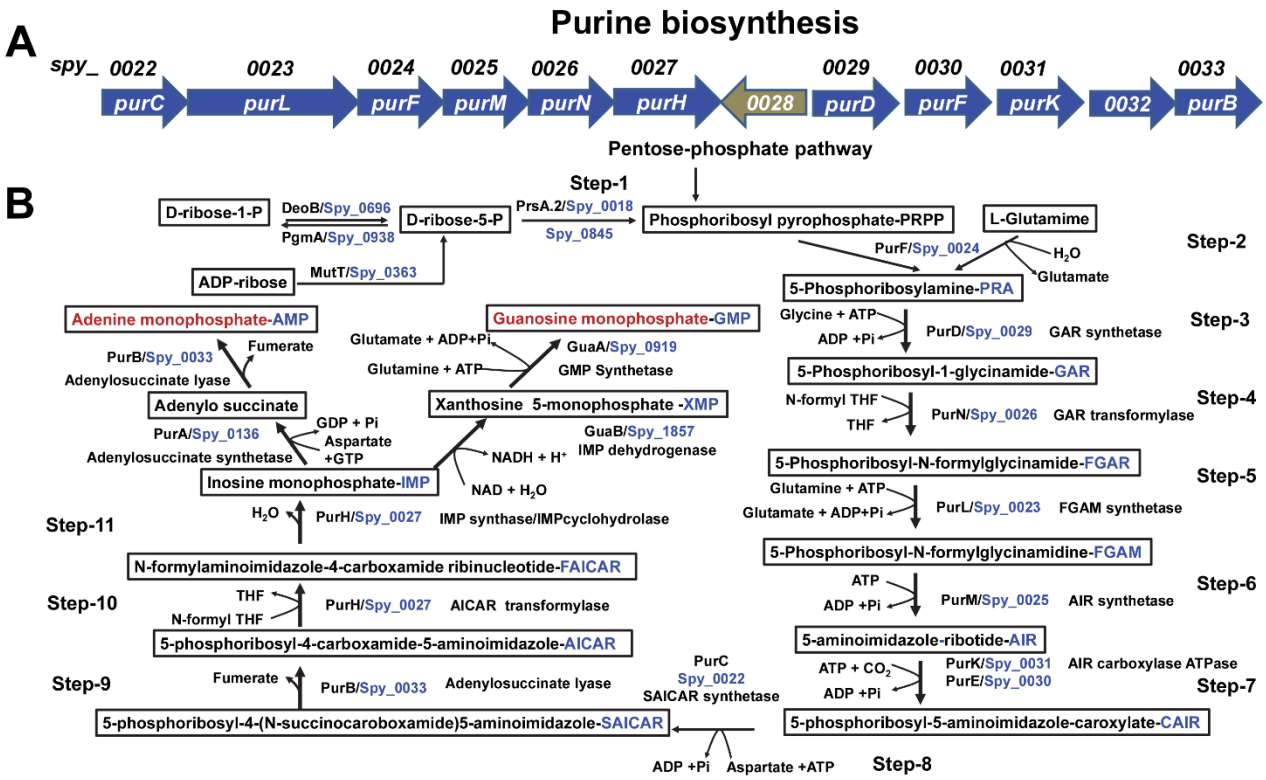

Figure 12: Schematic representation of the purine biosynthesis pathway in *S. pyogenes*. (A). The genomic organization of the 12-gene "*pur*" operon (*M5005_Spy_0022-0033*). 11 genes are involved in the purine biosynthesis. The role of *Spy_0028* is presently unknown. (B). The flow chart shows the steps 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 (Juttukonda & Skaar, 2015). 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; Juttukonda & Skaar, 2015). 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

