4 The Diversity of Bacteriocins in Gram-Positive Bacteria

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Summary

Gram-positive bacteria, and especially the lactic acid bacteria (LAB), are now increasingly studied for their production of bacteriocin-like inhibitory activity. This has yielded detailed insight into many unique features of this surprisingly heterogeneous array of antibiotic molecules, a group apparently united only by their proteinaceous composition and targeted killing of bacteria generally closely related to the producer bacterium. Contemporary developments in this field have included increased knowledge of factors influencing bacteriocin expression, mode of action and specific host-cell immunity. Much of the burgeoning interest in the bacteriocin-producing LAB is driven by their perceived potential practical applications either to food preservation or as probiotics. In this chapter, we propose that all of the currently confirmed bacteriocins of Gram-positive bacteria can be classified into four broad groups: (1) lantibiotics, (2) small non-modified peptides, (3) large proteins, and (4) cyclic peptides.

4.1 Introduction

4.1.1 Bacteriocins: A Historical Perspective

The term antibiotic is generically used to describe substances produced by organisms that selectively interfere with the growth of other organisms. Within this extremely broad category of bioactive molecules, the subset known as the bacteriocins comprises the ribosomally synthesized proteinaceous compounds released extracellularly by bacteria that can be shown to interfere with the growth of other bacteria, typically including some that are

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closely related to the producing bacterium and to which the producer cell expresses a degree of specific immunity.

The study of inter-bacterial inhibition, similarly to so many other fundamental facets of microbiology, can trace its origins to Louis Pasteur. In 1877 Pasteur, together with his assistant Joubert, in seeking a way to control the growth of the anthrax bacillus, reported both in vivo and in vitro inhibitory activity associated with co-inoculated "common bacteria" (probably *Escherichia coli*) isolated from urine. Pasteur's pioneering studies heralded several decades of investigations, predating the antibiotic era, which focused upon the dosing of patients with relatively harmless bacteria in an attempt to counter the proliferation of pathogens – the so-called bacterial interference strategy – an approach to infection control now experiencing a renaissance after the half century of neglect that followed the discovery of penicillin, and the associated smug dependence of clinicians on the profligate use of therapeutic non-ribosomally synthesized antibiotics to control bacterial infection. Most of the early successes in defining the nature of bacteriocins related to those of Gram-negative bacteria, especially the colicins, and much of this knowledge stemmed from the work of Gratia and Fredericq. It was Gratia who first described antagonism between strains of *E. coli* (Gratia 1925). Interestingly, the first documented inhibitory strain produces colicin V, a bacteriocin of the microcin class that, in many respects, more closely resembles bacteriocins typically produced by Gram-positive bacteria (Håvarstein et al. 1994). Fredericq used specific (receptor-deficient) colicin-resistant mutants to classify the colicins (Fredericq 1946). General characteristics of the colicins included (1) plasmid-encoded, large domain-structured proteins, (2) bacteriocidal activity via specific receptors, and (3) lethal SOS-inducible biosynthesis. The study of bacteriocins of Gram-positive bacteria got off to a relatively faltering start, largely focusing on the staphylococci, and with various attempts to apply similar principles of classification to those that had been established for the colicins. However, relatively few of the protein antibiotics of Gram-positive bacteria fit closely the classical colicin mold. Major differences include their relatively broad activity spectra, less defined specific producer cell self-protection (immunity), and absence of SOS-inducibility. In the past three decades, studies of bacteriocins of Gram-positive bacteria, especially those of the lactic acid bacteria (LAB), have come to dominate the bacteriocin-related literature, a change largely driven by commercial imperatives.

4.1.2 Bacteriocins of Gram-Positive Bacteria

A landmark observation in the investigation of bacteriocins of Gram-positive bacteria was the documentation in 1947 that some of the inhibitory activity of lactococci (group N streptococci) toward other LAB is due to a molecule characterized as a proteinaceous antimicrobial called "group N inhibitory

substance", or nisin (Mattick and Hirsch 1947). Now approved for use as a food additive in around 50 countries, nisin has led the way in the bacteriocin field, not only with regard to the accumulated knowledge of its chemical characteristics and genetic basis but also for the extent and variety of its practical applications. The spectacular commercial success of nisin stimulated a gold rush-like frenzy of prospecting activity for comparable inhibitory agents. In 1976, the first review specifically of the literature on bacteriocins of Gram-positive bacteria noted the beginnings of this groundswell of exploration for nisin-like molecules produced by Gram-positive bacteria, and predicted growing interest in their potential applications to bacterial interference and food preservation (Tagg et al. 1976). On the other hand, failure to find practical outcomes for the bacteriocins of Gram-negative bacteria had caused them to be rather disregarded by researchers and funding organizations.

Two decades later, the scientific literature in the bacteriocin field had become dominated by studies of the bacteriocins of LAB, although the vast majority of these studies did not progress beyond superficial descriptions of their activity spectra against random collections of indicator bacteria and lavish predictions of their potential practical applications (Jack et al. 1995). Some small groups of enthusiasts had continued to explore the use of bacterial interference throughout the early days of the antibiotic era, and most of these studies targeted *Staphylococcus aureus*, due to its predilection for antibiotic resistance development. Some success was achieved with the use of the relatively avirulent 502A strain of *St. aureus*, although the active inhibitory principle(s) was never characterized (Bibel et al. 1983). More recently, the concept of specific modulation of the oral microflora via the introduction of well-characterized bacteriocin producers has found application to the control of a variety of ailments and infections of the oral cavity, ranging from streptococcal pharyngitis and dental caries to otitis media and halitosis (Tagg and Dierksen 2003). Meanwhile, an impressive number of bacteriocin-producing, natural food-associated isolates, mostly LAB and many of GRAS (generally regarded as safe) status, continue to be touted for their potential ability to specifically influence the bacterial content (both beneficial and detrimental) of food – providing a so-called "rudimentary innate immunity to foodstuffs" (Cotter et al. 2005b). Nevertheless, there have still been no comparable commercial successes to that of the benchmark bacteriocin, nisin.

4.1.3 Why Produce Bacteriocins?

Although there has been considerable discussion about their role in nature, it seems that the overriding "raison d'être" for bacteriocins is to provide the producing organism with an ecological advantage over its most likely competitors (Riley and Wertz 2002). So, how common is bacteriocin production?

It has been speculated that all members of the Eubacteria and also of the Archaea, when freshly isolated from their natural ecosystems, are probably equipped with the capability of expressing bacteriocins. It has even been suggested that, where no bacteriocins have yet been found to be produced by a bacterial isolate, it is only because the researchers have not yet defined the expression or detection conditions appropriate to display that strain's bacteriocinogenicity in vitro (Tagg 1992). As many of these bacteriocins are structurally complex, and therefore undoubtedly come at a considerable genetic and biosynthetic cost to the producer cell, they must clearly be functionally indispensable for the existence and persistence in nature of bacterial and archaeal cell lineages. However, under laboratory conditions where bacteria are typically grown as monocultures (with no competitors) and under pampered (low stress) conditions of nutritional excess, the production of anti-competitor molecules (bacteriocins) can more readily be dispensed with. Indeed, exposure of laboratory cultures to chemical curing agents or growth at elevated temperature can lead to the elimination of bacteriocin-encoding plasmids. In other cases, insertion of transposons within bacteriocin loci can lead to loss of bacteriocin expression. Close linkage of bacteriocin structural and immunity determinants on plasmids encourages retention of these plasmids under conditions favorable for bacteriocin expression, since plasmid-cured (and thus bacteriocin-sensitive) derivatives are likely to be counter-selected either in natural ecosystems or in laboratory cultures containing the homologous bacteriocin. Bacteriocin production, however, must inherently be an unstable strain characteristic, otherwise all bacteria would be expected to express multiple bacteriocins – so, from the bacterium's perspective, there must be a tradeoff between the metabolic (and genetic) cost to the cell of bacteriocinogenicity and the survival benefits accrued from the expression of bacteriocin(s) and/or retention of the genetic capability of producing bacteriocin(s) as the need arises.

4.1.4 Detection of Bacteriocins of Gram-Positive Bacteria

Two simple, agar culture-based methods have been most commonly used for the detection of bacteriocin production in vitro – simultaneous and deferred antagonism. However, some bacteriocins such as streptocins STH_1 and STH_2 appear to be produced only in liquid media (Schlegel and Slade 1973; Tompkins et al. 1997). In simultaneous antagonism, the test and indicator bacteria are typically grown together on an agar surface, and detection of bacteriocin production is dependent on the release of the inhibitory agent(s) relatively early in the growth of the test culture (i.e., before overgrowth of the indicator bacterium). By contrast, the deferred antagonism test, which is most commonly used in bacteriocin typing procedures, allows for independent variation of the incubation parameters (time, temperature, atmosphere) of the test and indicator bacteria. In practice, it is important to test by both

methods when screening bacteria for bacteriocinogenicity. Optimal conditions for test strain growth do not necessarily coincide with optimal bacteriocin production conditions. Indeed, bacteriocin production can be enhanced when the producer cells are relatively stressed (nutritionally or environmentally). Specific medium supplements shown to markedly affect the production of bacteriocins by Gram-positive bacteria have included yeast extract (enhancing mutacin production), glucose (effecting catabolite repression of some streptococcal bacteriocins), and magnesium ions (repressing expression of some lantibiotics).

Screening tests for inter-bacterial inhibition on agar media do not, of course, distinguish the activities of bacteriocins from inhibition due to non-bacteriocin agents such as bacteriophage, primary metabolites such as H_2O_2 and lactic acid, or non-ribosomally synthesized antibiotics such as bacitracin. Nor can such tests discriminate inhibition attributable to nutrient depletion or to the combined activities of multiple bacteriocins and/or other inhibitory agents. We recommend the use of the acronym BLIS (for bacteriocin-like inhibitory substance) to refer to as yet uncharacterized inhibitory agents that appear "bacteriocin-like" in their activity; e.g., *St. aureus* BLIS H12 is the initial descriptor for bacteriocin-like activity found to be produced by *St. aureus* strain H12.

4.1.5 Nomenclature of Bacteriocins of Gram-Positive Bacteria

Assignment of a specific bacteriocin designation to an inhibitory agent should occur only after isolation, purification and sequencing of the peptide(s) and of the corresponding bacteriocin structural gene(s), and confirmation of the uniqueness of the active bacteriocin molecule(s) by reference to publicly available sequence databases. Over the years, the naming of bacteriocins of Gram-positive bacteria has been haphazard, being based sometimes upon the species and, at other times, upon the genus of the primary producer strain. Although, in theory, the first described example of a bacteriocin should be accorded naming priority, this is not always adhered to in practice. For example, the name "macedocin" was ascribed to a lantibiotic produced by a strain of *Streptococcus macedonicus* (Georgalaki et al. 2002), and this despite it being identical to the previously described SA-FF22 from *Streptococcus pyogenes* (Jack et al. 1994). Whereas for the colicins (with generally only one type produced per strain) the basis for subdivisions was receptor specificity, as defined by using specific colicin-resistant mutants, it has been more problematic to obtain bacteriocin receptor mutants in Gram-positive bacteria. Furthermore, strains producing multiple bacteriocins have created difficulties in several Gram-positive species, and in our experience this is particularly so for *Streptococcus mutans*, *Streptococcus salivariu*s, and *Streptococcus uberis*. Bacteriocins that have only minor conservative differences in the amino acid sequences of their propeptide components, resulting

in no significant change to (1) their secondary structure, (2) their activity spectra, and (3) the cross-specificity of their producer strain immunity, should be referred to as natural variants. For example, nisins Z, Q and U are natural variants of the first described nisin A. Similarly, the subsequently described variants of the *S. salivarius* lantibiotic salivaricin A, all of which exhibit differences in their propeptide sequences, have been named salivaricins A1, A2, A3, A4, and A5. All of the salivaricin A variants exhibit the same (putative) bridge pattern structure, cross-inducibility and cross-immunity characteristics. By contrast, the *S. mutans* bacteriocins successively characterized and named by the Caufield group include both lantibiotics (mutacins I, II, III; Chikindas et al. 1995; Qi et al. 1999, 2000) and a non-lantibiotic bacteriocin (mutacin IV; Qi et al. 2001).

