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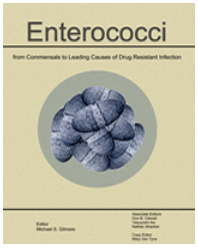
NLM Citation: Ness IF, Diep DB, Ike Y. Enterococcal Bacteriocins and Antimicrobial Proteins that Contribute to Niche Control. 2014 Feb 16. In: Gilmore MS, Clewell DB, Ike Y, et al., editors. Enterococci: From Commensals to Leading Causes of Drug Resistant Infection [Internet]. Boston: Massachusetts Eye and Ear Infirmary; 2014-.
Bookshelf URL: https://www.ncbi.nlm.nih.gov/books/



Enterococcal Bacteriocins and Antimicrobial Proteins that Contribute to Niche Control

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Created: February 16, 2014.

Introduction

Enterococci, which belong to the group of lactic acid bacteria (LAB), have received increased attention in recent years for various reasons (Fisher & Phillips, 2009; Franz, Huch, Abriouel, Holzapfel, & Gálvez, 2011; Leavis, Bonten, & Willems, 2006). While lactobacilli, another group of LAB, have been shown to confer numerous benefits and are often regarded as health-bringing organisms, enterococci have become more recognized as emerging human pathogens despite the fact that they are as numerous as the lactobacilli in our gastrointestinal tractgastrointestinal tract. *Enterococcus faecalis* is the dominant *Enterocuccus* in the gastrointestinal tract, followed by *E. faecium*; however, *E. avium* and *E. hirae*, as well as other enterococcal species, are frequently found in human stool samples. The commensal/probiotic role of enterococci in humans and animals has evolved through thousands of years in mutual coexistence—but the ability of the enterococci to behave in a way that causes problems to human health is only beginning to be understood. Virulence, which may evolved as an adaptation to the "modern lifestyle," including the profligate use of antibiotics in medical practice and animal husbandry, needs to be understood and limited where possible. On the other side, enterococci have many positive traits that have been appreciated in food fermentation and preservation, and may also serve as probiotics to promote health.

Bacteriocin-producing bacteria are found in all environments. In many LAB isolates, bacteriocin production has been examined by biochemical and genetic studies, and the bacteriocins produced by enterococci are often similar to those produced by other lactic acid bacteria. A classification scheme has been developed for bacteriocins produced by Gram-positive bacteria, and most of this information is based on findings from LAB. Although classification is still a disputed issue, two major classes of heat-stable, ribosomally synthesized antimicrobial peptides are well defined. Class I constitutes the lantibiotics, while Class II constitutes the unmodified non-lantibiotics.

Bacteriocins within different classes and subclasses also have been isolated and characterized in enterococci (Cotter, Hill, & Ross, 2005; Nes, Diep, Håvarstein, Brurberg, Eijsink, & Holo, 1996; Nes, Yoon, & Diep, 2007). One of the most striking findings so far is the almost complete absence of lantibiotics among enteroccci, with the

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only exceptions being cytolysin and enterocin W (Coburn & Gilmore, 2003; Cox, Coburn, & Gilmore, 2005; Sawa, et al., 2012). Most of the characterized enterocins belong to the Class II bacteriocins and a few are heat-labile lytic enzymes. The latter were previously classified as bacteriocins, but are now included in a distinct class of antimicrobials (Cotter, Hill, & Ross, 2005).

The hemolytic bacteriocin (cytolysin), the circular AS-48, and bacteriocin 21 have been known as *E. faecalis* bacteriocins for a long time, and they have been genetically and biochemically well characterized (Clewell, 1981; Gilmore, 1991; Gilmore, Coburn, Nallapareddy, & Murray, 2002; Gilmore, Segarra, Booth, Bogie, Hall, & Clewell, 1994; Haas, Shepard, & Gilmore, 2002; Ike, Clewell, Segarra, & Gilmore, 1990). Many bacteriocin producers have been identified and investigated from infection-derived *E. faecalis* or *E. faecium* isolates. From *E. faecalis* isolates, for example, bacteriocin 31(Tomita, Fujimoto, Tanimoto, & Ike, 1996) and bacteriocin 41 (Tomita, Kamei, & Ike, 2008) have been studied; and from *E. faecium*, there are bacteriocin 43 (Todokoro, Tomita, Inoue, & Ike, 2006), bacteriocin 32 (Nes, Diep, Håvarstein, Brurberg, Eijsink, & Holo, 1996), and bacteriocin 51(Yamashita, Tomita, Inoue, & Ike, 2011). Besides enterococcal bacteriocins of clinical origin, bacteriocins from enterococci of food origins have been studied, and several bacteriocins from *E.faecium* isolates have been identified and characterized. These include enterocin L50A/L50B (Cintas, Casaus, Holo, Hernandez, Nes, & Håvarstein, 1998), enterocin Q (Cintas L. M., et al., 2000; Criado, et al., 2006), enterocin A (Aymerich, Holo, Håvarstein, Hugas, Garriga, & Nes, 1996; Nilsen, Nes, & Holo, 1998), enterocin P (Cintas L. M., Casaus, Håvarstein, Hernández, & Nes, 1997; Kang & Lee, 2005), enterocin B (Casaus, Nilsen, Cintas, Nes, Hernández, & Holo, 1997) and others.

Many enterocins have also been characterized from various enterococcal species and from many environments, and the most thoroughly characterized enterocins are summarized in Table 1. Most of the characterized enterocins are from *E. faecium* and *E. faecalis*, but enterocins have also been isolated from *E. muntii*, *E. avium*, *E. hirae*, and *E. durans* (see Table 1). The bacteriocin-producing enterococci are by and large isolated from food, waste, and the feces and gastrointestinal tract of humans and animals, but may also be isolated from other sources. Fermented food, specimens from human infections, and feces from healthy babies seem to be particularly good niches for isolating bacteriocin-producing enterococci (see Figure 1). It seems likely that most enterococci originate from the digestive tract of humans and animals, a notion which is in line with the finding that the same bacteriocins are identified in enterococci isolated from many environments, which most often include those of human origin.