Fig. 13

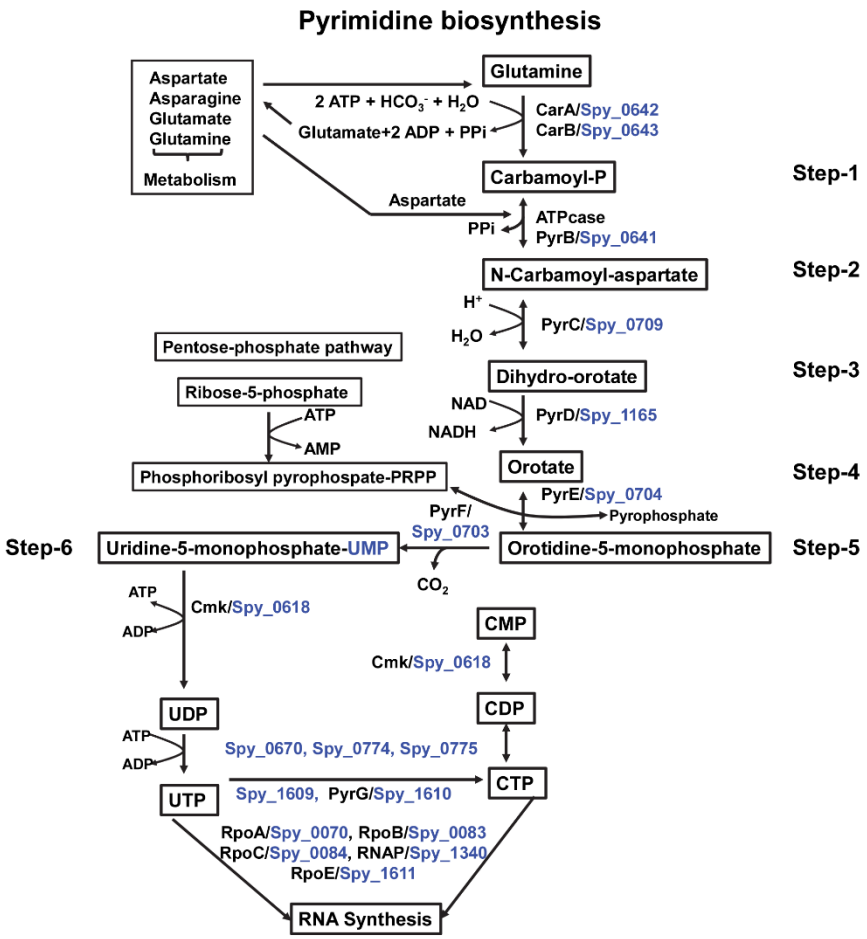

Figure 13: A schematic diagram of the pyrimidine biosynthesis pathway in *S. pyogenes*. This pathway involves six steps.
Step 1: Carbamoyl-P synthesis.
Step 2: The condensation of carbamoyl phosphate and aspartate to yield carbamoyl aspartate is catalyzed by aspartate transcarbamoylase or ATPcase.
Step 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.

(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, Pennati, Devlin, Huang, Gadda, & Eichenbaum, 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 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 which as a metalloprotein 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* (Reyes-Caballero, Campanello, & Giedroc, 2011; Merchant & Spatafora, 2014). In contrast, MtsR regulates both *mtsABC* and *htsABC*, but does so with distinct patterns, as both Fe^{3+} and Mn^{2+} regulate *mtsABC* (Do, et al., 2019), but only Fe^{3+} regulates *htsABC* (King, Horenstein, & Caparon, 2000; Reyes-Caballero, Campanello, & Giedroc, 2011; Merchant & Spatafora, 2014). The functional significance of this discrimination involves coordinate regulation between MtsR-regulated Mn^{+2} import by MtsABC and PerR-regulated Fe^{+2} efflux by PmtA. Together these act to ensure the optimal metallation of the SodA superoxide dismutase, which requires Mn^{2+} for its activity, but is inhibited by Fe^{2+} (Turner, et al., 2019). The balance of iron and manganese can differentially affect virulence in a host compartment-specific manner. For example, MtsR mutants were not attenuated in a model of murine soft tissue infection (King, Horenstein, & Caparon, 2000), but were attenuated in a murine model of invasive infection (Do, et al., 2019).