4.1.6 Classification of Bacteriocins of Gram-Positive Bacteria

In 1993, Klaenhammer attempted to put some order into the classification of the bacteriocins of LAB, by proposing four major classes (Klaenhammer 1993):

- Class I – post-translationally modified bacteriocins, i.e., the lantibiotics,
- Class II – small (<10 kDa) heat-stable membrane-active bacteriocins,
- Class III – larger (>30 kDa) heat-labile bacteriocins, and
- Class IV – complex bacteriocins composed of essential lipid or carbohydrate moieties in addition to protein.

Class II was further subdivided into IIa (anti-listerial peptides having the amino acid motif YGNGV/L in the N-terminal part of the peptide), IIb (two-component peptides), and IIc (thiol-activated peptides requiring reduced cysteine residues for activity).

This provisional scheme was adopted by most investigators in the field, although it was repeatedly noted that sustainable evidence was lacking for bacteriocins fulfilling the criteria for group IV. The lantibiotics have been generally divided into linear (type A) and globular (type B) subtypes, though additional subdivisions have also been mooted. The class II bacteriocins have occasionally been carved into additional subgroups either for convenience or because of the personal bias of some investigators. Class III, the small group of large cell wall-active bacteriocins, has typically either been dismissed or largely overlooked by those focused on the membrane-active small peptides. More recently, Kemperman et al. (2003a) recommended recognition of a new group (class V) comprising ribosomally synthesized, non-modified head-to-tail ligated cyclic antibacterial peptides.

As standard practice in this laboratory, we first test LAB for their production of bacteriocins by use of a three-step screening process:

1. deferred antagonism bacteriocin "fingerprinting", using a set of nine standard indicator strains (Tagg and Bannister 1979),
2. repeating the bacteriocin fingerprinting procedure, but incorporating a heating step (80 °C for 45 min) prior to application of the indicator (detector) bacteria, and
3. polymerase chain reaction (PCR)-based detection of lantibiotic processing genes (*lanM*, *lanB* and *lanC*).

This process can sometimes provide preliminary evidence for the production of multiple bacteriocins by the test strain, and also may hint to the possible class of inhibitory molecule(s) being produced. For example, the lantibiotics (class I) typically produce heat-stable inhibition of the *Micrococcus luteus* indicator strain, whereas inhibitory activity due to class III (large) bacteriocins is usually eliminated by the heating step. Our application of these procedures to many different species of LAB has shown that even use of only a single set of nine indicator strains can demonstrate a very high frequency of BLIS detection. *S. mutans*, *S. salivarius* and *S. uberis* exhibit a particularly high incidence of bacteriocinogenicity, with some strains producing combinations of bacteriocins belonging to different classes. Some notable examples include (1) bacteriocin-producing *S. salivarius* that harbor mega-plasmids (typically 160–220 kb), some of which encode no less than five different bacteriocins (Wescombe et al. 2006a), (2) *S. uberis* 42 that produces the lantibiotic nisin U and uberolysin, a circular (cyclic) bacteriocin (R.E. Wirawan et al. submitted), and (3) *S. mutans* UA140 that elaborates a lantibiotic (mutacin I) and a class II inhibitory agent (mutacin IV; Qi et al. 2001).

Cotter et al. (2005b) have recently proposed a more radical modification to the Klaenhammer classification scheme for LAB bacteriocins, in which there are essentially only two principal categories: lantibiotics (class I) and non-lanthionine-containing bacteriocins (class II). The former class III (large heat-labile murein hydrolases) are renamed bacteriolysins, and class IV (the lipid- or carbohydrate-containing bacteriocins) is withdrawn. It was further suggested that the Klaenhammer class II subgroups IIa (listeria-active peptides) and IIb (two-peptide bacteriocins) be retained, and that the class V (cyclic peptides) proposed by Kemperman et al. (2003a) be reassigned as class IIc. Class IId was proposed to be a repository for all of the remaining linear non-lanthionine-containing bacteriocins. It should be noted that Cotter et al. commented only upon those class III members that possess a lytic mode of action (e.g., lysostaphin), despite reports of non-lytic large heat-labile bacteriocins such as helveticin J (Joerger and Klaenhammer 1986) and streptococcin G-2580 (Tagg and Wong 1983). Although we concur with some elements of the revised classification scheme of Cotter et al. (2005b) for LAB bacteriocins, some of our own experiences with these molecules lead us to propose some further modifications for consideration (outlined in Fig. 4.1), which can generally be applicable to all bacteriocins from Gram-positive sources:

– In agreement with Cotter et al.:
  1. Klaenhammer's class IV (chemically complex bacteriocins) is eliminated, and
  2. Class IIc (thiol-activated peptides) is eliminated.
– In contrast to Cotter et al.:
  1. Class III (large bacteriocins) is retained, and is now subdivided into IIIa (bacteriolysins) and IIIb (non-lytic proteins),
  2. the cyclic bacteriocins (class IIc) now constitute a newly defined class IV, and
  3. as a consequence of upgrading the cyclic bacteriocins to their own class, type IIc now becomes the repository for all unmodified class II inhibitors other than the listeria-active (type IIa) and multi-component bacteriocins (type IIb).

Clearly, research in the field of LAB bacteriocins is still progressing exponentially, and it is not easy to formulate an enduring natural classification scheme that encompasses all of the existing bacteriocin-like proteins (the evolutionary origins of which appear quite independent) as well as potentially accommodating as yet unimagined novel members.

In this chapter, we attempt to provide the reader with an overview of some of the diversity of bacteriocins elaborated by Gram-positive bacteria, admittedly heavily biasing our attention toward the LAB, where much contemporary research in this field has been conducted. Wherever possible, the most recent developments within an existing class of bacteriocins, and information relating to previously undescribed classes of inhibitors will be given greatest emphasis.

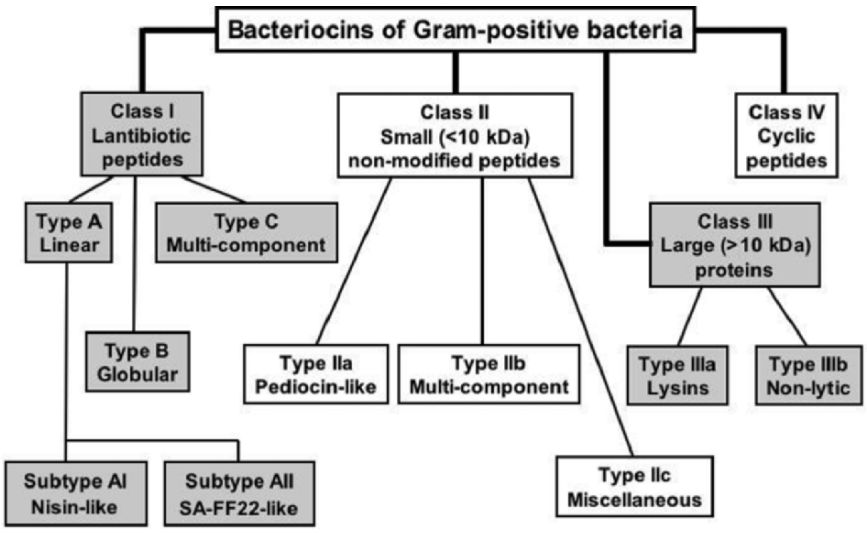

Fig. 4.1 Our proposed classification schema, based on that of Cotter et al. (2005b), but modified so as to be applicable to most, if not all, known bacteriocins of Gram-positive bacteria

4.2 Class I: The Lanthionine-Containing (Lantibiotic) Bacteriocins

The term lantibiotic (Schnell et al. 1988; Jung 1991) refers to the diverse array of bacterial antibiotic peptides that contain the non-genetically encoded amino acids lanthionine (Lan) and/or 3-methyllanthionine (MeLan), as well as various other highly modified amino acids, commonly including the 2,3-unsaturated amino acids dehydroalanine (Dha) and dehydrobutyrine (Dhb). All of the lantibiotics currently described are thought to be produced as ribosomally synthesized precursor peptides, which then undergo a series of post-translational modification reactions to produce the unusual amino acids described above as intrinsic components of the biologically active peptides. To date, lantibiotics have been found to be produced only by Gram-positive bacteria. Furthermore, they are generally considered to predominantly, if not exclusively, act on Gram-positive targets. As the family of lantibiotic molecules grew, the individual members were initially classified according to the topology of their ring structures and their biological activities (Jung and Sahl 1991), as either type A (elongated amphipathic structures) or type B (globular and more compact structures). In order to encompass the more recently described two-component varieties, the type C lantibiotics has been proposed. The type A lantibiotics are further divided into subtypes AI and AII based on the size, charge and sequence of their leader peptides (de Vos et al. 1995). It must be noted, however, that the lantibiotics are a difficult group to subdivide, and indeed it has been proposed that on the basis of similarities in their unmodified propeptide sequences, they could be split into 11 groups (Cotter et al. 2005a). The lantibiotics have been reviewed extensively over the last decade, and the reader is referred to some of these accounts for a more complete overview (McAuliffe et al. 2001b; Chatterjee et al. 2005). We propose to focus only on a selection of lantibiotics that we consider illustrate some of the significant diversity of these molecules, and to update the reader on recent developments in the field not covered by other reviews (cf. Table 4.1).

4.2.1 Type AI Lantibiotics

The prototype type AI lantibiotic, nisin, is perhaps the most extensively characterized of all bacteriocins. Produced by *Lactococcus lactis*, nisin has a long history of research, its discovery in 1928 (Rogers 1928) predating that of penicillin. Nisin has now been used safely in the food industry as a preservative for over 40 years without the appearance of significant bacterial resistance. Since the nisin biosynthetic pathway, requiring the coordinated expression and action of at least 11 gene products, is generally mirrored by most other lantibiotics, we will give a brief description of the processes involved. The precursor peptide, encoded by *nisA*, is acted upon by the proteins NisB and

Table 4.1 A selection of class I (lantibiotic) bacteriocins

Bacteriocin	Producer species	Distinctive characteristics	Recommended reference(s)
Type A			
Subtype AI			
Nisin A	*Lactococcus lactis*	Prototype AI lantibiotic, widely used as a food preservative for over 40 years; the best-characterized of the bacteriocins of Gram-positive bacteria; induces its own expression through binding to a two-component signal transduction system	Mattick and Hirsch (1947), Gross et al. (1971), McAuliffe et al. (2001b)
Nisin U	*Streptococcus uberis*	First nisin variant to be characterized from a non-lactococcal host bacterium	Wirawan et al. (2006)
Streptin	*Streptococcus pyogenes*	Utilizes a general bacterial protease (SpeB), rather than a dedicated protease (LanP) to remove the leader sequence from the prepeptide	Wescombe and Tagg (2003)
Subtype AII			
SA-FF22	*S. pyogenes*	First subtype AII lantibiotic to be characterized; has a two-component sensor-kinase histidine-response regulator system for which the inducing molecule (most likely a peptide) is not SA-FF22 itself	Jack et al. (1994)
Lacticin 481	*L. lactis*	Considered by some to be the prototype of this subtype; in contrast to SA-FF22, the lacticin 481 locus does not encode a two-component sensor-kinase histidine-response regulator	van den Hooven et al. (1996)
Salivaricin A	*Streptococcus salivarius*	Multiple SalA variants have been characterized from different species of streptococci; all tested M serotypes of *S. pyogenes* (other than M11 and M37) have the SalA structural gene variant *salA1*; each SalA variant has been shown to effect both auto- and heterologous induction of SalA production in their respective host strains	Ross et al. (1993), Wescombe et al. (2006b)