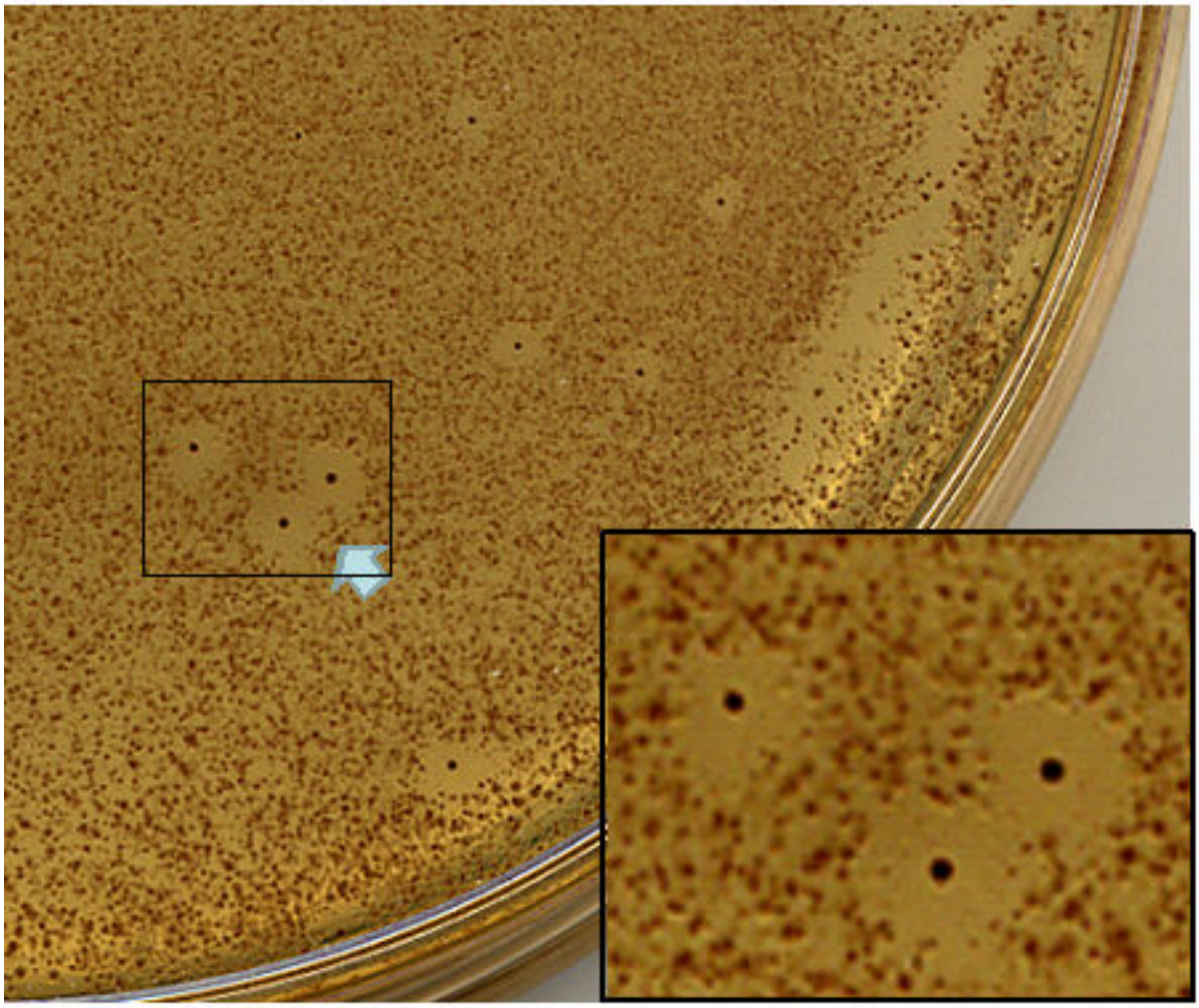

Figure 1. Plating of a fecal sample from a healthy 6-month-old breast fed baby on a MRS agar plate anaerobically. No indicator bacteria are added and only endogenous fecal lactic acid bacteria are grown on the plate. Some bacteriocin-producing fecal bacteria inhibit growth of other fecal bacteria. The arrow indicates bacterial growth inhibitory zones due to the bacteriocin producing endogenous lactic acid bacteria (enterococci) present in the feces. The insert shows magnification of some bacteriocin-producing colonies.

Table 1. Classification of Enterocins

			Source of isolation[a)]	Mol. Weight (amino acids)	References
Class I: Lantibiotics					
E. faecalis	Cytolysin Cyl_L, and Cyl_S	two-peptide lantibiotic	Clinical isolates	3,458 (38), 2,032 (21)	(Gilmore, Segarra, Booth, Bogie, Hall, & Clewell, 1994)
E. faecalis	Enterocin Wα and Wβ	two-peptide lantibiotic	Thai fermented fish	3,256 (30) and 2,728 (29)	(Sawa, et al., 2012)
Class II: Bacteriocins					
Class IIa: Antilisteria-Pediocin-like bacteriocins					
E. faecium	Enterocin A	double-glycine leader	Spanish dry fermented sausage	4,829 (47)	(Aymerich, Holo, Håvarstein, Hugas, Garriga, & Nes, 1996)
E. faecium	Enterocin P	sec -leader	Spanish dry fermented sausage	4,493 (44)	(Cintas L. M., Casaus, Håvarstein, Hernández, & Nes, 1997)

Table 1. continued from previous page.

Class II: Bacteriocins					
E. faecium	Bacteriocin GM-1	sec-leader	Feces infants	4,630 (44)	(Kang & Lee, 2005)
E. faecalis	Bacteriocin 31	sec-leader	Clinical isolate	(43)	(Tomita, Fujimoto, Tanimoto, & Ike, 1996)
E. mundtii	Mundticin KS, enterocin CRL35, mundticin QU2	double-glycine leader	Grass silage, artisanal cheese	4,287 (43)	(Kawamoto, et al., 2002; Saavedra, Minahk, de Ruiz Holgado, & Sesma, 2004)
E. faecalis	Enterocin SE-K4	sec-leader	Grass silage	5,356 (43)	(Eguchi, et al., 2001)
E. faecalis	Enterocin MC4-1	sec-leader	Macaque monkey	(43)	(Flannagan, Clewell, & Sedgley, 2008)
E. faecium	Bacteriocin T8	sec-leader	Vaginal secretion of children infected with HIV	5,090 (44)	(De Kwaadsteniet, Fraser, Van Reenen, & Dicks, 2006)
E. avium	Avicin A	double-glycine leader	Feces from babies	4,289 (43)	(Birri, Brede, Forberg, Holo, & Nes, 2010)
E. hirae	Hiracin JM79	sec-leader	Wild mallard duck	5,093 (44)	(Sánchez, Diep, Herranz, Nes, Cintas, & Hernández, 2007)
E. faecium	Bacteriocin RC714	sec-leader Identical to bacteriocin 31	Human exudate	(43)	(del Campo, et al., 2001)
E. faecium	Bacteriocin 43	sec-leader Identical to bacteriocin 31	Clinical isolate	(44)	(Todokoro, Tomita, Inoue, & Ike, 2006)
E. durans	Duracin GL			(43)	gb | ADW93772.1|
Class IIb: Two-peptide bacteriocins					
E. faecalis	Enterocin C	Human colostrum	4,286 (39) 3869 (35)	(Maldonado-Barragán, Caballero-Guerrero, Jiménez , Jiménez-Díaz, Ruiz-Barba, & Rodríguez, 2009)	
E. faecalis	Enterocin 1071 A and B	Feces from mini-pigs	4,286 (39), 3,899 (35)	(Balla, Dicks, Du Toit, Van Der Merwe, & Holzapfel, 2000)	
E. faecium	Enterocin X a and b	Sugar apples	4420 (40), 4068(37)	(Hu, Malaphan, Zendo, Nakayama, & Sonomoto, 2010)	
Class II: circular bacteriocins					
E. faecalis	AS-48, Bacteriocin 21, enterocin 4	Clinical isolate	7,166 (70)	(Joosten, Nunez, Devreese, Van Beeumen, & Marugg, 1996; Martínez-Bueno, et al., 1994; Samyn, et al., 1994; Tomita, Fujimoto, Tanimoto, & Ike, 1997)	
Class II: leaderless bacteriocins					
E. faecalis	MR10A MR10B / (343)Ent7A Ent7B	Bird uropyal glands, Beef	5,202 (44), 5,208 (43)	(Liu, et al., 2011; Martín-Platero, et al., 2006)	
E. faecium	Enterocin L50A, L50B / 62A , 62B	Dry fermented sausage, Human vagina	5,190 (44), 5,178 (43)	(Cintas L. M., Casaus, Holo, Hernandez, Nes, & Håvarstein, 1998; Dezwaan, Mequio, Littell, Allen, Rossbach, & Pybus, 2007)	
E. faecium	Enterocin Q	Dry fermented sausage,	3,980 (34)	(Cintas L. M., et al., 2000; Criado, et al., 2006)	
E. faecalis	Enterocin EJ97	Municipal waste water	5,328 (44)	(Gálvez, et al., 1998; Sánchez-Hidalgo, Maqueda, Gálvez, Abriouel, Valdivia, & Martínez-Bueno, 2003)	
E. faecium	Enterocin RJ-11	Rice bran	5,049 (44)	(Yamamoto, Togawa, Shimosaka, & Okazaki, 2003)	

Table 1. continued from previous page.

Class II: leaderless bacteriocins				
Class II: Other bacteriocins				
E. faecium	Bac 32, enterocin IT	Clinical isolate (VRE), ryegrass	6,390 (54)	(Inoue, Tomita, & Ike, 2006; Izquierdo, et al., 2008; Izquierdo, Cai, Marchioni, & Ennahar, 2009)
E. faecium	Bacteriocin 51	Clinical isolate (VRE)	105 aa heat stable	(Yamashita, Tomita, Inoue, & Ike, 2011)
E. faecium	Enterocin B	Spanish dry fermented sausage	5,479 (53)	(Casaus, Nilsen, Cintas, Nes, Hernández, & Holo, 1997)
E. faecalis	Enterocin 96	Munster cheese	5,179 (theoretical) (48)	(Izquierdo, Wagner, Marchioni, Aoude-Werner, & Ennahar, 2009)
Heat label enterolysins				
E. faecalis	Enterolysin A	Fish , milk	34,501 (343)	(Hickey, Twomey, Ross, & Hill, 2003; Nilsen, Nes, & Holo, 2003)
E. faecalis	Bacteriocin 41	Clinical isolate	(595)	(Tomita, Kamei, & Ike, 2008)

This indicates the first source of isolation.