Zinc

Investigation into Zinc homeostasis has provided new insights into the mechanisms of *S. pyogenes* pathogenesis. Zinc is the second most abundant transition metal found in human tissues, serving as a structural element or cofactor for several surface proteins, enzymes and immunoregulatory proteins (Blencowe & Morby, 2003; Coleman, 1998). The concentration of Zn is critical for *S. pyogenes* growth and the host responds using "nutritional immunity" to manipulate Zn concentrations. Proteins like calprotectin sequester and reduce the concentration of Zn available in the extracellular microenvironment, and immune cells like macrophages inundate *S. pyogenes* with toxic levels of Zn following phagocytosis (Sanson, et al., 2015; Djoko, Ong, Walker, & McEwan, 2015; Ong, Berking, Walker, & McEwan, 2018). Excess Zn impairs S. *pyogenes* glucose metabolism via inhibition of phosphofructokinase and GAPDH/SDH and through the inhibition of phosphoglucomutase, which can result in decreased capsule synthesis (Ong, Walker, & McEwan, 2015). In response, *S. pyogenes* switches its metabolism away from using glucose to using galactose (see above). Thus, to preserve homeostasis, *S. pyogenes* must precisely calibrate the expression of genes for Zn export, import, and those involved in Zn-sparing responses. An interesting feature of the *S. pyogenes* Zn regulatory network is that its genome lacks Zur, a transcriptional regulator that plays an important role in the regulation of Zn homeostasis in many other Gram-positive pathogens (Hantke, 2005; Hantke, 2005). Instead, functional and structural studies of the peroxide-stress response regulator PerR (a homolog of Zur) shows that it serves in a dual capacity by also functioning as the regulator of Zn^{2+} homeostasis (Brenot, Weston, & Caparon, 2007; Makthal, et al., 2013). PerR regulates the expression of PmtA (*Spy_1167*), a putative Zn efflux transporter. It also indirectly regulates several additional genes involved in Zn metabolism, presumably because de-repression of PmtA results in unregulated Zn efflux, which leads to Zn starvation.

Another cluster of genes critical for Zn homeostasis are those regulated by the MarR-family transcription repressor AdcR (adhesin competence repressor, *Spy_0077*) that directly monitors cytosolic Zn concentrations to modulate adaptive responses (Sanson, et al., 2015). These include genes that encode an ABC-family membrane transporter for Zn^{2+} import that include *adcB* (*Spy_0079)* and *adcC* (*Spy_0078)* and two genes in an unlinked operon encoding *lmb*/*lsp*/*adcAII* (*Spy_1711*) and *phtD* (*Spy_1710*) (Sanson, et al., 2015; Makthal, et al., 2017). Another unliked gene encodes the cell surface-exposed Zn-binding protein AdcA (*Spy_0543*) (Sanson, et al.,

2015; Makthal, et al., 2017). Two Zn-binding domains of AdcA stabilize its structure upon Zn-binding Zn^{2+} and are essential for increasing Zn affinity and transfer kinetics (Cao, et al., 2018). AdcB is a histidine triad family inner membrane permease and AdcC is a cytosolic cluster 9 family lipoprotein ATPase (Sanson, et al., 2015; Makthal, et al., 2017). Analysis of mutants lacking Lsp/AdcAII indicates that this protein is required for growth under conditions of Zn starvation, which raises the intriguing possibility that Lsp/AdcAII functions as the Zn^{2+}-binding component of the Adc transporter. Should PhtD have a similar function, AdcAII and PhtD may act as interchangeable high and low-affinity solute-binding components to fine-tune adaptor affinity to environmental Zn concentrations (Elsner, et al., 2002; Weston, Brenot, & Caparon, 2009). AdcAII-deficient mutants are highly attenuated in the murine soft tissue infection model (Elsner, et al., 2002; Weston, Brenot, & Caparon, 2009) (Figure 14). AdcR also regulates genes involved in the Zn-sparing response, which involves the expression of Zn-free paralogs of proteins critical for growth that replace their Zn-requiring counterparts. For an M3 *S. pyogenes* strain, these include *rpsN.2* (*SpyM3_1615*) and two Zn-free alleles of alcohol dehydrogenase (*SpyM3_0036*, *SpyM3_0037*) (Sanson, et al., 2015).