Sublancin 168	*Bacillus subtilis*	The only lantibiotic shown to have a disulfide bond in addition to the typical MeLan and Dha residues	Paik et al. (1998)
Type B			
Mersacidin	*Bacillus* spp.	Prototype type B lantibiotic, and the only lantibiotic of which the structure has been resolved by X-ray crystallography; effective in treating systemic staphylococcal (*St. aureus*) infections and nasal carriage in a mouse model system	Chatterjee et al. (1992)
Cinnamycin	*Streptomyces cinnamoneus*	The only characterized lantibiotic thought to be exported by the general secretory (Sec) pathway; has two MeLan rings in which the nucleophilic Cys is positioned N-terminally to the Dhb; may have use as an anti-inflammatory and anti-allergy drug	Kessler et al. (1987)
Type C			
Lacticin 3147	*L. lactis*	The prototype two-component lantibiotic; structures and functions of both peptide components have been defined; killing of sensitive cells requires initial binding of LtnA1 to lipid II, followed by complexing with LtnA2 to effect pore formation	Martin et al. (2004)
Smb	*Streptococcus mutans*	Regulated in response to the external levels of CSP; a variant (BHT-A) has been identified in *S. rattus* that appears to be closely associated with the locus for the class II bacteriocin BHT-B	Yonezawa and Kuramitsu (2005), Hyink et al. (2005)
Cytolysin	*Enterococcus faecalis*	A lantibiotic that exhibits toxicity for both bacterial and eukaryotic cells; cytolysin subunits are activated by a two-step process involving transport and initial GG specific cleavage by CylB (an ABC transporter), following which the cytolysin subunits are activated via the removal of an additional six amino acids by CylA (a serine protease)	Booth et al. (1996), Coburn and Gilmore (2003), Coburn et al. (2004)

NisC to dehydrate particular Ser/Thr residues, some of which are then used to form specific thioether bonds (i.e., Lan and MeLan) with Cys residues located (generally) further toward the C-terminus of the molecule. NisT is an ABC transporter responsible for export of the modified prepeptide, and NisP is a membrane-anchored protease able to cleave the leader peptide to release active nisin. NisI is involved in nisin immunity by an as yet ill-defined mechanism. Although the majority of NisI appears to be localized within the cytoplasmic membrane of the producer cell (Qiao et al. 1995), a significant amount is also secreted into the cytoplasm where it may bind to external nisin before it can aggregate at the cell surface (Koponen et al. 2004). Expression of *nisI* in nisin-sensitive *L. lactis* strains results in moderately decreased sensitivity to nisin (Qiao et al. 1995). However, full immunity levels are not achieved without the presence of NisFEG. NisFEG is an ABC transport protein complex, presumably contributing to nisin immunity in a manner similar to that used by multi-drug transporters, by reducing the concentration of nisin in direct contact with the cytoplasmic membrane. NisR and NisK together form a two-component sensor-kinase/response-regulator element involved in the regulation of nisin biosynthesis, which characteristically occurs late in the exponential phase of growth. Interestingly, since nisin itself is the specific ligand recognized by the sensor NisK, it up-regulates its own expression (Kuipers et al. 1995). The basic elements of the nisin biosynthetic pathway are conserved for all lantibiotics, with only minor variations such as the use of the LanM modification enzyme, rather than of the LanB/C complex for dehydratase and ring formation reactions and the encoding of LanD enzymes by a minority of lantibiotic loci to effect formation of the unusual amino acids S-[(Z)-2-aminovinyl]-D-cysteine (AviCys) and S-[(Z)-2-aminovinyl]-(3S)-3-methyl-D-cysteine (AviMeCys). For some lantibiotics, specific immunity appears attributable either only to LanI (e.g., Pep5; Reis et al. 1994) or only to the LanFEG system (e.g., lacticin 481; Rince et al. 1997). On the other hand, the epidermin and gallidermin gene clusters encode an additional accessory factor LanH, which enhances LanFEG-mediated immunity (Hille et al. 2001).

In addition to its widespread use as a food preservative, nisin and other members of the lantibiotic class have been investigated for their potential applications in medicine. The MICs of mutacin B-Ny266 and nisin A were shown to be comparable to those of vancomycin and oxacillin against various bacterial pathogens (Mota-Meira et al. 2000). Both lantibiotics were active against vancomycin- and oxacillin-resistant strains of *Helicobacter pylori* and *Neisseria* spp., making them potential candidates for treatment of infections caused by these bacteria (Hancock 1997; Mota-Meira et al. 2000). A novel potential application of nisin is as a spermicidal contraceptive. Studies with rabbits indicated that vaginal administration of 1 mg of nisin stopped sperm motility completely, none of the treated animals having become pregnant (Reddy et al. 2004). Complete histopathologic evaluation of the vagina indicated no adverse effects resulting from the intravaginal application of nisin,

in terms of either tissue damage or subsequent reproductive performance (Aranha et al. 2004; Reddy et al. 2004). A future direction for lantibiotic application may involve the rational design of new peptides based on desirable structural features of some well-characterized biologically active peptides such as nisin. An analysis of 37 known lantibiotics indicated that although there were no hard and fast rules, Ser/Thr residues were more likely to be dehydrated when flanked by hydrophobic amino acids than by hydrophilic residues. To test the predicted dehydration sequence rules, hexapeptide-encoding sequences were fused to the nisin leader peptide, and expressed in a *L. lactis* strain containing the nisin modification and export enzymes. Analysis of the composition of the hexapeptide products confirmed the designers' predictions, demonstrating the feasibility of rational design of novel peptides having specific dehydrated amino acid residues (Rink et al. 2005).

As ever more lantibiotics are being detected, it has become increasingly obvious that a continuum of natural variants exists, some exhibiting only a single amino acid residue difference from previously documented lantibiotics, but others having multiple sequence variations. A variant of nisin produced by *Streptococcus uberis* strain 42 has recently been identified (Wirawan et al. 2006), the first of the nisin family not produced by a *Lactococcus* strain. The biologically active 31-amino acid (aa) nisin U differs from the 34-aa nisin A in 12 of its amino acids (82% similarity of the propeptides; Fig. 4.2a). Nisin U is predicted to share the same bridging pattern as nisin A, and the producer strains of nisin A and nisin U are cross-immune. This apparent cross-immunity to the two nisin peptides is particularly interesting, since the putative immunity peptide for nisin U, NsuI, shares only 55% homology with NisI. By contrast, there is no indication of cross-immunity between subtilin (from *Bacillus subtilis*) and nisin, despite them having 60% propeptide sequence similarity (McAuliffe et al. 2001b). In addition, the antimicrobial spectrum of nisin U appears to match closely that of nisin A, although there appears to be some relative reduction in the activity of nisin U against *S. pyogenes* and *L. lactis* strains. Significantly, nisin U and nisin A exhibited both auto-inducing and cross-inducing activity when added to cultures of the respective nisin-producing *Lactococcus* and *Streptococcus* strains, further emphasizing the close functional identity of the two peptides and justifying the classification of the *S. uberis* lantibiotic as a nisin variant. The nisin U genetic locus comprises 11 open reading frames, closely similar to their nisin A counterparts, but with *nsuPRKFEG* located upstream of *nsuA* rather than downstream of *nsuI*, as in the nisin A locus (Fig. 4.2b). The nisin U locus is flanked by transposon-related sequences, and also has a 742-bp region between *nsuG* and *nsuA* encoding remnants of a transposase (Fig. 4.2b), indicating that a rearrangement of the locus has occurred. Streptin is a 23-aa type AI lantibiotic produced by *S. pyogenes* strain M25 exhibiting similarity in its first two ring structures with the corresponding region in nisin (Karaya et al. 2001; Wescombe and Tagg 2003). The streptin locus appears similar to that of subtilin, in that it does not encode a specific protease (LanP) for propeptide

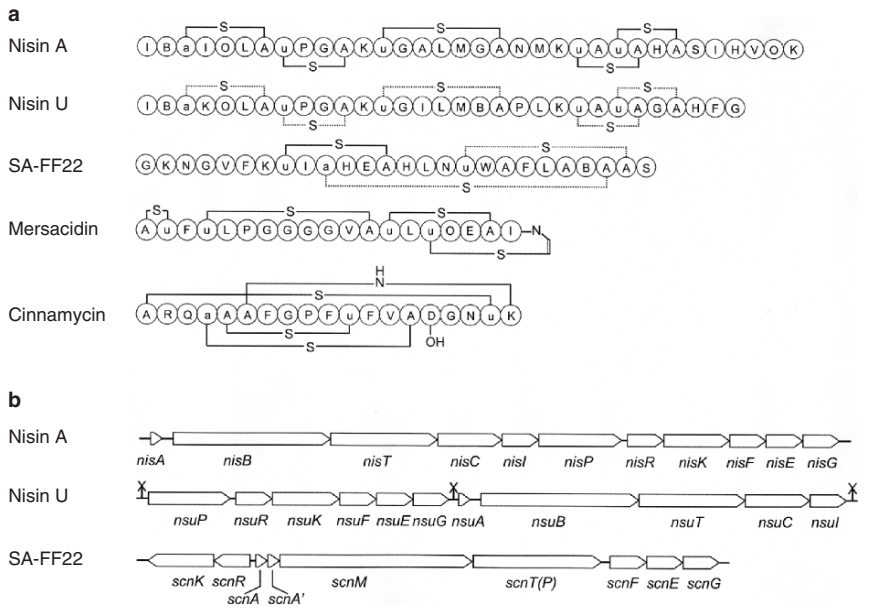

Fig. 4.2 **a** The primary structures of the type A lantibiotics nisin, nisin U and SAFF-22, and the representative type B lantibiotics mersacidin and cinnamycin. Modified amino acid abbreviations: a, D-alanine; B, 2,3-dihydrobutyrine; O, 2,3-dihydroalanine; u, D-α-aminobutyric acid; a-S-A, lanthionine (Lan); u-S-A and A-S-u, methyllanthionine (MeLan); A-NH-K, lysinoalanine; D-OH, *erythro*-3-hydroxyaspartic acid. All other conventional amino acids are given in one-letter code. The *solid lines* represent the Lan and MeLan bridges that have been confirmed experimentally, whereas those with *dotted lines* are predicted. **b** Organization of the biosynthetic loci of nisin A, nisin U and SA-FF22. Note the different order of the *lanPRKFEG* genes between the nisin A and nisin U loci. The *X symbols* in the nisin U locus represent remnants of mobile genetic elements (see text)

activation (Stein and Entian 2002). Rather, it appears that the producers of subtilin and streptin utilize other host cell proteases to remove the lantibiotic leader sequences, probably following prepeptide export. In the case of streptin, the *S. pyogenes* cysteine proteinase, SpeB, has been implicated in the prepeptide cleavage reaction, since proteinase-negative mutants of strain M25 concomitantly lose the ability to express the streptin phenotype (Hynes and Tagg 1986; S. O'Brien and J.R. Tagg, unpublished data). The utilization of host cell proteases for the processing of lantibiotics could be viewed as an efficient way to reduce the metabolic burden of lantibiotic production, although it may limit the dissemination of the locus to other species.

4.2.2 Type AII Lantibiotics

The type AII lantibiotics differ from those in type AI in that their thioether ring formation is effected by bifunctional LanM enzymes, rather than by the

combined action of LanB and LanC, and also because they generally have the conserved consensus sequences E(L/V)S and E(L/M) in their leader peptides. Furthermore, their leader sequences resemble more closely those of class II bacteriocins, in that they contain a "double-glycine" (GG/GA/GS) motif immediately preceding the cleavage site (McAuliffe et al. 2001a; Chatterjee et al. 2005). This group of lantibiotics also includes a most unusual member, sublancin 168 produced by a *B. subtilis* strain, which appears to be the first bacteriocin to contain both lanthionine ring structures and stabilizing disulfide bonds (Paik et al. 1998).