Class I: Lantibiotics

Lantibiotics are rarely found in enterococci. Until now, only two two-peptide lantibiotics have been purified and genetically characterized, both from *E. faecalis* isolates (Booth, Bogie, Sahl, Hatter, & Gilmore, 1996; Coburn, Hancock, Booth, & Gilmore, 1999; Sawa, et al., 2012).

The cytolysin is the most thoroughly characterized enterococcal lantibiotics, and it exerts antimicrobial activity against a broad range of Gram-positive bacteria, but it also antagonizes certain eukaryotic cells, such as erythrocytes from various animals, and is therefore often referred to as a hemolysin. Cytolysin also lyses retinal cells, polymorphonuclear leukocytes, and human intestinal epithelial cells (Coburn & Gilmore, 2003). The role of cytolysin in the pathogenesis of enterococcal infection has been reported in mice (Ike, Hashimoto, & Clewell, 1984). Isogenic mutants of cytolysin, which is encoded by the pheromone-responsive plasmid pAD1, have been used in a murine intraperitoneal lethal challenge model. *E. faecalis* strains that express cytolysin of wild type pAD1 are more than 10 times more toxic than that of the *E. faecalis* cytolysin-negative mutant. The 50% lethal dose of *E. faecalis* strains that express cytolysin was one order of magnitude lower than that of a non-cytolysin producing strain. Experiments using a rabbit endocarditis model, in which valvular damage was induced by catheter insertion, demonstrated that the mortality was higher when both the plasmid-determined aggregation substance and the cytolysin were expressed (Chow, et al., 1993; Galli & Wirth, 1991). In a rabbit experimental endophthalmitis model, it was shown that the cytolysin significantly determined the course and severity of the disease (Jett, Jensen, Nordquist, & Gilmore, 1992).

The activity of cytolysin entails two unique peptides that possess modifications and physiochemical characteristics of lantibiotic bacteriocins. Both peptides are needed for antimicrobial activity. Expression of the cytolysin is tightly controlled by a two-component regulatory system, which is often referred to as quorum-sensing regulation (Coburn, Pillar, Jett, Haas, & Gilmore, 2004; Gilmore, Coburn, Nallapareddy, & Murray, 2002; Haas, Shepard, & Gilmore, 2002). In this regulatory network, the smaller peptide of the cytolysin induces the high-level expression of the cytolysin genes through binding to a membrane-bound histidine protein kinase (receptor protein), followed by a phosphorylation relay resulting in a phosphorylated response activator that, in turn, activates genes involved in the biosynthesis of the cytolysin (Coburn, Pillar, Jett, Haas, & Gilmore, 2004).

The second two-peptide lantibiotic characterized so far is enterocin W, which shares the strongest homology to plantaricin W, a bacteriocin from *Lactobacillus plantarum* (Sawa, et al., 2012). The amino acid sequences of the two prepeptides of enterocin W are 63.3% and 44.7% identical to plantaricin Wα and Wβ prepetide counterparts, respectively (Sawa, et al., 2012). Enterocin W is active against several Gram-positive bacteria and its optimal antimicrobial activity is achieved at equimolar concentration of the two peptides. The target bacteria are killed at peptide concentrations below 1 μM.

The recent advent of high through-put DNA sequencing technologies has generated a wealth of genomic information in public domains that represents an important source for bioprospecting of bioactive substances. Indeed, in silico screening has identified a gene cluster of a one-peptide lantibiotic within the genome of *E. faecalis* Fly1 (Marsh, O'Sullivan, Ross, Cotter, & Hill, 2010). This lantibiotic shows significant homology to a number of epidermin-like (type 1) lantibiotics (Marsh, O'Sullivan, Ross, Cotter, & Hill, 2010), but has not been purified or studied further.

Class II Bacteriocins

Class II comprises a diverse group of bacteriocins that still is under debate with respect to their classification. The Class II bacteriocins are defined as non-modified and heat stable bacteriocins, although some modifications (e.g., disulfide bridging, circularization, and methionine formylation) are actually found within some of these bacteriocins. However, there is a general consensus that the Class IIa (the pediocin-like) and IIb (the two-peptide bacteriocins) are well-defined groups. This is also the case for the circular bacteriocins, but it has been proposed that this group should be included in a separate class (Maqueda, et al., 2004). In addition, a subgroup of leaderless bacteriocins (enterocins) has been defined; and finally, some enterocins do not fall into any of the recognized subgroups.

Class IIa: the pediocin-like and strong anti-listerial enterocins

Class IIa constitutes the largest subclass of Class II bacteriocins, which are apparently the most abundant bacteriocins in LAB. They are found among many species of enterococci, while identical and closely related enterocins are encountered in different enterococcal species, though they have been given different names. Presently, Class IIa bacteriocins have been identified in six enterococcal species: *E. faecalis. E. faecium, E. mundtii, E. avium, E. hirae*, and *E. durans* (Table 1). Class IIa bacteriocins from all genera vary in length between 37 to 58 amino acid residues, while the Class IIa enterocins vary from 43–47 residues, but they all share the following consensus sequence in their N-terminal half: KYYGNGL/VXCXKXXCXVDW (Drider, Fimland, Héchard, McMullen, & Prévost, 2006; Eijsink, Skeie, Middelhoven, Brurberg, & Nes, 1998; Nes, Diep, Håvarstein, Brurberg, Eijsink, & Holo, 1996).

The two cysteines in the N-terminal consensus sequence form a disulfide bridge, which is a prerequisite for antimicrobial activity. These bacteriocins are of particular interest because they strongly inhibit the growth of *Listeria monocytogenes* and enterococci. At least thirteen different Class IIa bacteriocins have been isolated and characterized in enterococci, and *E. faecium* is the most frequent species found to be in the Class IIa enterocin producers (Table 1). It is interesting to note that enterocin A is among the most potent antimicrobial peptides in this subgroup (Eijsink, Skeie, Middelhoven, Brurberg, & Nes, 1998). It has been shown that the high antibacterial potency of enterocin A is due to the presence of two disulfide bridges, where the second bridge is located in the C-terminal part (Eijsink, Skeie, Middelhoven, Brurberg, & Nes, 1998; Fimland, et al., 2000; Uteng, et al., 2003). This structural feature is also found in some of the other Class IIa bacteriocins, which all exert strong antimicrobial activity. NMR analysis has shown that the C-terminal disulfide bridge stabilizes a folded-back structure in this region, which is required for enhanced antimicrobial activity. In fact, introducing a C-terminal disulfide bridge in similar position in Class IIa bacteriocins that originally enclose only the N-terminal disulfide bridge significantly enhances their antimicrobial activity (Fimland, et al., 2000). Temperature studies

have also shown that the Class IIa bacteriocins without the C-terminal disulfide bond are 30-50 times less active at 37ºC, as compared to the activity at 25ºC, while bacteriocins with the C-terminal disulfide bond do not exhibit such a temperature-dependent antimicrobial activity (Fimland, et al., 2000; Kaur, Andrew, Wishart, & Vederas, 2004; Uteng, et al., 2003).

Class IIa bacteriocins can use two different secretion systems. Some are secreted by a dedicated ABC transporter that recognizes the double-glycine leader in the N-terminal part of the prebacteriocin while others are synthesized with a *sec*-dependent leader, and are consequently secreted by a *sec*-system (see Table 1).

Some of the Class IIa bacteriocins with a double glycine leader, which also includes a few enterocins, are regulated by a three-component regulatory system that encompasses a peptide pheromone, also referred to as an autoinducing peptide (which often resembles a bacteriocin molecule but with fewer amino acid residues and have very low, if any, antimicrobial activity), a membrane-bound histidine protein kinase that serves as receptor for the peptide pheromone, and finally, a response regulator protein that activates the operons participating in the bacteriocin biosynthesis upon phosphorylation (96,97). Among the Class IIa enterocins, it has been shown that such a regulatory system is involved in the production of enterocin A and B, as well as avicin A (Birri, Brede, Forberg, Holo, & Nes, 2010; Nilsen, Nes, & Holo, 1998).