Zn transport is also influenced by czcD (Spy_0653), which encodes a cation transporter that imparts resistance to Zn and several other heavy metals, including copper and cadmium. Originally characterized in *Relstonia* (Anton, Grosse, Reissmann, Pribyl, & Nies, 1999), it was subsequently identified in *S. pyogenes* (Sumby, et al., 2005). The gene is The upstream of *czcD* gene is the *gczA* (*Spy_0652*) serving as its positive transcriptional regulator that responds to the presence of Zn, but not to other divalent heavy metal ions. Mutants that lack CzcD and/or GczA grow poorly in the presence of high concentrations of Zn and survive poorly in neutrophils and in a mouse infection model (Ong, Gillen, Barnett, Walker, & McEwan, 2014). Together, these studies on the extensive network of Zn regulators and transporters demonstrate that the Zn homeostasis network is critical for *S. pyogenes* virulence.

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 several mechanisms, including the generation of ROS by participation in the Fenton reaction. The principal mechanism by which Gram-positive bacteria protect themselves from copper toxicity is by removing copper from the cytosol via a dedicated P-type ATPase 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 three genes- containing *cop* operon in *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 copper 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). Recently, the role of this transport system in *S. pyogenes* has been unraveled (Stewart, et al., 2020). When cultured *in vitro* in the presence of increasing concentrations of $CuSO_4$/ $CuCl_2$, *S. pyogenes* shows defective growth and viability, decreased fermentation, and decreased GAPDH activity in a delayed, rather than a time-dependent fashion concomitant with increased expression of CopZ and a decreased intracellular concentration of glutathione. However, mutants that lack the efflux pump do not show reduced virulence in a mouse model of invasive disease. In contrast, the copper-associated reduction in glutathione and its associated deleterious effects can be

rescued by supplementation with glutathione, which indicates that glutathione buffers excess intracellular Cu^{2+} and plays an important role in Cu tolerance in *S. pyogenes*. Interestingly, *S. pyogenes* lacks the gene required for synthesis of glutathione, indicating that it is dependent on glutathione import (Brenot, King, Janowiak, Griffith, & Caparon, 2004). In this regard, Spy_0270 (Glutathione solute-binding protein) with 70% similarity /59% identity to GshT of *S. mutans* may function in the transport of externally added glutathione to rescue Cu toxicity (Vergauwen, et al., 2013). However, unlike *S. pyogenes*, cop deletion mutants of *S. mutans* become hypersensitive to copper toxicity and fail to form biofilm (Singh, Senadheera, Lévesque, & Cvitkovich, 2015).

Sodium and protons (H^+) transport

Genomic analysis of *S. pyogenes* reveals 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 15). 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*, 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*, 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 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.

Post-translational Modifications and *S. pyogenes* metabolism.