Although lacticin 481 is largely touted as the prototype of this subclass, the first of this group to be characterized was actually the 26-aa lantibiotic streptococcin A-FF22 (SA-FF22) produced by *S. pyogenes* strain FF22 (Jack and Tagg 1991, 1992). Lacticin 481, now extensively characterized, is a 27-aa lantibiotic containing two Lan, one MeLan and one Dhb residue (Piard et al. 1992). Interestingly, the lacticin 481 genetic locus, unlike that of most type AI lantibiotics, appears not to encode a two-component sensor-kinase response-regulator system, rather being regulated at the transcriptional level by pH control of P1 and P3 promoters located upstream of the structural gene *lctA* (Hindre et al. 2004). By contrast, the locus encoding SA-FF22 in *S. pyogenes* does have a two-component sensor-kinase response-regulator system, but this responds not to the inhibitory lantibiotic SA-FF22 but to another putative signal molecule (P.A. Wescombe, unpublished data). In fact, the molecular mechanisms of lantibiotic regulation are strikingly diverse, with examples of

1. negative regulation of members of the type AII (e.g., lactocin S; Rawlinson et al. 2002) and two-component lantibiotics (e.g., lacticin 3147, McAuliffe et al. 2001a; cytolysin, Haas et al. 2002),
2. no genes encoding regulatory elements within the locus (e.g., lacticin 481; Hindre et al. 2004),
3. homologous (auto) regulation (e.g., nisin, Kuipers et al. 1995; salivaricin A, Upton et al. 2001),
4. heterologous regulation using a signal peptide differing from the induced lantibiotic (e.g., SA-FF22; P.A. Wescombe, unpublished data), and
5. regulation by two peptides, each influencing expression of different genes within the locus (for example, the mersacidin locus encodes the response regulators MrsR1 and MrsR2, where MrsR1 regulates immunity gene expression and MrsR2 regulates lantibiotic biosynthesis; Guder et al. 2002).

In our laboratory, we have conducted extensive research on salivaricin A (SalA), a 22-aa type AII lantibiotic produced by *S. salivarius* (Ross et al. 1993). Five closely related variants of SalA have recently been described (Wescombe et al. 2006b), each shown to effect both auto- and heterologous induction of SalA production in the respective host strains. The novelty of SalA lies in the wide distribution of the SalA (and variant) locus in *Streptococcus* species, having now been detected in *S. salivarius*, *S. pyogenes*,

Streptococcus dygalactiae and *Streptococcus agalactiae*. Oddly, the structural gene *salA1* was detected in *S. pyogenes* of 65 different M serotypes (Simpson et al. 1995). Only two strains (of serotypes 11 and 37) did not harbor *salA1*. At first glance, this appears to be anomalous, since the majority of *S. pyogenes* are inhibited by SalA when tested in vitro (Ross et al. 1993). However, it has now been demonstrated that, other than in serotype M4T4 *S. pyogenes*, all SalA1 loci are non-functional, due (at least in part) to either deletions in the genes encoding SalM and SalT, or frameshift mutations in the *salT* gene (Wescombe et al. 2006b). It is tempting to speculate that this very common retention, especially of the immunity-associated components of the SalA locus in *S. pyogenes*, may be ecologically driven, as both *S. pyogenes* and *S. salivarius* are inhabitants of the human oral cavity. The *S. pyogenes* serotype M11 and M37 prototype strains are unusual in that they do not possess the SalA immunity genes (P.A. Wescombe et al., unpublished data). The M11 strain is an A-variant *S. pyogenes*, thought to have lost the ability to assemble intact group A carbohydrate during the course of prolonged serial subculture in vitro (D. Johnson, personal communication). The lack of an obvious selective advantage associated with SalA immunity for *S. pyogenes* strains grown for prolonged periods as laboratory monocultures could favor the loss of immunity-related components of the locus. The M37 prototype strain is also very unusual, in that no other examples of strains of this serotype appear to have been isolated (D. Johnson, personal communication). Both of these observations are consistent with a survival advantage for *S. pyogenes* in situ being linked to their retention of at least the immunity-related components of the *salA* locus. Hence, in this case, the acquisition and retention of lantibiotic genetic elements may have contributed to the adaptation and survival of a bacterial species.

It has recently been observed that the SalA locus is borne on large (>150 kb) plasmids in *S. salivarius*, whereas in *S. pyogenes* the locus appears typically to be chromosomally located. The ubiquitous presence of *salA* in *S. pyogenes* indicates that the acquisition of this locus was an early event in the establishment of the species, or at least that only strains of *S. pyogenes* that are capable of expressing SalA immunity have maintained associations with the human host. The large plasmids in bacteriocin-producing *S. salivarius* have been found to harbor loci for various combinations of streptococcal lantibiotics including salivaricin A, salivaricin B, streptin, and a variant of SA-FF22. The lantibiotic loci appear to be juxtaposed in contiguous segments, separated by no more than ca. 4 kb of non-lantibiotic-related DNA. Moreover, genes encoding Tra-like proteins (potentially involved in conjugative transfer of the plasmids) have also been identified. These observations support the hypothesis that cassettes of lantibiotic loci could be disseminated together, thereby rapidly expanding the antimicrobial arsenal of the recipient strain. Indeed, in vivo transfer of the entire 180 kb of *S. salivarius* K12 bacteriocin-associated plasmid to indigenous *S. salivarius* has been demonstrated to occur in the oral cavity of subjects colonized with the probiotic

S. salivarius K12 (Wescombe et al. 2006a). The wide distribution of closely related lantibiotic loci throughout different oral streptococcal species indicates a high frequency of horizontal gene transfer. In the case of *S. salivarius*, the large plasmids appear to have been particularly effective at acquiring additional bacteriocin loci, and our preliminary findings indicate that most BLIS-producing *S. salivarius* strains have plasmids of size >40 kb.

4.2.3 Type B (Globular) Lantibiotics

The type B lantibiotics are more globular and compact in shape than those of type A, and generally are either uncharged or negatively charged at neutral pH. Mersacidin, the prototype for this group, is a 20-aa peptide (mass 1,825 Da) and its distinctive features include three MeLan rings, one Dha and a S-[(Z)-2-aminovinyl]-(3S)-3-methyl-D-cysteine (AviMeCys) residue (Chatterjee et al. 1992). Mersacidin, which derives its name from its potent activity against methicillin-resistant *St. aureus* (MRSA, the hospital-acquired "superbug"), is also the only lantibiotic of which the structure has been resolved by X-ray crystallography (Schneider et al. 2000). Mersacidin does not form pores in bacterial membranes, but rather inhibits peptidoglycan synthesis through a specific interaction with the peptidoglycan precursor lipid II (Brotz et al. 1997). The sequestering of lipid II prevents its utilization by the transpeptidase and transglycosylase enzymes that install the crosslinked network of the bacterial cell wall. Both nisin and mersacidin appear to bind to a different portion of lipid II than does vancomycin (the antimicrobial of last resort for the treatment of multiply antibiotic-resistant *St. aureus*), indicating that these molecules may prove to have important chemotherapeutic applications (Brotz et al. 1995; Breukink et al. 1999). Indeed, mersacidin has been shown to be very effective for the treatment of systemic staphylococcal infections, and in eliminating nasal carriage of *St. aureus* in a mouse model system (Chatterjee et al. 1992; Kruszewska et al. 2004). Lipid II, however, does not serve as a docking molecule for all lantibiotics, since Pep5 and epilancin K7 have been shown specifically not to bind lipid II. These molecules presumably have an alternative docking molecule or receptor, since they have greater activity than other pore-forming molecules against certain indicator bacteria (Brotz et al. 1998; Pag et al. 1999).

Cinnamycin is a 19-aa type B lantibiotic produced by *Streptomyces cinnamoneus*, and has also been purified as Ro 09-0918 (Kessler et al. 1987) and lanthiopeptin (Naruse et al. 1989; Palmer et al. 1989). Its structure is novel in that it has two MeLan residues in which the nucleophilic cysteine is positioned N-terminally to the Dhb. Although also found in some other type B lantibiotics, this direction of ring formation has so far not been observed in any of the type A lantibiotics other than the LtnA1 peptide of the lacticin 3147 two-component lantibiotic system. Another unusual feature of cinnamycin is a head-to-tail lysinoalanine bridge, the formation of which has so far not

been ascribed to any particular gene product in the comprehensive array of putative ORFs identified in the cinnamycin locus. Intriguingly, the CinA prepeptide has a much longer leader peptide than that of other lantibiotics, and it has been proposed that cinnamycin may be secreted by a more general export mechanism such as the general secretory (Sec) pathway, once again illustrating the broad diversity of the lantibiotic class (Widdick et al. 2003). Cinnamycin has been shown to be a potent inhibitor of phospholipase A2 (an enzyme involved in the synthesis of prostaglandins and leukotrienes in the human immune system) through the sequestration of its substrate phosphatidylethanolamine. Due to this activity, cinnamycin may prove to have a useful application as an anti-inflammatory and anti-allergy drug (Marki et al. 1991).

4.2.4 Type C (Multi-Component) Lantibiotics

Each of the multi-component lantibiotic consortia described to date comprise two post-translationally modified peptides that individually have little or no activity, but display strong synergistic antibacterial action. Lacticin 3147 produced by *Lactococcus lactis* DPC3147 is arguably the most intensively studied member of this group, and both component peptides (LtnA1 and LtnA2) have been structurally characterized (Ryan et al. 1999). Features of the peptides include Lan and MeLan residues, and also a D-Ala residue derived from L-Ser by post-translational modification. Interestingly, the structure of LtnA1 bears some resemblance to that of the type B lantibiotic mersacidin, and the LtnA2 peptide displays some similarity to lactocin S (a type AII lantibiotic). The obvious structural differences between LtnA1 and LtnA2 therefore require that the genetic locus for lacticin 3147 encode two LanM enzymes, each of which is presumably responsible for the post-translational modification of one of the peptides (McAuliffe et al. 2000). The mechanism of action of lacticin 3147 has recently been shown to result from the sequential action of the two peptides, on condition that LtnA1 be added prior to LtnA2 (Morgan et al. 2005). It was therefore inferred that LtnA1 binds lipid II (a reaction responsible for the independent inhibitory activity displayed by LtnA1), following which LtnA2 interacts with the LtnA1–lipid II complex to bring about more effective insertion into the target membrane and pore formation, with an associated 30-fold increase in inhibitory activity compared to that obtained by LtnA1 alone (Morgan et al. 2005).

Smb (*Streptococcus mutans* bacteriocin) was recently shown to be a two-component lantibiotic (Yonezawa and Kuramitsu 2005). Expression of SmbA and SmbB by the *smb* locus appears to be regulated in response to the external levels of a competence-stimulating peptide (the peptide that activates the development of competence for genetic transformation; see below). It is possible that the production of the lantibiotic has been coupled to the competence cascade to ensure that there is an abundance of exogenous DNA available for uptake by the newly competent bacteria. Alternatively, it may be

that the apparent co-regulation is purely a consequence of the insertion of the lantibiotic locus into a region having the competence promoter upstream. A variant of the Smb lantibiotic, named BHT-A, was recently identified in *Streptococcus rattus* strain BHT, and shown to be composed of the two peptides BHT-Aα and BHT-Aβ (Hyink et al. 2005). Interestingly, the Smb/BHT-A locus appears to be closely linked to the locus for BHT-B, a class II bacteriocin, in all *S. rattus* strains examined to date (Hyink et al. 2005).

Cytolysin, produced by *Enterococcus faecalis*, consists of two lantibiotic subunits ($CylL_L$ and $CylL_S$), and is the only lantibiotic confirmed to exhibit toxicity for both bacterial and eukaryotic cells. Although not all strains of *E. faecalis* are hemolytic, the occurrence of hemolysis is higher among clinical isolates, especially those from the bloodstream (Booth et al. 1996). As many as 60% of infection-associated *E. faecalis* elaborate cytolysin, and it has been shown to lower the LD_{50} of *E. faecalis* for mice and to contribute to toxicity in experimental endocarditis and endophthalmitis models. In addition, cytolysin-positive strains are associated with a fivefold increased risk of acute terminal outcome in patients with nosocomial enterococcal bacteremia (Coburn et al. 1999). Interestingly, cytolysin is encoded on large pheromone-responsive conjugative plasmids, which may, at least in part, account for the high prevalence of the cytolysin locus in enterococci.