Mundticin KS-producing *E. mundtii* was originally isolated from grass silage and its genetic determinants are encoded on the 50 Kbp-plasmid pML1 (Kawamoto, et al., 2002). In contrast to the enterocin A and avicin A strains, the accessory protein for the ABC transporter is apparently missing, and the three-component regulator system for the bacteriocin production is not found in the mundticin KS-producing strains. It has also been shown that mundticin KS is produced by an *E. faecium* isolate obtained from Peruvian cheese (Aguilar-Galvez, Dubois-Dauphin, Campos, & Thonart, 2011).

The *sec*-dependent leader Class IIa bacteriocins, except enterocin P, share a high degree of amino acid sequence homology (more than 70% identity) to bacteriocin 31. The peptide sequence of enterocin P shares less identity with other bacteriocins that have a *sec*-dependent leader, which suggests that enterocin P belongs to another sub-group of the *sec*-leader dependent bacteriocins. The genetic organization of *sec*-dependent Class IIa bacteriocin is included in one operon that consists of the structural gene encoding the bacteriocin preprotein and its dedicated immunity gene, while the *sec*-dependent transporter system is situated in a different location on the bacterial genome.

The crystal structure of the immunity protein of enterocin A and mundticin KS has been determined (Jeon, Noda, Matoba, Kumagai, & Sugiyama, 2009; Johnsen, Dalhus, Leiros, & Nissen-Meyer, 2005). The immunity proteins of enterocin A (ImEntA) and mundticin KS (ImMunKS) consist of 98 and 103 amino acid residues, respectively. Based on sequence homology, Class IIa bacteriocins have been classified into three subgroups (A, B, and C) (Eijsink, Skeie, Middelhoven, Brurberg, & Nes, 1998; Fimland, Eijsink, & Nissen-Meyer, 2002). The two structurally determined immunity proteins belong to different subgroups: ImEntA to subgroup A and ImMunKSto subgroup B. The structures of both immunity proteins fold into an antiparallel four-helical bundle with a flexible C-terminal part, which is also shown with the immunity proteins of other Class IIa bacteriocins. This finding indicates that this is a conserved structural feature common for all pediocin-like immunity proteins, independent of their subclassification. The current model of mode of action suggests that the C-terminal half of the immunity protein contains a region that recognizes the C-terminal half of the cognate bacteriocin, and the flexibility in the C-terminal end of the immunity protein might therefore be an important characteristic that enables the immunity protein to interact directly or indirectly with its cognate bacteriocin (Fimland, Johnsen, Dalhus, & Nissen-Meyer, 2005). It has also been shown that the Class IIa immunity proteins, together with their cognate bacteriocins, form a strong ternary complex with the mannose-phosphotransfer-system (manPTS), and thereby prevent permeabilization and killing of the target cell, as seen in Figure 2 (Diep, Skaugen, Salehian, Holo, & Nes, 2007).

It was recently observed that the conjugative plasmid antibiotic-resistant pAMS1 encodes a Class IIa bacteriocin, designated MC4-1 (Sedgley, Clewell, & Flannagan, 2009). Interestingly, the production of the bacteriocin from its original host *E. faecalis* MC4-1 was not detectable in plate assays but when pAMS1 was transferred to an *E. faecalis* JH2-2 host, production of bacteriocin MC4-1 was observed. It was also shown that transconjugants of only some *E. faecalis* strains gave rise to detectable bacteriocins. It was not detectable in plasmid hosts that produced gelatinase (protease), which is not produced in the JH2-2 strain. It was also found that while bacteriocin production and related immunity occurs readily as cells enter the stationary phase, production is not expressed during exponential growth; thus, strains growing on plates as an early lawn (i.e., exponentially growing) exhibit sensitivity to bacteriocins produced by stabs from sibling colonies (Flannagan, Clewell, & Sedgley, 2008; Sedgley, Clewell, & Flannagan, 2009). This phenomenon is referred to as siblicidal activity (sibling killing).

Enterocin A is most often produced in conjunction with other bacteriocins, and often in combination with enterocin B (Casaus, Nilsen, Cintas, Nes, Hernández, & Holo, 1997). Occasionally, enterocin P, which is another Class IIa bacteriocin, and the leaderless enterocins L50 and Q, as well as other enterocins, are coproduced with enterocin A (Aguilar-Galvez, Dubois-Dauphin, Campos, & Thonart, 2011; Cintas L. M., et al., 2000; Strompfová, Lauková, Simonová, & Marcináková, 2008). Enterococci seem to commonly have the genetic ability to produce more than one bacteriocin, as also seen among some other LAB (Diep, Håvarstein, & NEs, 1996; van Belkum, Kok, & Venema, 1992). It is interesting to note that many Class II bacteriocins use a typical double-glycine leader for export, which is facilitated by a dedicated ABC transporter, but many Class IIa enterocins have a *sec* leader and consequently apply a *sec*-dependent secretion.

Bacteriocins target a defined group of bacteria that are often closely related to the producers. This constriction in the inhibitory spectrum suggests that a specific receptor is being targeted in sensitive cells. However, very little is known about receptors for most LAB bacteriocins. Some lantibiotics, including nisin, employ the cell wall precursor lipid II as a docking molecule. Depending on their concentration, nisin and related bacteriocins can either inhibit cell wall biosynthesis (at low bacteriocin concentrations) or form lethal pores on target cells (at high bacteriocin concentrations) (Bierbaum & Sahl, 2009). For Class II bacteriocins, the sugar transporter man-PTS has been shown to serve as a receptor for the pediocin-like bacteriocins (Class IIa) and for some linear non-pediocin-like bacteriocins, such as lactococcins A and B. The man-PTS, which is a major permease for glucose in most bacteria, is composed of three proteins where the IIC and the IID proteins form a membrane-embeded complex, and the cytosolic IIAB entity is reversibly associated with its membrane-located partners. Only the membrane-located components (IIC and IID) are required for the receptor to function (83). Moreover, a region of about 40 amino acid residues which encloses a predicted extracellular loop of IIC appears to enclose the specific interaction site with the Class IIa bacteriocins (see Figure 2A). This region contains a sequence signature present only in bacterial species/genera that are known to be sensitive to pediocin-like bacteriocins, but is absent in the corresponding IIC protein of the man-PTS in Gram-negative cells, such as bacilli, clostridia, and other bacterial cells that are known to be insensitive to the pediocin-like bacteriocins (Kjos, Salehian, Nes, & Diep, 2010). Thus, this region is definitely involved in defining the spectrum of sensitive cells targeted by pediocin-like bacteriocins.

It has been clearly demonstrated that pediocin-like bacteriocins not only require man-PTS as a target molecule, but also that they destroy the functionality of man-PTS by keeping it irreversibly open as depicted in Figure 2B. However, this finding has not yet been experimentally proven—but there are some strong pieces of additional evidence that favor this model. First, each producer cell expresses an immunity protein to protect itself from self-destruction. Upon purification of this immunity protein, it was found that it was tightly associated and co-purified with components of the receptor (man-PTS) and the bacteriocin (Diep, Skaugen, Salehian, Holo, & Nes, 2007; Kjos, Nes, & Diep, 2009), which suggests that this bacteriocin was most likely trapped within the protein complex, as shown in Fig. 1C. This complex formation is probably needed to prevent the bacteriocin from causing the irreversible leakage of intracellular solutes, as seen in the absence of an immunity protein. Second,

upon exposure to higher concentrations of bacteriocin, an immunity clone (expressing the immunity gene) appeared to grow less efficiently on glucose than on galactose (Diep, Skaugen, Salehian, Holo, & Nes, 2007; Kjos, Nes, & Diep, 2011); the second sugar does not use the man-PTS for transport. This result strongly indicates that man-PTS is impaired or less functional when bacteriocin immune cells are challenged by the bacteriocin. This mechanism of action is applied not only to the pediocin-like bacteriocins but also to lactococcin A—but lactococcin A recognizes only lactococcal man-PTS, and not the man-PTS that are recognized by the pediocin-like bacteriocins (Diep, Skaugen, Salehian, Holo, & Nes, 2007; Kjos, Nes, & Diep, 2009; Kjos, Salehian, Nes, & Diep, 2010).