Post-translational modifications (PTM) of proteins are covalent alterations to polypeptide primary structure that function to increase the functional diversity of proteins (Macek, et al., 2019). PTMs include proteolytic processing and modifications that add new functional groups onto individual amino acids. These modifications are often introduced in response to various extra- or intracellular environmental cues and are essential to enhance cellular survival, fitness, growth, and development. Since *S. pyogenes* must adapt to numerous nutritional and environmental stresses during contact with inert abiotic and living tissue environments, the study of PTMs has been increasingly recognized as critical for understanding *S. pyogenes* pathogenesis. PTM of proteins is a dynamic process that can be reversible or irreversible, may involve a single or multiple modifications, and may occur sequentially or concurrently at multiple amino acid side chains. Typical amino acids targeted include serine, threonine, tyrosine, histidine, aspartate, asparagine, lysine, arginine, and cysteine (Macek, et al., 2019), and modification results in structural and functional changes that regulate protein activity, localization, and interaction with other cellular molecules. Combinatorial modifications within a metabolic

Fig. 15

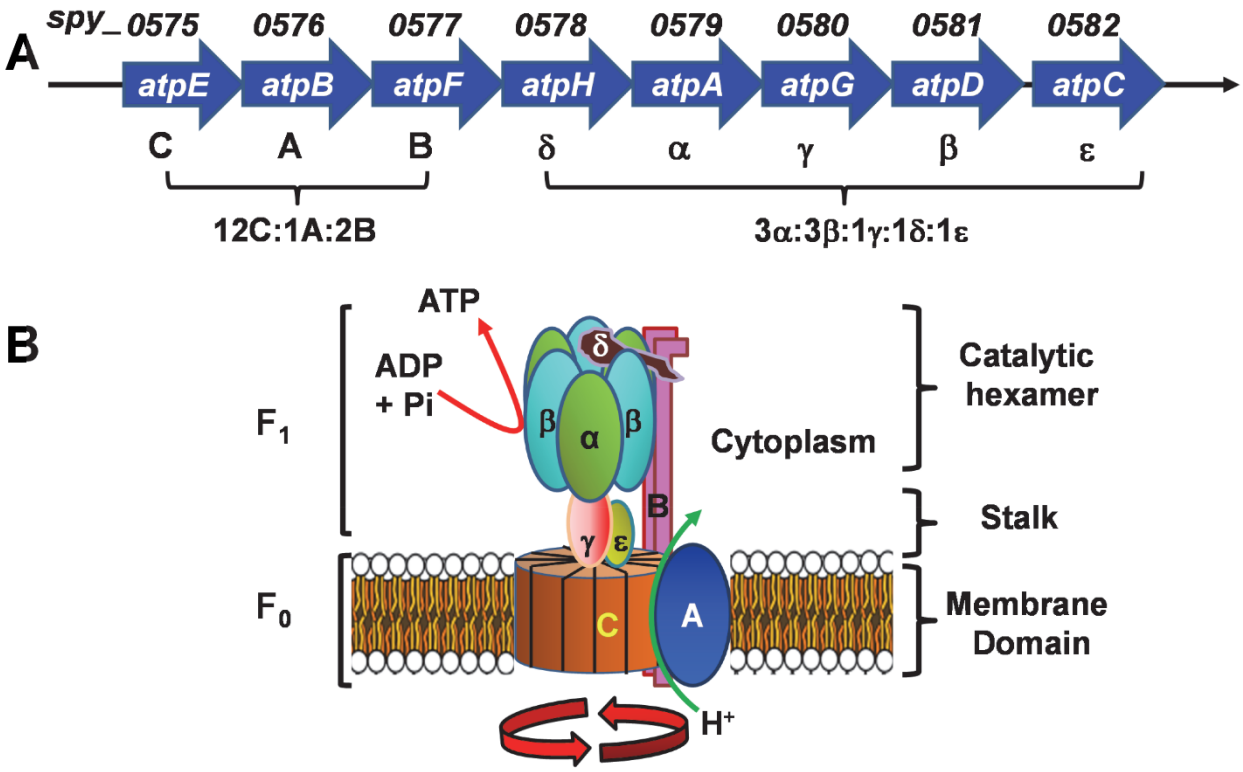

Figure 15: A schematic diagram of the predicted F_1-F_0 (F)-ATPase transport system in *S. pyogenes*. (A) The F-ATPase proton pump and its eight components are encoded by an operon constituted of eight genes: *M5005_SPy_0575-0582*. (B) A schematic diagram of the F_1-F_0 (F)-ATPase pump is based on the 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.