The $CylL_L$ and $CylL_S$ subunits are activated by a two-step process involving initial transport and GG site-specific cleavage (to $CylL_L'$ and $CylL_S'$) mediated by the ABC transporter CylB, followed by removal of a further six amino acids (forming $CylL_L''$ and $CylL_S''$) by the serine protease CylA. It was shown that in order for the cytolysin to efficiently lyse erythrocytes and bacterial cells, both subunits need to be fully processed by CylA (Booth et al. 1996).

Expression of the cytolysin locus is directly regulated by the synergistic action of two repressor proteins CylR1 and CylR2, both of which lack homologues of known function (Haas et al. 2002). De-repression occurs at a specific cell density when one of the cytolysin subunits ($CylL_S''$) reaches an extracellular threshold concentration. These observations form the basis for a model of cytolysin auto-induction by a quorum-sensing mechanism involving a novel two-component regulatory system (Haas et al. 2002). $CylL_L$ and $CylL_S$ expression is further regulated in response to aerobiosis, with transcription being up-regulated under anaerobic conditions (Day et al. 2003).

Comparison of the cytolysin determinants with those of the type AII lantibiotic lactocin S (from *Lactobacillus sake*) indicates they may share a common ancestry (Gilmore et al. 1996). Although no cytotoxicity for eukaryotic cells has been reported for lactocin S, it seems prudent to perform toxicity tests on any lantibiotics (particularly two-component forms) that may have human or veterinary applications to assess their potential for disruption of eukaryotic membranes. Indeed, this revelation of the dual toxicity of cytolysin sounds a timely warning for those contemplating the engineering of novel lantibiotics, since it demonstrates the potential for these molecules to exhibit toxicity for eukaryotic cells, perhaps sometimes by forming

multi-component membrane poration complexes in combination with heterologous bacteriocins produced by indigenous bacteria (Wescombe et al. 2005).

4.3 Class II: The Unmodified Peptide Bacteriocins

Class II essentially encompasses all of the currently described, small (<10 kDa) unmodified (i.e., non-lantibiotic and non-cyclic) peptide bacteriocins of Gram-positive bacteria (Eijsink et al. 2002). As a result, this class comprises over 50 members with diverse origins, ranging from genera inhabiting the oral cavity and gastrointestinal tract (of humans and other animals) to species best known for their involvement in the dairy and food industries. As with the lantibiotics, class II includes inhibitors either functioning as single peptides or requiring the coordinated activity of two or more component peptides. Furthermore, some bacteriocin-like peptides that conform to the class II definition do not appear to possess intrinsic activity of their own, but function to activate bacteriocin biogenesis (Eijsink et al. 2002).

4.3.1 Type IIa: The Pediocin-like Peptides

The largest single collection of class II bacteriocins, at present consisting of over 20 members, is type IIa, the so-called pediocin-like bacteriocins that are epitomized by their particular effectiveness in killing the food-borne pathogen *Listeria monocytogenes* (for recent and comprehensive reviews, refer to Rodriguez et al. 2002 and Fimland et al. 2005). It is this characteristic that has provided the impetus for research of this family of molecules, due to their potential applications as food biopreservatives (Rodriguez et al. 2002). Despite the moniker of this bacteriocin family, it was actually leucocin A from *Leuconostic gelidum* that was the first member to be described (Hastings et al. 1991). Nevertheless, pediocin PA-1, a 44-aa peptide produced by *Pediococcus acidilactici*, is the best-characterized member of this family and therefore justifiably the prototype of this group (Fimland et al. 2005). Moreover, pediocin PA-1 is the only type IIa bacteriocin to be used commercially, i.e., as the active ingredient in Alta™*2341*, an anti-*Listeria* food preservation product (Rodriguez et al. 2002).

Aside from their characteristic anti-listerial activity, the pediocin-like peptides (which vary in length from 37 to 58 residues) are typified by (1) the highly conserved amino acid motif Tyr–Gly–Asn–Gly–Val/Leu (also known as the YGNGV motif or "pediocin box") near their N-termini, and (2) the presence of two cysteine residues forming a disulfide bond. With few exceptions, the members of this group have a similar spectrum of bacterial inhibition, although the MIC values toward the target organisms vary considerably.

In general, the peptides possessing four cysteine residues (forming two disulfide bridges) have a much broader inhibitory spectrum than those with only one disulfide bond (Eijsink et al. 1998). While the conserved YGNGV motif has historically been considered to be the *Listeria*-active part of type IIa bacteriocins, exceptions have been noted, namely acidocin A from *Lactobacillus acidophilus*, which is still anti-listerial despite possessing a slightly altered pediocin box (YGTNGV; Kanatani et al. 1995). In some cases, substitutions within the YGNGV motif strongly reduce the activity of the molecules not only against *Listeria* but also toward other lactic acid bacteria (Miller et al. 1998). Similarly, experiments involving hybrid molecules of four pediocin-like peptides have demonstrated that the C-terminal module of each peptide plays an important role in determining the inhibitory spectrum (Fimland et al. 1996). Therefore, while the pediocin box appears to correlate with anti-*Listeria* activity, the specifics of the biological activity of each type IIa peptide are probably determined by the collective contributions of its amino acid constituents.

The bacteriocidal mode of action of pediocin PA-1 appears to involve three basic steps: (1) binding to the cytoplasmic membrane, (2) insertion of the bacteriocin molecules into the membrane, and (3) formation of the poration complex that permeabilizes the membrane, thereby disrupting the proton motive force and leading to cell death (Rodriguez et al. 2002). Through analyses of bacteriocin-resistant *Lis. monocytogenes* strains, it appears that the receptor for type IIa bacteriocins (including pediocin PA-1) is a mannose-specific phosphotransferase (PTS) system (Ramnath et al. 2000; Gravesen et al. 2002; Vadyvaloo et al. 2004). Although the molecular mechanism of how a pediocin-like peptide interacts with its putative receptor remains to be elucidated, it is possible that the PTS complex acts as a docking molecule that stabilizes the pediocin-mediated pore, not unlike the role of lipid II in the mode of action of nisin.

The genetic determinants for pediocin PA-1 production have been determined to be plasmid-borne in all producing strains examined to date. The pediocin (*ped*) locus consists of four genes *pedA*, *pedB*, *pedC* and *pedD* transcribed as a single polycistronic unit, with *pedA* and *pedB* encoding the pediocin PA-1 prepeptide and its immunity protein, respectively (Venema et al. 1995; Rodriguez et al. 2002). Interestingly, the pediocin PA-1 prepeptide is approximately 80% as active as the mature bacteriocin (Venema et al. 1995). The first 18 amino acids of PedA, the 62-aa pediocin PA-1 precursor, constitute the signal peptide that is cleaved off at the C-terminal side of two glycine residues (the so-called double-glycine or GG motif) during export (Rodriguez et al. 2002; Fimland et al. 2005). This event is carried out by the ATP-binding cassette (ABC) transporter complex (also known as a type I secretion system) composed of the proteins encoded by *pedC* and *pedD*. PedD (724 aa), the actual ABC transporter, is composed of three domains: (1) an N-terminal peptidase domain, which presumably cleaves the prepeptide at the GG motif, (2) a central cell membrane-spanning domain, and (3) a C-terminal

ATP-hydrolyzing domain (Fimland et al. 2005). The role of PedC, the so-called "ABC transporter accessory protein", is not entirely clear, but it may function to aid the passage of the bacteriocin precursor through PedD (Rodriguez et al. 2002).

The core class II bacteriocin-associated genetic locus, which can be found in most unmodified Gram-positive bacteriocin systems irrespective of origin, is essentially composed of the genes encoding

- the bacteriocin precursor peptide (including the GG motif),
- the immunity-associated protein, and
- components of the ABC transporter complex.

Naturally, variations on this theme do exist, such as (1) the organization of the genes, e.g., the ABC transporter gene located upstream of its accessory factor, (2) the absence of a genetic determinant encoding an accessory factor, or (3) the disparate locations of the genes encoding the ABC transporter and the GG-motif-containing peptides. It is noteworthy that the prepeptide of colicin V, an 8-kDa *E. coli* microcin, possesses a GG motif and its biosynthetic locus clearly conforms to the definition of a "core class II locus" (Håvarstein et al. 1994).

An example of the diversity within type IIa is exemplified by the biosynthesis of certain members of the pediocin family such as listeriocin 743A, bacteriocin 31 and enterocin P. These bacteriocins have been shown to be exported via the general secretory (Sec-dependent) pathway, and hence possess Sec-type signal peptides (Cintas et al. 1997; Kalmokoff et al. 2001; Fimland et al. 2005). Such a mechanism of export is potentially beneficial to the host due to its apparent metabolic and genetic economy, i.e., eliminating the need to commit resources to the synthesis of two sets of transport systems.

While most type IIa molecules have been isolated from bacteria usually associated with food products, we have recently identified, from *S. uberis* strain E, a 5.3-kDa anti-listerial bacteriocin possessing a typical pediocin box (G.A. Burtenshaw et al., unpublished data). This, to our knowledge, is the first report of a pediocin-like molecule being produced by a member of the genus *Streptococcus*, and indicates that the production of molecules of this family may be more widespread than previously recognized.

4.3.2 Type IIb: Multi-Component Bacteriocins

Some non-lantibiotic bacteriocins, as for their lantibiotic counterparts, can require two or more peptides to effect optimal inhibitory activity. For detailed descriptions of the numerous two-component bacteriocins characterized, the reader is referred to the recent comprehensive review by Garneau et al. (2002). It has been proposed that two-component bacteriocins be subdivided into synergistic (S)- and enhancing (E)-type inhibitory agents

(Marciset et al. 1997). S-type two-component bacteriocin activities are dependent on the concerted action of both peptides, and neither component appears inhibitory on its own (Marciset et al. 1997; Garneau et al. 2002). Examples of S-type bacteriocin systems include lactococcin G from *L. lactis*, lactacin F from *Lactobacillus johnsonii*, and lactocin 705 from *Lactobacillus casei* (Nissen-Meyer et al. 1992; Allison et al. 1994; Cuozzo et al. 2000). Conversely, for an E-type two-component bacteriocin, either each component peptide or only one peptide of the duet possesses inhibitory activity, but combination of the components results in greatly enhanced killing action toward the target species. Thermophilin 13 from *Streptococcus thermophilus*, enterocin L50 from *Enterococcus faecium*, and ABP-118 from *Lactobacillus salivarius* are representatives of E-type two-component bacteriocins (Marciset et al. 1997; Cintas et al. 2000; Flynn et al. 2002).

Although very uncommon, reports of three- or four-component bacteriocin systems have also arisen (Donvito et al. 1997; Netz et al. 2001). For example, the SLUSH β-hemolysin produced by *Staphylococcus lugdunensis*, which also exhibits antimicrobial activity, apparently consists of three peptides (Donvito et al. 1997). Similarly, aureocin A70 elaborated by *St. aureus* is proposed to comprise four peptides (Netz et al. 2001). Whilst definitive classification of the SLUSH peptides as either an S- or an E-type system has not been possible, as each component peptide has not been individually purified, aureocin A70 can be regarded as an E-type system due to the intrinsic inhibitory activity of AucA, AucB and AucC, but not AucD (Netz et al. 2001).