Class IIb: The two-peptide bacteriocins

The first two-peptide, lactococcin G, was isolated from *Lactococcus lactis*, while the first identified two-peptide enterocin was enterocin 1071, which originates from *E. faecium* (Franz, et al., 2002; Nissen-Meyer, Holo, Håvarstein, Sletten, & Nes, 1992). The two peptides in a two-peptide bacteriocin do not share sequence identity toward each other, but significant peptide sequence identity was observed between the homologous peptides of lactococcin G and enterocin 1071. A two-peptide bacteriocin needs both peptides, which are genetically co-localized, in order to exert antimicrobial activity. In a few cases, individual peptides show some antimicrobial activity, but this activity is far less when compared to the combined activity of both peptides in equal molar concentration. It is worth noting that the peptides of a two-peptide bacteriocin are different from the leaderless peptide bacteriocins (which have been occasionally and incorrectly assigned as two-peptide bacteriocins) in that, the leaderless peptides within a bacteriocin share high sequence identity with each other and the individual peptides exert significant antimicrobial activity (see below). It has therefore been suggested that the genes that encode a two-peptide bacteriocin probably evolved independently, while genes for the leaderless peptides of a bacteriocin evolved by gene duplication. Another important criterion of a two-peptide bacteriocin is that only a single immunity protein is involved to protect the producer from committing suicide.

Enterocin 1071, enterocin C, and enterocin X are included in the group of two-peptide bacteriocins. Enterocin 1071, which is composed of the peptide enterocin 1071A, and enterocin 1071B were first characterized in *E.faecalis* BFE1071, an isolate obtained from fecal samples of a minipig. The enterocin-1071–encoding genes are situated on a 50kbp conjugative plasmid (Balla, Dicks, Du Toit, Van Der Merwe, & Holzapfel, 2000). Enterocin C, which is composed of the two peptides enterocin C1 and C2, is produced by *E.faecalis* C901 (Maldonado-Barragán, Caballero-Guerrero, Jiménez , Jiménez-Díaz, Ruiz-Barba, & Rodríguez, 2009). The genes encoding Ent C1, Ent C2, and the putative immunity protein Ent CI are located on the 9-kbp plasmid pEntC1. Full antimicrobial activity requires the complementary action of Ent C1 and Ent C2. The Ent C1 peptide is identical to the Ent 1071A peptide, while the Ent C2 peptide diverges from the Ent 1071 peptide by a single amino acid residue in position 17, where alanine is replaced by threonine. Based on antimicrobial testing with a cell-free supernatant, it was shown that enterocin C differs from enterocin 1071 in a few major aspects, which include the complementary activity of the two peptides as well as its broader inhibitory spectrum (Maldonado-Barragán, Caballero-Guerrero, Jiménez , Jiménez-Díaz, Ruiz-Barba, & Rodríguez, 2009). However, such investigations should be performed on purified peptides, since bacteria often produce multiple bacteriocins, which might affect the bacteriocin activity measurements and hamper the results and conclusions.

Enterocin X is composed of two peptides, EntX_α and EntX_β, and is produced by *E. faecium* KU-B5 (Hu, Malaphan, Zendo, Nakayama, & Sonomoto, 2010). There are no homologies between Ent X_α/X_β and other reported bacteriocins. When X_α and X_β were mixed beforehand in equimolar amounts, the combined antibacterial activity displays variable antibacterial activity toward a panel of indicators compared to the individual peptides.

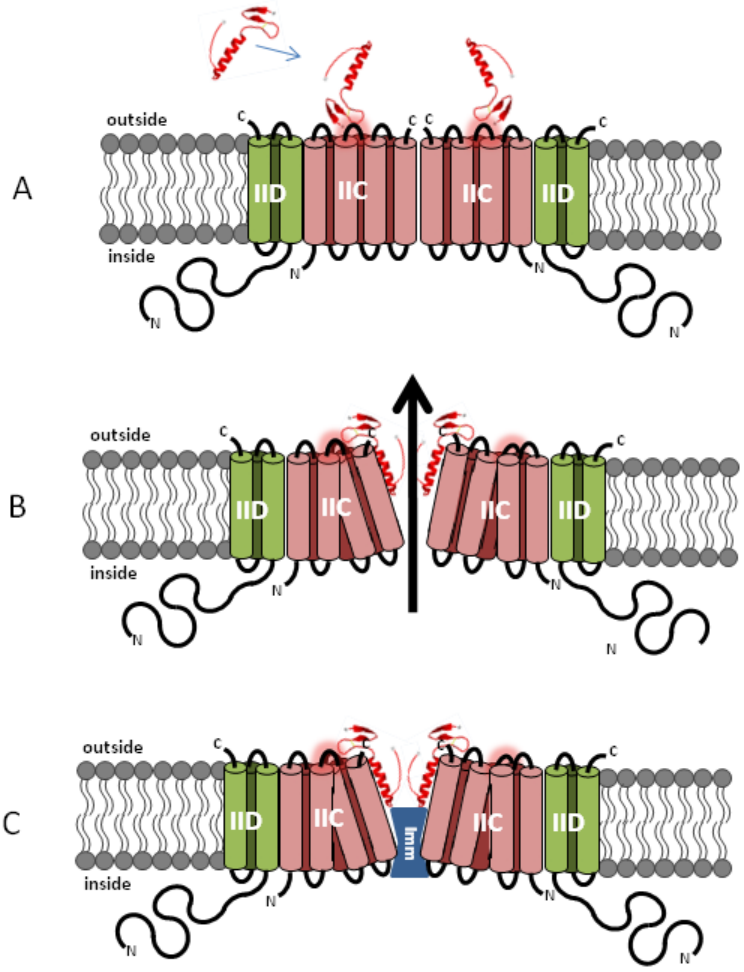

Figure 2. Model of target recognition, mode of killing, and immunity of Class IIa bacteriocins. A) a Class IIa bacteriocin specifically targets an extracellular loop of IIC, one of the two membrane-embedded components (IIC and IID, also called ManCD) of man-PTS; and B) the initial interaction leads to further interactions with some membrane helices of man-PTS, somehow causing the channel of the sugar permease to remain open, leading to leakage of solutes, destruction of membrane integrity, and eventually cell death. C) In producer cells, the cognate immunity protein binds to IICD and locks the bacteriocin in a tight complex, thereby preventing the bacteriocin from opening the pore.

Class II: Circular bacteriocins

Circular bacteriocins have often been included in the Class II bacteriocins, but grouping them into a separate class has been proposed (Class IV bacteriocins) (Maqueda, Sánchez-Hidalgo, Fernández, Montalbán-López, Valdivia, & Martínez-Bueno, 2008). These bacteriocins differ from most Class II bacteriocins in that they do not have free ends and are circularized by the α-amino group of one residue linked to the carboxyl group of the terminal residue of the peptide as a peptide bond. Circular bacteriocins are synthesized with a leader sequence that varies between 3 and 20 amino acids. After removal of the leader, the resulting N-terminal residue is covalently linked to the C-terminal residue and finalized in a compact structure of four or five helical bundles structured with a hydrophobic core.

Not many circular bacteriocins are found among LAB, but enterocin AS-48 is among them (Cobos, et al., 2001; Gálvez, Giménez-Gallego, Maqueda, & Valdivia, 1989; González, et al., 2000). AS-48, which was originally isolated from *E. faecalis*, has been studied in great detail with respect to genetics, biochemical features, and

structure, as well as its mode of action. AS-48 has also been called bacteriocin 21 in some studies (Tomita, Fujimoto, Tanimoto, & Ike, 1997; Tomita, Fujimoto, Tanimoto, & Ike, 1996). The genetic foundation of AS-48 production and its immunity function relies on the coordinated expression of ten genes located in two operon structures (Maqueda, et al., 2004).