network, along with intra- and inter-protein cross-talk regulated by PTM, can coordinate complex biological functions essential for virulence (Macek, et al., 2019). Analysis of PTM has been advanced by new qualitative and quantitative methods for mass spectrometry that integrate robust bioinformatics with genomic and proteomic databases. It is now possible to obtain accurate identification of PTM at single amino acid resolution over a global protein landscape (Leutert, Entwistle, & Villén, 2021; Macek, et al., 2019; Choudhary & Mann, 2010). Among the PTMs described in bacteria, phosphorylation has been most extensively studied (Macek, et al., 2019), primarily in the context of two-component signal transduction systems (TCS) (Stock, Robinson, & Goudreau, 2000). Other PTMs include the phosphoenolpyruvate:carbohydrate transport system (PTS) (Deutscher, Francke, & Postma, 2006; Deutscher, et al., 2014), bacterial protein-tyrosine (BY-kinase) and Ser/Thr kinases (Hank's /eukaryote-type) with mono or dual-specificity (Pereira, Goss, & Dworkin, 2011; Mijakovic, Grangeasse, & Turgay, 2016). The impact of PTM on *S. pyogenes* metabolism is not fully appreciated; however, metabolic enzymes are often the targets for phosphorylation by Ser/Thr andTyr kinases (Schastnaya, et al., 2021). Glycolytic enzymes in particular are substrates for Ser/Thr kinases (Dannelly, Duclos, Cozzone, & Reeves, 1989; Lomas-Lopez, Paracuellos, Riberty, Cozzone, & Duclos, 2007; Pietack, et al., 2010) and while the impact of

PTM on glycolytic function is unknown, it has been recently found that a *S. pyogenes* UbK-type (universal bacterial kinase) tyrosine kinase (Nguyen, et al., 2017) with a novel dual specificity (SP-TyK/Spy_1476) directly phosphorylates multiple Ser, Thr, and/or Tyr residues of a metabolic enzyme (surface dehydrogenase, SDH/Plr/Spy_0233) and a transcription regulator (WalR/Spy_0435 and CovR/Spy_0282), both *in vitro* and/or *in vivo* (Kant & Pancholi, 2021). Mutants lacking SP-TyK have altered expression of multiple metabolism and cell division genes, along with defects in virulence and biofilm formation (Kant & Pancholi, 2021). Several of these SP-TyK genes are also regulated by the *S. pyogenes* orphan protein tyrosine phosphatase (SP-PTP/ Spy_0036) (Kant, Agarwal, Pancholi, & Pancholi, 2015), which suggests that SP-TyK and SP-PTP function as a cognate pair to regulate a common subset of genes via a reversible phosphorylation cycle. However, SP-TyK itself is not dephosphorylated by SP-PTP (Kant & Pancholi, 2021), and indicates that it is also possible that these two enzymes may act independently. In contrast, Ser/Thr kinase (SP-STK/ Spy_1335) and phosphatase (SP-STP/ Spy_1336) do function as cognate enzymes (Jin & Pancholi, 2006). Analysis of STK-STP deficient mutants indicates that this system modulates the activity of many key regulatory proteins of *S. pyogenes* affecting carbohydrate and cell wall metabolism, as also observed in *Streptococcus mutans* (Banu, et al., 2010) and *S. suis* (Zhang, et al., 2017). Together, these studies have shown that environmental stress-sensitive Ser/Thr and Tyr kinases and phosphatases form a complex network to fine-tune the ability of *S. pyogenes* to adhere to and invade host cells, to modulate inflammatory responses and to form biofilm. Much additional work will be required to identify the full range of targets affected by PTMs, supported by focused protein chemistry, enzymological analyses, and studies *in vivo* to understand the physiological relevance of these PTMs systems for *S. pyogenes* pathogenesis.