4.3.3 Type IIc: Miscellaneous Unmodified Bacteriocins

Due to the absence of any constraints imposed by either physical structure or characteristics of their genetic loci, all single-peptide non-modified bacteriocins that do not fulfill the criteria of type IIa or type IIb are automatically members of type IIc (formerly class IId, according to the scheme of Cotter et al. 2005b). Type IIc is a menagerie of inhibitory agents produced by strains from many ecological sources. As a result, its members are by far the most diverse, for example, with regard to the post-translational processing of the prebacteriocins and export of the biologically active agents. A recent intriguing example of a type IIc bacteriocin is sakacin Q, in which the prebacteriocin and immunity-related protein are translationally coupled (Mathiesen et al. 2005). The identification of new type IIc bacteriocins has been further facilitated by the availability – for data-mining – of a multitude of complete genome sequences of Gram-positive bacteria. For example, bioinformatics and mutational analyses were used to detect type IIc mutacins produced by *S. mutans* UA159, the genome sequence reference strain (Hale et al. 2005b).

Many type IIc bacteriocins share common features such as a prepeptide possessing a GG-containing leader sequence, an associated three-domain ABC transporter, and an immunity-related protein. However, some type IIc

members are exported, apparently via ABC transport systems, but without a recognizable N-terminal signal peptide. Examples include enterocin Q (from *E. faecium*), aureocin A53 (from *St. aureus*), and BHT-B (from *S. rattus*; Cintas et al. 2000; Netz et al. 2002; Hyink et al. 2005). It should also be noted that bacteriocin systems categorized as type IIb, such as the two-component enterocin L50 and the four-component aureocin A70 (Cintas et al. 2000; Netz et al. 2001), are also exported without identifiable signal peptides, indicating that this particular mode of export may be a common phenomenon among Gram-positive bacteria.

In the following subsections, we wish to digress from the bacteriocins of LAB by (1) describing the unusual type IIc bacteriocins from the propionic acid bacteria, and (2) highlighting the roles of type IIc-like non-inhibitory peptides in the so-called three-component signal transduction systems.

4.3.3.1 The Propionic Acid Bacteria: Producers of Novel Bacteriocins

While most of the bacteriocins described in this chapter originate from LAB, several novel bacteriocins produced by members of the propionic acid bacteria are of special interest (Table 4.2). These bacteria produce propionic acid as the primary end-product of glucose fermentation. The prominent bacteriocinogenic species belong to the genus *Propionibacterium*, principally those involved in the production of Swiss-style cheeses (Faye et al. 2000; Brede et al. 2004). Despite numerous reports of bacteriocinogenicity by this group of bacteria (Faye et al. 2002), only five propionibacterial bacteriocins have been characterized to date, four of which are non-lantibiotic peptides (Table 4.2) and the fifth a large, ca. 20-kDa protein (Miescher et al. 2000). The first to be described biochemically was propionicin PLG-1, a ca. 10-kDa bacteriocin produced by *Propionibacterium thoenii* P127 (Lyon and Glatz 1993), a strain that elaborates two propionicin activities (Ben-Shushan et al. 2003). However, neither the N-terminal amino acid sequence nor the genetic determinant of PLG-1 have been reported.

By contrast, the second inhibitory agent elaborated by *P. thoenii* P127, designated GBZ-1 (Ben-Shushan et al. 2003), has been characterized at the genetic level and is highly homologous to a novel protease-activated antimicrobial protein (PAMP) produced by *Propionibacterium jensenii* (Faye et al. 2002). Based on sequence analysis of its structural gene, it has been hypothesized that PAMP is initially synthesized as a 225-aa precursor protein containing a 27-residue Sec-dependent signal peptide (Faye et al. 2002). Upon export, an additional processing step involving proteolytic cleavage (at a specific Arg–Arg site) by an as yet unidentified extracellular protease yields the mature, biologically active 64-aa PAMP molecule (Faye et al. 2002). It is interesting to note that the post-export processing of closticin 574, a non-lantibiotic bacteriocin produced by *Clostridium tyrobutyricum* (Kemperman et al. 2003a), is analogous to that of PAMP. In addition to PAMP, certain strains of

Table 4.2 Some distinctive class II bacteriocins and their characteristics

Bacteriocin	Producer species	Distinctive characteristics	Key reference(s)
Type IIa: pediocin-like peptides			
Pediocin PA1	*Pediococcus acidilactici*	Prototype anti-listerial bacteriocin; biosynthetic locus is plasmid-borne; contains YGNGV motif that typifies this bacteriocin family; mode of action and mechanism of immunity are well-defined; first bacteriocin shown to be exported by an ABC transporter with concomitant cleavage of a signal peptide immediately following a double-glycine (GG) sequence motif	Rodriguez et al. (2002), Fimland et al. (2005)
Carnobacteriocin B2	*Carnobacterium piscicola*	First pediocin-like bacteriocin shown to be exported via a Sec-dependent transport system	Rodriguez et al. (2002), Fimland et al. (2005)
Listeriocin 743A	*Listeria innocua*	First bacteriocin to be described for *Listeria* spp.; displays the typical characteristics of pediocin-like peptides and is exported via the Sec-dependent route	Kalmokoff et al. (2001)
Ubericin A	*Streptococcus uberis*	First streptococcal pediocin-like inhibitory agent to be characterized; chromosomally encoded biosynthetic locus	G.A. Burtenshaw, unpublished data
Type IIb: multi-component inhibitors			
Lactococcin G	*Lactococcus lactis*	First two-component and S (synergistic)-type class II bacteriocin to be characterized	Nissen-Meyer et al. (1992)
Thermophilin 13	*Streptococcus thermophilus*	Prototype E (enhancer)-type two-component bacteriocin; ThmA* is the primary inhibitory peptide and ThmB* (no intrinsic activity of its own), when added, enhances the inhibitory activity; export mechanism unknown, although GG motif is present in the putative leader sequences of the precursor peptides	Marciset et al. (1997)

(*Continued*)

Table 4.2 Some distinctive class II bacteriocins and their characteristics—Continued

Bacteriocin	Producer species	Distinctive characteristics	Key reference(s)
Enterocin L50	*Enterococcus faecium*	An E-type bacteriocin system in which the component peptides are secreted without recognizable N-terminal signal peptides	Cintas et al. (1998, 2000)
Streptocins STH_1 and STH_2	*Streptococcus gordonii*	A novel two-component bacteriocin/hemolysin system of which the biosynthesis is dependent upon development of natural competence for genetic transformation; expression of the structural genes appears to be activated by the competence-specific sigma factor, and the prepeptides are processed and exported via the same transport system required for secretion of the competence-stimulating peptide	N.C.K. Heng et al., submitted
Plantaricin NC8	*Lactobacillus plantarum*	A two-component bacteriocin of which the biogenesis is activated by a three-component signal transduction system that appears to be responsive to interspecies cell-to-cell contact	Maldonado et al. (2004)
SLUSH peptides	*Staphylococcus lugdunensis*	A putative three-component bacteriocin/hemolysin system; the individual peptides have not been purified	Donvito et al. (1997)
Aureocin A70	*Staphylococcus aureus*	A putative E-type four-component bacteriocin, but inhibitory activity can be demonstrated only with three individual component peptides; each component peptide appears to be secreted without a signal peptide	Netz et al. (2001)
Plantaricin C11	*Lb. plantarum*	A multi-component bacteriocin system encoded by two operons; genetic regulation of the bacteriocin locus is unique, in that two transcriptional regulators with antagonistic functions appear to operate, promoting environment-dependent temporal and spatial fine-tuning	Diep et al. (2003)

Type IIc: miscellaneous bacteriocins			
Aureocin A53	*Staphylococcus aureus*	A single-peptide bacteriocin that, similarly to aureocin A70, is exported despite not possessing an identifiable signal peptide	Netz et al. (2002)
Sakacin Q	*Lactobacillus sakei*	A novel bacteriocin in which the precursor peptide and its immunity-related protein are translationally coupled	Mathiesen et al. (2005)
Mutacins IV and V	*Streptococcus mutans*	Identified by a combination of protein purification, bioinformatic analyses and genetic dissection methods; structural genes are located in different segments of the genome but both bacteriocins are exported by a single ABC transport system	Qi et al. (2001), Hale et al. (2005a, 2005b)
Sakacins T and X	*Lb. sakei*	Two bacteriocin systems encoded by divergent genetic loci that are activated by a single three-component regulatory system; the component peptides of both bacteriocins are exported by the same ABC transporter without the need for an accessory factor	Vaughan et al. (2003)
Bacteriocins produced by propionic acid bacteria (PAB)			
Propionicin PLG-1	*Propionibacterium thoenii*	First bacteriocin from PAB to be characterized biochemically (but not genetically), with an estimated molecular mass of ca. 10 kDa	Lyon and Glatz (1993)
Propionicin T1 (thoeniicin 447)	*P. thoenii*	Sec-dependent bacteriocin active against *Propionibacterium acnes* (causative agent of acne vulgaris)	Faye et al. (2000), van der Merwe et al. (2004)
Protease-activated antimicrobial protein (PAMP)/propionicin GBZ-1	*Propionibacterium jensenii* and *P. thoenii*	A novel bacteriocin that is exported (as a 198-aa precursor protein) via the Sec-dependent pathway, but is only activated when an additional 134 aa is cleaved off by extracellular proteases	Faye et al. (2002), Ben-Shushan et al. (2003)
Propionicin F	*Propionibacterium freudenreichii*	A novel bacteriocin, in that the biologically active peptide is the central portion of a much larger preprotein that is doubly processed	Brede et al. (2004)

P. thoeniii have also been shown to produce propionicin T1 (thoeniicin 447), a Sec-exported non-lantibiotic peptide that is active against the acne-causing bacterium *Propionibacterium acnes* (Faye et al. 2000; van der Merwe et al. 2004).

One of the more unique bacteriocins encountered to date, and the most extensively studied of the propionicins, is propionicin F produced by *Propionibacterium freudenreichii* (Brede et al. 2004). Propionicin F constitutes the central 43-aa peptide portion, namely, residues Trp^{102} to Pro^{145}, of a much larger 255-aa precursor protein, PcfA (Brede et al. 2004). As the propionicin F genetic locus has been completely sequenced, the proteolytic processing steps (between Cys^{101} and Trp^{102}, and between Pro^{145} and Gly^{146}) are considered to be mediated by the gene products of *pcfB* and *pcfC*, respectively (Brede et al. 2004). Since PcfA does not contain an identifiable N-terminal signal peptide, the mechanism of export of propionicin F remains unknown, although PcfD, a putative ABC transporter (which lacks the peptidase domain), has been implicated here (Brede et al. 2004). However, while not explicitly stated by Brede et al. (2004), we have noticed that the amino acid sequence of mature propionicin F does contain a GG motif. Based on the analogy that the pediocin PA-1 prepeptide is 80% active (Sect. 4.3.1), it is therefore feasible that despite being exported by PcfD with an intact GG motif, propionicin F peptide itself is inherently biologically active. However, it is tempting to speculate whether a further processed propionicin F (i.e., minus the putative GG-containing signal peptide) may have higher levels of antimicrobial activity.

4.3.3.2 Bacteriocin-like Peptides as Signaling Molecules

As described in Section 4.2.1, bacteriocins such as nisin can influence expression of their biosynthetic operons through binding to their cognate two-component histidine kinase-response regulator signal transduction systems, which in turn regulates transcription of the bacteriocin operon. In recent years, it has become apparent that such "three-component regulatory mechanisms" are also involved in cell density-dependent phenomena (quorum sensing) and non-lantibiotic bacteriocin biosynthesis (see reviews by Kleerebezem and Quadri 2001; Morrison 2002). Activation of these processes is usually mediated by the binding of a specific inducing peptide to its cognate sensor histidine kinase, which in turn phosphorylates a dedicated response regulator. The latter then proceeds to up- or down-regulate the expression of genes under its control. Interestingly, the inducing peptides, which do not appear to exhibit intrinsic inhibitory activity, are synthesized as prepeptides containing signal peptides with double-glycine motifs. Secretion of these prepeptides into the extracellular milieu is invariably facilitated by dedicated ABC transporters not unlike those involved in the export of pediocin-like peptides (Sect. 4.3.1).