Studies on modes of action have been performed on the circular bacteriocins, including AS-48, by using liposomes and lipid bilayers, and it was concluded that membrane receptors were not engaged in antimicrobial activity (Gálvez, Maqueda, Martínez-Bueno, & Valdivia, 1991). Furthermore, it has been shown that AS-48 activity is not dependent on a membrane potential (Gálvez, Maqueda, Martínez-Bueno, & Valdivia, 1991). It is important to note that these mode-of-action studies were performed on synthetic membranes/lipids, in which a potential receptor was absent, and that the active concentrations of bacteriocins used on synthetic systems were far higher (100–1000 fold) than the active concentrations used on live sensitive cells. Thus, it is not possible to rule out that a specific receptor/target/mediator for a high sensitivity of live cells is implicated in some killing mechanisms. Indeed, it was quite recently shown that another cyclic bacteriocin, garvicin ML from *Lactococcus garvieae*, needs a membrane-located maltose transporter to exert maximum killing efficiency (Gabrielsen, Brede, Hernández, Nes, & Diep, 2012). It remains to be seen whether this is true for other cyclic bacteriocins, including AS-48.

Class II: Leaderless bacteriocins

Most Class II bacteriocins contain an N-terminal leader peptide, which directs the secretion of the bacteriocin and is cleaved off during the secretion process. Their leaders belong to the so-called double-glycine leader type, or, in some cases, to a *sec*-dependent leader type, and as a result, the *sec* secretion system is employed for the latter. However, some bacteriocins are not synthesized with a leader-peptide, and their secretion seems to be performed by dedicated ABC transporters with sequence features that are notably different from the ones that externalize the double-glycine leader and the *sec*-leader bacteriocins. A bacterium can produce a single or multiple leaderless bacteriocins. When several leaderless bacteriocins are produced by the same strain, the bacteriocins often share strong sequence homology and their genes are located next to each other. The individual peptides possess antimicrobial activity, but when combined, they exhibit increased potency. However, enhanced antimicrobial activity among peptides varies, and are target-dependent, as seen with the two leaderless peptides (L50A and L50B) of enterocin L50 (Cintas, Casaus, Holo, Hernandez, Nes, & Håvarstein, 1998). In one strain of *Staphylococcus aureus*, up to five homologous antimicrobial peptides have been found (100). Among enterococci, single leaderless peptides and two homologous leaderless peptides have been identified so far (Cintas, et al., 2000; Cintas, Casaus, Holo, Hernandez, Nes, & Håvarstein, 1998). Dedicated ABC transporters seem to manage the transport of such bacteriocins (Criado, et al., 2006).

Ent L50A and L50B, which are encoded on the plasmid pCIZ1(50kbp), are produced by *E. faecuim* L50 isolated from Spanish dry-fermented sausage. Enterocin L50A and Enterocin L50B display 72% sequence identity and consist of 44 and 43amino acids, respectively. Both bacteriocins possess individual antimicrobial activity, with EntL50A being the most active. A synergistic antimicrobial activity was observed by combining the two bacteriocins (Ent L50A and Ent L50B). In addition to the production of EntL50, *E. faecium* L50 also produces enterocin Q, which is a single leaderless peptide whose genes are located on another plasmid pCIZ2 (7.4kbp), as well as enterocin P, which is chromosomally encoded (Cintas, et al., 2000; Criado, et al., 2008).

Several characterized leaderless enterocins have been shown to be identical or highly homologous to Ent L50A/L50B (Dezwaan, Mequio, Littell, Allen, Rossbach, & Pybus, 2007; Izquierdo, et al., 2008; Kang, et al., 2009; Liu, et al., 2011; Yamamoto, Togawa, Shimosaka, & Okazaki, 2003). The two respective peptides of the leaderless enterocins 7A/7B and MR10A/10B are identical and share strong homology to EntL50A and Ent L50B peptides (Kang, et al., 2009). However, amino acid sequences of enterocin Q and enterocin EJ97 have no strong homology to other bacteriocins.

A recent study has shown that leaderless bacteriocins might possess a formyl group in their N-terminal methionine (Liu, et al., 2011). The two leaderless bacteriocin peptides, enterocin 7A (Ent7A) and enterocin 7B (Ent7B), were analyzed by matrix-assisted laser desorption ionization-time-of-flight mass spectrometry and electrospray infusion tandem mass spectrometry analyses. The data and DNA sequence analysis showed that both peptides are produced without N-terminal leader sequences but the observed masses for Ent7A and Ent7B were 5200.80 and 5206.65 Da, respectively, which are larger than the theoretical masses. Further experiments provided evidence that both Ent7A and Ent7B are formylated in the N-terminal methionine residue (Liu, et al., 2011). It seems likely that N-terminal formylation is a general feature for such leaderless bacteriocins.

Other small heat-stable bacteriocins

Some bacteriocins fall outside the classification scheme described above, because they do not share the basic classification features of bacteriocins from any of the above mentioned classes/subclasses. Two such enterocins have presently been characterized. Enterocin B is a 53-amino-acid residue, linear, non-pediocin-like peptide that employs a double glycine-leader for export. This bacteriocin is often co-expressed with enterocin A and they act synergistically (Casaus, Nilsen, Cintas, Nes, Hernández, & Holo, 1997; Ennahar, Asou, Zendo, Sonomoto, & Ishizaki, 2001; Franz, et al., 1999). The second one is bacteriocin 32, which is a sec-dependent bacteriocin encoded on the mobile plasmid pTI1 (12.5kbp) of the vancomycin-resistant *E. faecium* (VRE) 200 strain. It is active against *E. faecium, E. hirae*, and *E. durans*, but is not active against *L. monocytogenes* (Inoue, Tomita, & Ike, 2006). The *bac32* operon is composed of two genes, *bacA* and *bacB*, which encode the bacteriocin preprotein with a *sec*-dependent leader peptide and the immunity protein, respectively (Inoue, Tomita, & Ike, 2006; Yamashita, Tomita, Inoue, & Ike, 2011).

Bacteriolysins (previously named Class III bacteriocins)

Years ago, a group of heat-labile bacteriocins from LAB were included in the separate Class III of heat-labile bacteriocins (Klaenhammer, 1993). Since then this class has been redefined to include heat-labile antimicrobial proteins that performed enzymatic degradation of the cell wall of targeted bacteria, and it seemed reasonable to name this group of antimicrobial proteins bacteriolysins (Cotter, Hill, & Ross, 2005). Such antimicrobial proteins are apparently easy to define because of their heat-labile characteristics, as well as their ability to degrade the cell walls of susceptible target strains.

Enterolysin A has been characterized as a Class III bacteriocin in *E. faecalis* (Hickey, Twomey, Ross, & Hill, 2003; Nilsen, Nes, & Holo, 2003). The mode of action of enterolysin A is quite different from the previous mentioned heat-stable bacteriocins, because it attacks susceptible bacteria by degrading the cell wall structure, which eventually leads to lysis of the cells. Enterolysin A exhibits a similar identity to cell wall-degrading enzymes that are produced by different Gram-positive bacteria. Sequence analysis of enterolysin A suggested that this bacteriocin consists of two separate domains: an N-terminal catalytic domain, and a C-terminal substrate recognition domain. The specificity of enterolysin A indicates that the target of its putative recognition domain is different from the previously described targets, and this is consistent with the fact that the putative, non-catalytic C-terminal part of enterolysin A shows no sequence homology to the proteins active against staphylococci and streptococci. However, the C-terminal domain of enterolysin A exhibits high levels of sequence identity to a lysin from bacteriophage A2 and an *N*-acetomuramoyl-L-alanine amidase from bacteriophage PL-1, both of which are bacteriophages of *L. casei*. The regions of these lysins that are homologous to the C-terminal part of enterolysin A are thought to be responsible for its binding to their cell wall substrate. It is also worth mentioning that the N-terminal part of enterolysin A is linked to a putative C-terminal recognition domain by a threonine-proline-rich region, which shows significant sequence identity to the two previously mentioned bacteriophage lysins (Klaenhammer, 1993).