S. pyogenes Biofilms and metabolism

It is becoming increasingly clear that *S. pyogenes*, like many other bacteria, forms both microcolony-dependent and -independent biofilms, although marked strain-dependent variations in biofilm formation have been observed (Lembke, et al., 2006; Baldassarri, et al., 2006; Marks, Mashburn-Warren, Federle, & Hakansson, 2014; Fiedler, Köller, & Kreikemeyer, 2015; Young, Holder, Dubois, & Reid, 2016; Vyas, Proctor, McArthur, Gorman, & Sanderson-Smith, 2019; Matysik & Kline, 2019). Transcriptomic studies have provided substantial insight into the metabolic status of *S. pyogenes* biofilms. Comparison of exponential and stationary phase cultures to biofilm for an M14 serotype strain revealed that carbohydrate metabolism and capsule biosynthesis are essential to establish a solid biofilm when bacteria are surrounded by an extracellular matrix of polysaccharides (Cho & Caparon, 2005) composed essentially of sugar components L-glucose and D-mannose (Shafreen, Srinivasan, Manisankar, & Pandian, 2011). Simultaneously, certain biofilm dispersing agents such as the SpeB cysteine protease are down-regulated. A comparative analysis of time and growth stage-dependent biofilm formation in M1T1 strain 5448 revealed that carbohydrate metabolism- and PTS transport-related genes are down-regulated during biofilm formation (Le Breton, et al., 2016). However, for many genes, a parallel proteomic analysis failed to establish a direct correlation between changes in protein levels and changes in transcription (Le Breton, et al., 2016), indicating that additional factors are also important for controlling expression of the proteome in *S. pyogenes* biofilm. These likely include PTM by Ser/Thr and Tyr kinases (see above) which can directly modulate metabolic enzyme activity (Kant & Pancholi, 2021) or modulate the activity of transcription regulators, such as CovR and WalR, to alter expression of multiple genes involved in cell wall biosynthesis and cell division (Kant, Agarwal, Pancholi, & Pancholi, 2015; Kant & Pancholi, 2021; Boisvert, et al., 2013; Dubrac, Boneca, Poupel, & Msadek, 2007). Further combined transcriptomic, proteomic, and metabolomic studies will continue to unravel the complex relationship between metabolic reprogramming and biofilm formation in *S. pyogenes*, which may produce leads for the development of novel bacterial and host-directed therapeutics.

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.