A classic biological phenomenon in Gram-positive bacteria that involves quorum sensing and a three-component signal transduction system is the development of natural competence for genetic transformation (reviewed by Morrison 2002; Lacks 2004). Competence is defined as the transient physiological state that allows a bacterium to take up DNA from the environment. Transformation of the recipient cell occurs when the exogenous DNA is integrated into the genome by homologous recombination and subsequently expressed. In the archetypal, naturally transformable Gram-positive bacterium, *Streptococcus pneumoniae*, the competence cascade is initially activated by binding of a 17-mer peptide, called the competence-stimulating peptide (CSP), to its cognate histidine kinase ComD, followed by phosphorylation of the response regulator ComE (Morrison 2002; Lacks 2004). Phosphorylated ComE perpetuates the signal by up-regulating expression of the *comCDE* and *comAB* operons as well as activating the expression of ComX, the alternative sigma factor involved in the expression of the genes encoding the DNA uptake machinery. The ComAB operon encodes the ABC transporter responsible for the export of CSP.

In our laboratory, we have recently shown that the production of two bacteriocins (STH_1 and STH_2) by the naturally competent oral bacterium *Streptococcus gordonii* is not only dependent upon activation of the competence cascade, but is also under the control of ComR, the homologue of the *S. pneumoniae* competence-specific sigma factor ComX (N.C.K. Heng, J.R. Tagg, G.R. Tompkins, submitted). Moreover, the bacteriocin precursor peptides, SthA and SthB, each contain a GG cleavage motif, and are exported via the ComAB (CSP-secreting) ABC transporter. Competence-associated lantibiotic (e.g., Smb; see Sect. 4.2.4) and non-lantibiotic (e.g., mutacin IV) bacteriocin production has also been shown to occur in *S. mutans* (van der Ploeg 2005; Yonezawa and Kuramitsu 2005; Kreth et al. 2005). However, in contrast to *S. gordonii*, (1) the promoters of the bacteriocin-encoding genes appear to contain nucleotide motifs for DNA-binding response regulators, possibly ComE (van der Ploeg 2005; Yonezawa and Kuramitsu 2005), and (2) export of the non-lantibiotic bacteriocins is mediated by an ABC transporter that differs from that involved in CSP secretion (Hale et al. 2005a).

In *Lactobacillus plantarum*, peptide-activated expression of bacteriocin (plantaricin) production has been very well characterized. For example, the complex plantaricin C11 biosynthetic locus comprises five distinct operons (*plnABCD*, *plnEFI*, *plnJKLR*, *plnMNOP* and *plnGHSTUV*), the first of which (*plnABCD*) encodes a unique three-component signal transduction system, but with two response regulators PlnC and PlnD (Diep et al. 2003). The *plnEFI* and *plnJKLR* operons encode peptides exhibiting bona fide inhibitory activity (and their associated immunity proteins), and *plnGHSTUV* specifies the components of the bacteriocin export machinery. The function of the last operon *plnMNOP* is unknown. It is believed that both PlnC and PlnD are antagonistic in their regulatory activity, which facilitates temporal and spatial fine-tuning of plantaricin production according to the environmental

situation (Diep et al. 2003). A recent exciting development, demanding further investigation, has been the discovery of plantaricin biosynthesis (in *Lb. plantarum* strain NC8), which is activated by a three-component signal transduction system that appears to be responsive to interspecies cell–cell contact (Maldonado et al. 2004).

Bacteriocin (sakacin) biosynthesis mediated by three-component signal transduction systems has also been extensively characterized in various strains of the food-associated species *Lactobacillus sakei* (Vaughan et al. 2003), and although their mechanisms of genetic regulation are not as elaborate as that of plantaricin C11, they are no less intriguing. Notable examples include (1) sakacin A biosynthesis, which has been shown to be profoundly affected at 34–37 °C but not at 30–33 °C, due to temperature-dependent synthesis of the inducer peptide Sap-Ph (Diep et al. 2000), and (2) activation, by a single three-component system (encoded by *stxPRK*), of two adjacent bacteriocin systems (the two-component and single-peptide sakacins T and X, respectively) that are encoded by divergent operons (Vaughan et al. 2003, 2004). Moreover, in the latter case, the inducing peptide and the sakacins are exported by the same ABC transporter that, unlike that of many such secretion systems, does not appear to require an accessory factor (Vaughan et al. 2003).

4.4 Class III: The Large (>10 kDa) Bacteriocins

While the bacteriocins characterized from Gram-positive species are predominantly small (< 10 kDa) peptides, several large antimicrobial proteins have been described at both the biochemical and genetic level. These bacteriocins typically manifest as heat-labile proteins, but one apparent exception is propionicin SM1, a heat-stable inhibitory agent produced by *P. jensenii* (Miescher et al. 2000). It should be noted, however, that aggregates of small peptides, for example, staphylococcin 1580 (Sahl 1994), have caused confusion in the past with regard to estimation of protein size. The bona fide large bacteriocins of Gram-positive bacteria can generally be subdivided into two distinct groups: (1) the bacteriolytic enzymes (or bacteriolysins), which facilitate the killing of sensitive strains by cell lysis, and (2) the non-lytic antimicrobial proteins. However, in some cases, such as propionicin SM1 and albusin B (from *Ruminococcus albus*; Chen et al. 2004), the lack of mode of action data precludes them at present from placement in this classification scheme (cf. Table 4.3).

4.4.1 Type IIIa: The Bacteriolysins (Bacteriolytic Enzymes)

4.4.1.1 Lysostaphin – The Prototype Bacteriolysin

Originally described more than 40 years ago (Schindler and Schuhardt 1964), lysostaphin (produced by *Staphylococcus simulans* biovar *staphylolyticus*)

Table 4.3 The Class III (>10 kDa) bacteriocins of Gram-positive bacteria

Bacteriocin	Producer species	Distinctive features	Relevant reference(s)
Bacteriolytic enzymes (bacteriolysins)			
Lysostaphin	*Staphylococcus simulans* subsp. *staphylolyticus*	Prototype and best-characterized bacteriolytic enzyme; plasmid-encoded; comprises two distinct domains separated by a linker; post-translational processing events, mode of action and mechanism of immunity well-defined	Schindler and Schuhardt (1964), King et al. (1980), DeHart et al. (1995), Thumm and Gotz (1997), Ehlert et al. (2000)
Zoocin A	*Streptococcus equi* subsp. *zooepidemicus*	First chromosomally encoded (and streptococcal) bacteriolysin to be characterized; attacks the interpeptide crosslink of streptococcal peptidoglycan; similarly to lysostaphin, is composed of two domains; function of zoocin immunity factor (Zif) similar to lysostaphin counterpart (Lif/Epr); biosynthesis of zoocin A may be subject to catabolite repression	Simmonds et al. (1996, 1997), Beatson et al. (1998), Lai et al. (2002), O'Rourke et al. (2003)
Millericin B	*Streptococcus milleri*	Novel broad-spectrum bacteriolysin that cleaves not only the interpeptide crosslinks but also the stem peptide of peptidoglycan	Beukes et al. (2000), Beukes and Hastings (2001)
Enterolysin A	*Enterococcus faecalis*	Relatively broad-spectrum bacteriolytic enzyme that may also cleave the stem peptide; C-terminal domain appears very similar to that of bacteriophage lysins	Nilsen et al. (2003)
Other large bacteriocins			
Helveticin J	*Lactobacillus helveticus*	Possibly the first chromosomally encoded and largest non-lytic bacteriocin (37 kDa) to be described; exact mode of action and mechanism of immunity unknown	Joerger and Klaenhammer (1986, 1990)
Dysgalacticin	*Streptococcus dysgalactiae* subsp. *equisimilis*	Plasmid-encoded non-lytic 21-kDa bacteriocin with a narrow spectrum of activity; possesses a novel predicted secondary structure containing a single essential disulfide bond	Wong et al. (1981), Heng et al. (2006)

(*Continued*)

Table 4.3 The Class III (>10 kDa) bacteriocins of Gram-positive bacteria—Continued

Bacteriocin	Producer species	Distinctive features	Relevant reference(s)
Streptococcin A-M57	*Streptococcus pyogenes* M-types 57 and 69	First plasmid-encoded large (17 kDa) streptococcal bacteriocin to be reported; exhibits an unusual inhibitory spectrum, but possesses similar predicted secondary structure to that of dysgalacticin	Simpson and Tagg (1984), Heng et al. (2004)
Propionicin SM1	*Propionibacterium jensenii*	20-kDa heat-stable bacteriocin identified in the propionic acid bacteria; mode of action and mechanism of immunity unknown	Miescher et al. (2000)
Albusin B	*Ruminococcus albus*	First large bacteriocin to be identified in a ruminal bacterium; mode of action and immunity mechanism unknown; structural gene encodes a prebacteriocin containing an unusually long secretion signal peptide	Chen et al. (2004)

represents the prototype bacteriolysin and is probably the most extensively studied large bacteriocin elaborated by any Gram-positive bacterium. Lysostaphin is a plasmid-encoded glycylglycine endopeptidase that kills sensitive cells by specifically hydrolyzing the pentaglycine crossbridges in peptidoglycan (Robinson et al. 1979; King et al. 1980). A homologue of lysostaphin, ALE-1 from *Staphylococcus capitis*, has also been characterized (Sugai et al. 1997a). Due to the ability of lysostaphin to kill members of virtually all staphylococcal species, including those that impact on human and animal health, such as *St. aureus* and *Staphylococcus epidermidis*, various reports over the years have recommended its use in a variety of medical and agricultural applications (Oldham and Daley 1991; Wu et al. 2003; Shah et al. 2004).

Lysostaphin is initially synthesized as a 493-aa precursor protein (prepro-lysostaphin), which comprises the 246-aa mature form of the bacteriocin plus the following N-terminal extensions: (1) a 36-aa secretion signal peptide at its N-terminus, followed by (2) 195 aa organized into 15 tandem repeats of a 13-aa sequence (Heinrich et al. 1987; Thumm and Gotz 1997). Following export (with concomitant removal of the signal peptide), the tandem repeats are removed by a cysteine protease to yield the fully activated lysostaphin molecule (Neumann et al. 1993; Thumm and Gotz 1997). The lysostaphin molecule is predicted to consist of two distinct domains separated by a short linker sequence: (1) a N-terminal peptidase domain responsible for the catalytic activity of the protein, and (2) a C-terminal targeting domain involved in binding to the peptidoglycan substrate (Wang et al. 1991; Simmonds et al. 1997).

Further studies showed that the genetic determinant conferring immunity to lysostaphin was also located on the plasmid (Heath et al. 1989). The immunity factor, designated *lif* or *epr*, encodes a protein that displays homology to the FemAB complex responsible for adding glycine residues to the pentaglycine crosslinks (Heath et al. 1989; Thumm and Gotz 1997). However, Epr adds serine residues, rather than glycine, and this change in the amino acid composition of the crosslinks is sufficient to protect the cell from the lytic effects of lysostaphin (Robinson et al. 1979; DeHart et al. 1995; Thumm and Gotz 1997; Sugai et al. 1997b; Ehlert et al. 2000). Overall, the findings arising from the studies on lysostaphin and its immunity factor have provided invaluable knowledge not only to researchers working on bacteriocins but also to those trying to elucidate the complexities of cell wall construction.