A new antimicrobial compound, named bacteriocin 41, was isolated in a recent study (Tomita, Kamei, & Ike, 2008). This bacteriocin consists of three components: a lysin-like molecule (BacL1), an activator (BacL2) of

BacL1, and a third protein, which is needed for the antimicrobial activity (BacA). In addition, an immune protein (BacI) that prevented lysis of the producer was identified. While BacL1 showed homology to several bacteriophage lysins and a muramidase, Bac L2 was homologous to the holin of a bacteriophage. BacA, which is the largest protein (726 aa) in the antimicrobial protein complex, showed homology to two *B. subtilis* proteins (Ybfg and YkuG) of unknown function. A putative peptidoglycan binding domain was identified in the N-terminal part of BacA. The mode of action of bacteriocin 41 is not presently known, but its antimicrobial activity is certainly heat-labile. However, the target is most likely the cell wall, and killing apparently occurs by cell wall degradation. Consequently, it seems most reasonable to include bacteriocin 41 in the class of bacteriolysins.

Because of the presence of conserved domain-like structures involved in the translocation, receptor binding, and antimicrobial activity, homologous bacteriolysins can readily be identified in genome databases by performing homology searches.

The Ecology of Enterocins

Bacteriocin-producing enterococci are isolated from a broad range of environments. It is of interest to note that food is a common source of enterococci (*E. faecium* in particular), but *E. faecalis* is also frequently found in fermented food commodities (Giraffa, 2003; Hugas, Garriga, & Aymerich, 2003). Enterococci isolated from different environments, including food, most likely originate from the commensal gut flora of humans and animals. Enterococci are one of the dominant fecal LAB in humans, though a great fluctuation in numbers, depending on age and lifestyle of the host (above 10^8 enterococcal cells per gram of feces in babies, but usually significant less in adults, have been observed). Several studies have shown that there is a high frequency of bacteriocin production among enterococci, and it has also been reported that a major fraction of the isolated enterococci from stool samples do produce bacteriocins (Forberg, 2005; Herrera, Brede, Salehin, Holo, & Nes, 2006).

Hospital isolates of vancomycin-resistant enterococci (VRE) have been shown to be frequent producers of bacteriocins (del Campo, et al., 2001). In one study, 636 VRE isolates were tested for bacteriocin production, and 44% of them were shown to be bacteriocinogenic (Inoue, Tomita, & Ike, 2006). The frequency of bacteriocinogenic enterococci appears to be higher for certain bacteriocins (Bac41, Bac32) among clinical isolates than those of non-clinical isolates (Ike, Inoue, Yamashita, & Tomita, 2010; Inoue, Tomita, & Ike, 2006). It is important to note that many of those clinical isolates are not clonal. In one study, approximately 40% of clinical *E. faecium* / *E. faecium* VRE produced Bac32, while this was the case for only 2% of *E. faecium* isolates from stools samples of healthy students. In another study, about 50% of *E.faecalis* clinical isolates were Bac41 producers (Ike, Inoue, Yamashita, & Tomita, 2010).

An increased hemolytic phenotype among clinical-derived *E. faecalis* has been reported (Ike, Hashimoto, & Clewell, 1987). About 60% of *E. faecalis* clinical isolates are cytolysin (Hly/Bac) producers. In contrast to the high frequency of hemolysin producers among parenteral isolates, isolates derived from fecal specimens of healthy individuals exhibited a low (17%) incidence of hemolysin production. In one study, *E. faecalis* blood isolates were probed for the serine protease activator of cytolysin (*cylA*) (Huycke & Gilmore, 1995), and it was found that *cylA* occurred more frequently among bacteremia isolates [34 of 68 (50%)] than isolates from endocarditis [4 of 35 (11%)] or stool samples [0 of 14]. But when clonality was taken into account, it was concluded that no significant enrichment for *cylA* was present among clonally unrelated bloodstream isolates (Huycke & Gilmore, 1995). However, the clonally related hemolytic strains demonstrated an increased propensity to cause bloodstream infections (Huycke & Gilmore, 1995). Bacteriocin-activities are also observed in other hemolytic peptides obtained from staphylococcus isolates (Donvito, Etienne, Denoroy, Greenland, Benito, & Vandenesch, 1997; Watson, Yaguchi, Bisaillon, Beaudet, & Morosoli, 1988).

As previously mentioned, LAB, including enterococci, often produce multiple bacteriocins. It has been shown that enterocins A and B are commonly found in the same isolates (Nilsen, Nes, & Holo, 1998), but they are also

found together with other enterocins, such as enterocins P, Q, and L50 (Cintas L. M., et al., 2000). It has also been shown that the multiple-enterocin-producing *E. faecium* L50 strain expressed bacteriocin activity within a broad temperature interval, with different combinations of bacteriocins produced at different temperatures. *E. faecium* L50 produced EntP and EntQ in the temperature range between 16 ^{O}C to 47 ^{O}C, and maximal activity was observed between 37 ^{O}C to 47 ^{O}C, while EntL50A and EntL50B are maximally synthesized between16 ^{O}C to 25 ^{O}C, and no activity was detected at 37 ^{O}C or above (Cintas, et al., 2000). The mechanism and the biological significance of temperature-regulated bacteriocin production are not known and should be investigated further.

Many of the same bacteriocins are found to be produced by enterococci isolated from quite different environments, which suggests that the bacteriogenicity is not a feature to promote growth in specific environments, but might be advantageous for a bacterium in any environment. The presence of identical bacteriocins in strains from different environments may indicate that the enterococci originate from a common source. For example, bacteriocin31 or bacteriocin31-type bacteriocins have been isolated from enterococci isolates from human sources (i.e., Bac31, Bac RC714, Bac43 and BacT8), grass silage from Thailand (Ent SE-L4), and from a wild Mallard duck (Hiracin JM79). Enterocin 7A/7B, enterocinMR10A/10B, enterocin 62-6A/B, and enterocin RJ-10, which are enterocin L50A/L50B-type bacteriocins, have been found among enterococci isolated from diverse environments. On the other hand, cytolysin-producing *E. faecalis* strains are predominantly located in human clinical isolates, and bacteriocin 32 and bacteriocin 41 determinants seem to be more frequently identified in human clinical isolates of *E. faecium* or *E. faecalis*. Enterocin A and enterocin B were originally isolated from *E. faecium* isolates from Spanish dry fermented sausage, but they are also among the most common bacteriocins found in enterococci obtained from stool samples of healthy babies. These findings suggest that some of the dominant type of bacteriocins in enterococcal clinical isolates might differ from the dominant type of bacteriocins found in enterococcal isolates that are predominantly found in fermented foods and commensal enterococci, and that the type of bacteriocins that dominate a given environment is influenced by nutritional and microbial factors.

Such a prevalence of bacteriocin-producing enterococci implies an efficient way for bacteriocins to transfer genes horizontally. It has been known for years that conjugative plasmids often carry bacteriocin genes and such mobile elements might promote the dissemination of bacteriocins. Three types of conjugative plasmids have been identified and analyzed in enterococci. Two of these plasmid types include pheromone responsive plasmids and non-pheromone responsive plasmids, which are efficiently transferred in broth mating (Clewell, 1981; Clewell, 1993; Ike, Tanimoto, Tomita, Takeuchi, & Fujimoto, 1998; Tomita, et al., 2003). The pheromone-responsive plasmids were originally identified in *E. faecalis* strains (Dunny, Brown, & Clewell, 1978) and comprise a narrow host range; they appear to transfer primarily within *E. faecalis* strains. The pheromone-responsive plasmid pAD1 (60kbp) that encodes cytolysin is representative, and is one of the most well-analyzed pheromone-responsive plasmids (Clewell, 1993; Clewell & Dunny, 2002; Dunny, Brown, & Clewell, 1978; Galli & Wirth, 1991). The bacteriocin determinants of *E. faecalis* are frequently encoded on pheromone-responsive plasmids. For example, cytolysin, AS48, bacteriocin 21, bacteriocin 31, bacteriocin 41, and enterocin EJ97 are encoded by pheromone-responsive plasmids pAD1 (60kbp) (Clewell, 1981; Gilmore, 1991; Gilmore, Coburn, Nallapareddy, & Murray, 2002; Ike, Clewell, Segarra, & Gilmore, 1990), pMB2 (58kbp) (Martínez-Bueno, Gálvez, Valdivia, & Maqueda, 1990), pPD1 (59kbp) (Fujimoto, Tomita, Wakamatsu, Tanimoto, & Ike, 1995; Tomita, Fujimoto, Tanimoto, & Ike, 1997), pYI17 (57.5kbp) (Tomita, Fujimoto, Tanimoto, & Ike, 1996), pYI14 (61kbp) (Tomita, Kamei, & Ike, 2008), and pEJ97 (60kbp) (Gálvez, et al., 1998), respectively. About 90% of conjugative plasmids in *E. faecalis* resemble the pAD1- type (Ike, Hashimoto, & Clewell, 1987).