Fig. 16

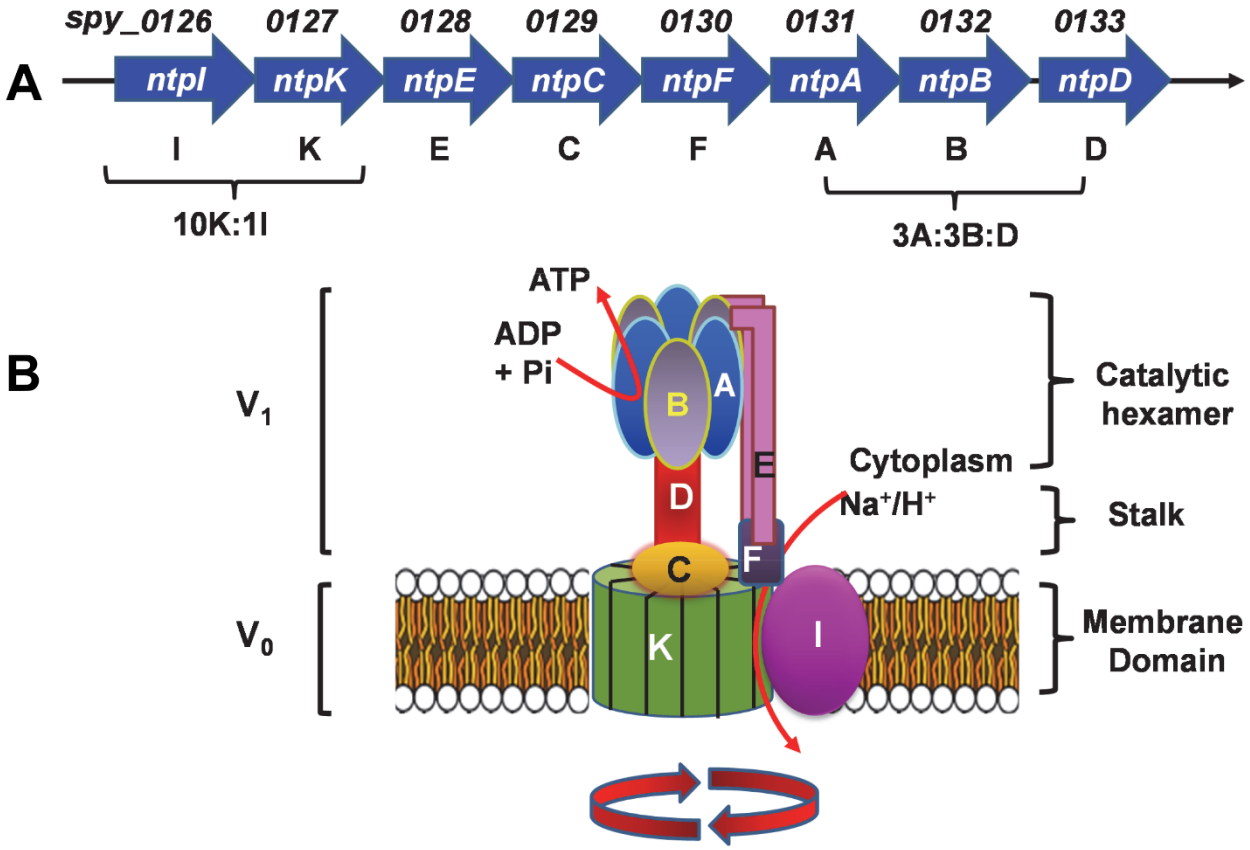

Figure 16: A schematic diagram of the predicted V_1-V_0 (V)-ATPase transport system in *S. pyogenes*. (A) The V_1V_0-ATPase complex is encoded by an operon that contains eight genes (*M5005_Spy_0126-0133*) and is annotated as shown. (B) The diagram of the predicted structure of the V_1-V_0 ATPase is 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.

Since metabolism is intimately linked to virulence, it is a burgeoning question and a matter of important 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 revised 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, 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.

Similarly, little progress has been made toward understanding how anchorless metabolic enzymes are exported and then displayed on the surface of *S. pyogenes* and the role of V_0V_1 and F_0F_1-ATPase pumps in *S. pyogenes* metabolism and pathogenesis. More work is required to understand how PTM of proteins, including metabolic enzymes and transcription regulators targeted by Ser/Thr and Tyr kinases and phosphatases, function to fine-tune key cellular activities. Furthermore, the role of a substantial number of genes of unknown function in the *S. pyogenes* genome still needs to be explored. Hence, the continued efforts towards high-resolution genome-based, proteome, and metabolomic analyses may reveal novel 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/ single-cell 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, these approaches should be combined with NMR and/or mass spectrometry-based PTM and metabolomics studies of *S. pyogenes* in its planktonic and biofilm forms to provide snapshots of the metabolic fingerprint of the cell in these different environments. A challenge for this systems biology approach will be to develop bioinformatic pipelines to efficiently integrate multiparameteric proteomic, functional genomic, transcriptomic, and metabolomic information to provide a complete picture of how *S. pyogenes* adapts in response to pathophysiological stimuli. The analysis of metabolism, once demoted to the backwaters in the face of revolutionary advances in molecular genetics, is now remerging to become a critical component of how we understand *S. pyogenes* pathophysiology.

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