The cytolytic phenotype is common in *E. faecalis* clinical isolates. About 60% of *E. faecalis* clinical isolates are hemolytic (Ike, Hashimoto, & Clewell, 1987). The cytolysin of about 50% of the cytolytic strains is encoded on pheromone-responsive conjugative plasmids. The cytolysin is also found to be encoded by a pathogenicity island found on the bacterial chromosome (Ike & Clewell, 1992; Shankar, Baghdayan, & Gilmore, 2002).

Enterocin 1071A/B isolated from *E.faecalis* BFE1071 was encoded on plasmid EF1071(50kbp), which can be transferred by filter mating. Enterocin L50A/B and Bac T8 from *E. faecium*, and mundticin KS from *E.mundtii* are encoded by the non-conjugative plasmids pCZ1(50kbp), 7kbp plasmid, and pML1(50kbp), respectively. Bacteriocin 32, bacteriocin RC714, bacteriocin 43, and bacteriocin 51, which were all isolated from *E. faecium* strains, are encoded by conjugative plasmids and are mobilized to the recipient strain by filter mating.

What is the biological role of bacteriocins? It is presently believed that the production of bacteriocins make their producer more competitive in certain ecological niches (Eijsink, Axelsson, Diep, Håvarstein, Holo, & Nes, 2002). This might be reflected by the high frequencies of bacteriocin-producing enterococci in the confined environment of the GI tract.

Large populations of bacteriocin-producing enterococci exist in human intestinal flora. As previously mentioned, the frequency of bacteriocin-producing isolates was reported to be higher among human clinical enterococcal isolates than among isolates from human fecal samples. In contrast, just a few clinical *E. faecium* bacteriocin producers were reported.

A different bacteriocinogenic profile was observed among enterococci obtained from fecal samples of babies. In one study, it was observed that among healthy Norwegian babies, bacteriocin-producing *E. faecium* was frequently observed, while significant fewer *E. faecalis* isolates were shown to produce bacteriocins (Birri, Brede, Forberg, Holo, & Nes, 2010; Nes, Forberg, Salehian, & Holo, 2005). Avicin A is produced by two different *E. avium* strains (XA83 and 208) isolated from feces of two healthy human infants from Norway and Ethiopia, respectively. The genetic determinants are located on the chromosome. Though avicin A producers are rare in babies, they have been isolated from two fecal stool samples of babies from such disparate areas as Ethiopia and Norway (Birri, Brede, Forberg, Holo, & Nes, 2010). This observation indicates that the bacteriocin producers have become a dominating LAB in the GI-tract of these babies (Birri, Brede, Forberg, Holo, & Nes, 2010). These results imply that the bacteriocins might provide a competitive advantage and promote dominance and colonization of the bacteriocinogenic strains in bacterial ecological environments, such as the human intestine. It is possible that bacteriocinogenic strains contribute to the pathogenic potential of virulent enterococci, while bactericnogenic commensal enterococci promote and maintain a healthy entercoccal flora.

Bacteriocin genes in *E. faecalis* are often carried by mobile genetic elements, including conjugative plasmids/transposons, which might serve an efficient way to disseminate such traits to a larger population of bacteria. Antibiotic-resistance genes and other accessory genes which are necessary for the bacterial host in certain growth conditions or ecological niches are often located on low-copy-number plasmids, and these traits can easily be lost during non-selective conditions. In such circumstances, it is feasible to think that their co-located bacteriocin genes might act as a toxin/antitoxin system to allow the low copies of plasmids to endure and pass on to daughter cells.

In some bacteria, bacteriocins may play a role to supply naked DNA for uptake and genetic recombination. It has been proposed that for certain streptococci, such as *Streptococcus mutants*, the production of bacteriocins is activated during DNA competence development (Claverys & Håvarstein, 2007; Perry, Jones, Peterson, Cvitkovitch, & Lévesque, 2009). It is suggested that the bacteriocins, in combination with certain lytic enzymes, lyse a subpopulation of the culture to make DNA available for the competent bacteria, a process referred to as fratricide. However, it has not been conclusively shown that competence development occurs in enterococci, and therefore the role(s) of bacteriocins in enterococci are likely to be involved in other functions.

Future Perspectives

Many bacteriocins have been purified or genetically analyzed in numerous enterococci (Table 1), and even more bacteriocinogenic isolates have been found, but their active entities have yet to be characterized. It is surprising to observe that among the fecal LAB flora, *E. faecium* is probably the most frequent producer of bacteriocins

(Nes, Forberg, Salehian, & Holo, 2005). In addition, enrichment of bacteriocinogenic *E. faecium* is found among vancomycin-resistant (VRE) hospital isolates (Inoue, Tomita, & Ike, 2006; Todokoro, Tomita, Inoue, & Ike, 2006). The many genome sequences that have been published the last few years have also confirmed the great abundance of bacteriocins that encode genes in enterococci. It is interesting to note that identical bacteriocins are encoded by *E. faecalis* and *E. faecium*. The genome sequences also suggest that bacteriocins that encode genes are found not only in hospital *E. faecalis*, but also in hospital isolates of *E. faecium*.

The reason why enterococci have developed such an ability to produce antimicrobial peptides is not known, but one might speculate that bacteriocin production is a beneficial trait (probiotic trait) in some environments (such as the human gut). However, the co-enrichment of antibiotic resistance and certain bacteriocinogenic traits among enterococci of clinical origins suggests that bacteriocin production might be a beneficial asset that allows enterococcal adaption to various environmental conditions. The bacteriocin system might serve as a toxin-antitoxin system to increase plasmid stability, thereby preventing the loss of the plasmid-encoding antibiotic-resistance genes under non-selective conditions. The characterized bacteriocins associated with antibiotic resistance seem to be different from the bacteriocins found in enterococci isolated from healthy individuals and babies (Birri, Brede, Forberg, Holo, & Nes, 2010). In the former case, bacteriocin 41 was frequently found in clinical isolates, but was absent in non-clinical isolates. Furthermore, the bacteriocin 32 was found at high frequencies among resistant VRE and clinical isolates, but at much lower frequencies among fecal isolates of healthy students. The genetic constituents of bacteriocin 32 were localized on a different plasmid than the vancomycin resistance genes, and it has also been shown that this bacteriocin can be co-localized with enterocin L50 (Inoue, Tomita, & Ike, 2006; Izquierdo, Cai, Marchioni, & Ennahar, 2009).

It is tempting to speculate that transferable antibiotic resistance takes advantage of bacteriocin systems to make such bacteria more competitive for growth and survival in an environment with other closely related bacteria—but more studies must be performed to support such a hypothesis.

The human gastrointestinal tract appears to be an excellent environment for bacteriocin-producing enterococci. We and others have shown that bacteriocin-producing enterococci are found in most stool samples of healthy babies at relatively high frequencies, suggesting that bacteriocin-producing enterococci are important in the development of the gut flora in infants.

Though most of the focus of enterococci today is on their pathogenicity and VRE, enterococci are a major LAB component in our gastrointestinal tract, and they are also a major bacterial entity of many endogenous dairy and meat products, as well as fermented food products (Hugas, Garriga, & Aymerich, 2003).

The biological role(s) of bacteriocins in nature are still not well understood. Are bacteriocins important for a healthy gut flora, could they be considered to be probiotics, or is their presence an indicator of virulence or antibiotic resistance? These are some of the urgent questions and scientific challenges that should be investigated. Only detailed insight in host-*Enterococcus* interaction can give us answers for how to safely appreciate their potential positive traits and how to deal with their problematic features.

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