4.4.1.2 Zoocin A and Other Bacteriolysins

Within the last 10 years, much progress has also been made in the characterization of bacteriolysins produced by lactic acid bacteria, mainly from members of the genera *Streptococcus* and *Enterococcus*. The prototype streptococcal bacteriolytic enzyme is zoocin A, which is specified by a chromosomally located gene (*zooA*) in *Streptococcus equi* subsp. *zooepidemicus*. Despite exhibiting limited amino acid sequence similarity, zoocin A and Zif

(the zoocin A immunity factor) share common properties with lysostaphin and Epr, respectively, such as (1) the hydrolysis of streptococcal interpeptide crossbridges, (2) a modular structure consisting of an N-terminal M37-like peptidase domain and a C-terminal substrate-binding domain, and (3) Zif, similarly to Epr, resembles a FemAB-like protein that, when expressed in a heterologous host such as *S. gordonii* (a zoocin A-susceptible species), confers the expected zoocin-resistant phenotype (Simmonds et al. 1996, 1997; Beatson et al. 1998; Liang et al. 2004). Intriguingly, Zif does not appear to alter the glycine–serine ratios of the interpeptide chain (Beatson et al. 1998), and therefore the exact mechanism of immunity to zoocin A remains enigmatic. A more recent and exciting development is the novel observation that the biosynthesis of zoocin A may be influenced by glucose levels, i.e., it may be catabolite-repressed (O'Rourke et al. 2003). In our laboratory, we have recently identified stellalysin, a new zoocin A-like antimicrobial protein produced by the oral bacterium *Streptococcus constellatus* subsp. *constellatus*. Preliminary analyses indicate that stellalysin biosynthesis may also be catabolite-repressed (N.C.K. Heng et al., unpublished data).

Aside from zoocin A and stellalysin, only two other bacteriolytic enzymes produced by lactic acid bacteria have been described, namely, millericin B from *Streptococcus milleri* and enterolysin A from *E. faecalis* (Beukes et al. 2000; Nilsen et al. 2003). Millericin B is distinctive in its ability to hydrolyze the cell walls of species such as *M. luteus*, *Staph. aureus* and non-millericin B-producing strains of *S. milleri*, all of which possess different interpeptide crosslinks (Beukes et al. 2000). It was further shown that millericin B could cleave peptidoglycan either in the stem peptide (which is common to the above-listed three species) or in the interpeptide crosslinks (Beukes et al. 2000). Moreover, the mechanism of immunity to millericin B, similarly to that of lysostaphin, involves amino acid substitution (leucine for threonine) in the interpeptide crosslinks of peptidoglycan (Beukes and Hastings 2001).

Enterolysin A is the first large bacteriocin to be described from *E. faecalis*, and similarly to millericin B, exhibits a rather diverse inhibitory spectrum. Although the common element in the peptidoglycan of all enterolysin A-sensitive species appears to be the stem peptide (L-Ala-D-Glu-L-Lys-D-Ala; Nilsen et al. 2003), the exact mode of action of enterolysin A remains to be determined. Enterolysin A is composed of the two-domain structure typical of other bacteriolysins (Nilsen et al. 2003). Interestingly, while the N-terminal domain of enterolysin A, similarly to that of other bacteriolysins, is of the M37-like peptidase type, the C-terminal domain displays significant homology to the lysins of *Lactobacillus casei* bacteriophages (Nilsen et al. 2003).

4.4.2 Type IIIb: The Non-Lytic Bacteriocins

As the antithesis to the bacteriolysins, several large bacteriocins have been shown to kill target cells by non-lytic means. This could involve dissipation of the proton motive force, leading to ATP starvation and ultimately cell death.

The first non-lytic bacteriocin to be described at the biochemical and genetic level was helveticin J, a 37-kDa bacteriocin produced by *Lactobacillus helveticus* that primarily targets other *Lactobacillus* species (Joerger and Klaenhammer 1986, 1990). However, the precise mode of action of helveticin J remains unknown.

Dysgalacticin (21 kDa) and streptococcin A-M57 (SA-M57; 17 kDa) are secreted bacteriocins produced by *Streptococcus dysgalactiae* subsp. *equisimilis* and M-type 57 *S. pyogenes*, respectively (Wong et al. 1981; Heng et al. 2004, 2006). The inhibitory spectrum of dysgalacticin is fairly narrow and is limited to strains of Lancefield serogroups A, C and G (Wong et al. 1981; Tagg and Wong 1983). On the other hand, the range of organisms inhibited by SA-M57 is unusual, consisting mainly of non-streptococcal Gram-positive species including *M. luteus*, *L. lactis*, all tested species of *Listeria* (including *Lis. monocytogenes*), *Bacillus megaterium* and *St. simulans* (Simpson and Tagg 1983; Heng et al. 2004). Both bacteriocins appear to kill sensitive cells in a non-lytic fashion (Wong et al. 1981; Simpson and Tagg 1983; Heng et al. 2006), although the exact mechanism remains unclear.

At first glance, the similarities between dysgalacticin and SA-M57 appear superficial (Heng et al. 2004, 2006): (1) the structural genes for both dysgalacticin (*dysA*) and SA-M57 (*scnM57*) are plasmid-borne, and (2) both bacteriocins are exported via Sec-dependent systems. Dysgalacticin does not display any similarity either to proteins of known function or to hypothetical proteins in publicly available databases. Conversely, SA-M57 exhibits primary amino acid sequence similarity with two hypothetical, potentially secreted proteins, EF1097 and YpkK, from *E. faecalis* and *Corynebacterium jeikeium*, respectively (Heng et al. 2004).

Despite the obvious lack of sequence similarity between dysgalacticin, SA-M57, EF1097 and YpkK, all four proteins possess similar predicted secondary structures consisting of (1) a fairly unstructured N-terminal portion, (2) a C-terminal region that appears to contain a helix-loop-helix motif, and (3) two cysteine residues that are predicted to form a disulfide bond. We have subsequently shown, for both dysgalacticin and SA-M57, that the two cysteines do indeed form a disulfide bond essential for antimicrobial activity (N.C.K. Heng et al., unpublished data). Furthermore, we have successfully expressed the EF1097 and YpkK structural genes in *E. coli*, and found that both recombinant proteins exhibit antimicrobial activity, with the former displaying a much broader inhibitory spectrum (P.M. Swe and H.J. Baird, unpublished data). Taken collectively, dysgalacticin, SA-M57, EF1097 and YpkK potentially constitute a novel family of antimicrobial proteins.

4.5 Class IV: The Cyclic Bacteriocins

Based on our proposed classification scheme for antimicrobial proteins produced by Gram-positive bacteria, the fourth and arguably the most unique class of bacteriocins is that encompassing the cyclic bacteriocins (Table 4.4).

Table 4.4 Known circular (cyclic) bacteriocins produced by Gram-positive bacteria

Bacteriocin	Producer species	Distinguishing features	Key reference(s)
Enterocin AS-48	*Enterococcus faecalis*	Prototype and most well-characterized circular bacteriocin, both biochemically and genetically	Maqueda et al. (2004)
Gassericin A	*Lactobacillus gasseri*	The first cyclic bacteriocin to be reported that contains a mixture of D- and L-amino acids (at least D-Ala)	Kawai et al. (2004b)
Reutericin 6	*Lactobacillus reuteri*	Identical primary amino acid sequence to gassericin A but different inhibitory spectrum due to altered composition of D-alanine	Kawai et al. (2004b)
Circularin A	*Clostridium beijerinckii*	Well-characterized cyclic bacteriocin with unusual post-translational processing; genetic locus (*cir*) contains genes homologous to those found in the AS-48 locus	Kemperman et al. (2003a, 2003b)
Butyrivibriocin A	*Butyrivibrio fibrisolvens*	The first cyclic bacteriocin to be identified from a rumen bacterium; similar to gassericin A	Kalmokoff et al. (2003)
Uberolysin	*Streptococcus uberis*	First cyclic bacteriocin to be characterized (biochemically and genetically) from the genus *Streptococcus*; unusual due to its thermolability and lysis of actively growing cells	R.E. Wirawan et al., submitted

These circular inhibitory agents are ribosomally synthesized peptides, which are post-translationally processed such that the first and last amino acids of the mature peptide are covalently bonded, corresponding to the so-called head-to-tail ligation (Maqueda et al. 2004). To date, this class comprises only a handful of members, the prototype being enterocin AS-48 (extensively reviewed by Maqueda et al. 2004).

4.5.1 Enterocin AS-48

More than 20 years ago, a new heat-stable inhibitory agent (designated enterocin AS-48) produced by *E. faecalis* subsp. *liquefaciens* strain S-48 was first described (Galvez et al. 1985). The broad inhibitory spectrum of enterocin AS-48 includes Gram-positive as well as certain Gram-negative species (Maqueda et al. 2004). Due to an inconsistency of nomenclature, AS-48 and its natural variants are also known by other names such as enterocin 4 and bacteriocin 21 (Maqueda et al. 2004). The cyclic nature of AS-48 caused initial attempts to obtain the amino acid sequence of the peptide to fail, since the N-terminus is essentially blocked. This limitation was eventually overcome by endopeptidase digestion followed by N-terminal sequencing of the internal fragments (Maqueda et al. 2004). AS-48 has since become one of the most extensively characterized bacteriocins in terms of its biochemistry (including a three-dimensional structure) and genetics.

Enterocin AS-48 is plasmid-encoded and its biosynthetic locus contains ten genes (Martinez-Bueno et al. 1998; Maqueda et al. 2004). The AS-48 structural gene, *as-48A*, specifies a 105-aa prepeptide containing a 35-residue leader peptide. Whereas *as-48BC*$_1$*D* are believed to be responsible for the maturation and secretion of AS-48, *as-48D*$_1$*EFGH* have been assigned roles in producer self-protection or immunity (Diaz et al. 2003). Upon cleavage of the leader peptide (by an as yet unidentified process), the Met^1 and Trp^{70} residues of the 70-aa linear form of AS-48 are then covalently bonded, with the concomitant loss of a water molecule (Maqueda et al. 2004). The three-dimensional NMR structure of AS-48 reveals that it has five alpha-helices that fold into a very compact structure, with the head-to-tail union (between Met^1 and Trp^{70}) residing in helix 5 (Maqueda et al. 2004). The latter is perceived to contribute to the heat stability and structural integrity of the molecule (Maqueda et al. 2004). It is noteworthy that the secondary structure of enterocin AS-48 resembles that of a mammalian lysin found in natural killer cells, which is a non-circular protein composed of five helices stabilized by disulfide bridges (González et al. 2000).

More recently, the biochemical and genetic characteristics of circularin A, a new cyclic bacteriocin produced by *Clostridium beijerinckii*, have been reported (Kemperman et al. 2003a, 2003b). The *cir* locus shares some elements with the *as-48* locus, albeit with limited similarity at the amino acid

level. For example, *cirBCDE* are the putative counterparts of *as-48BCDD*$_1$ (Kemperman et al. 2003b). Circularin A itself possesses an unusual leader peptide of only three amino acids, and exhibits limited amino acid similarity to AS-48 (Kawai et al. 2004b).

4.5.2 Gassericin A and Reutericin 6

Gassericin A and reutericin 6 are cyclic peptides produced by *Lactobacillus gasseri* and *Lactobacillus reuteri*, respectively, which possess identical primary amino acid sequences deduced from their structural genes (Kawai et al. 2004a). However, their inhibitory spectra as well as their killing kinetics against selected indicator bacteria differ (Kawai et al. 2004a). The basis for these phenotypic differences was revealed by partial composition analysis of the D- and L-amino acids of both peptides. It was determined that gassericin A and reutericin 6 both contain D- and L-amino acids, a novel finding in itself, but differing in their D-Ala:L-Ala ratios (Kawai et al. 2004a). It is not known whether other differences exist between the two peptides, as the evaluation of D- or L-status was carried out with only five of the 17 amino acids known to be present in both bacteriocins (Kawai et al. 2004a). Two other bacteriocins, butyrivibriocin AR10 (from *Butyrivibrio fibrisolvens*) and acidocin B (from *Lactobacillus acidophilus*), display significant amino acid similarity to gassericin A (Leer et al. 1995; Kalmokoff et al. 2003). Whereas butyrivibriocin AR10 has been shown to be circular, the physical properties of acidocin B remain to be confirmed. Interestingly, the structural gene of acidocin B (*acdB*; Leer et al. 1995) was reported prior to that of gassericin A (Kawai et al. 1998). It should be noted that of the four cyclic peptides described in this section, genetic data beyond that of the structural gene encoding each peptide exist only for butyrivibriocin AR10 (Kalmokoff et al. 2003). A detailed comparative analysis of the maturation- and transport-associated genes of these bacteriocins would greatly aid our understanding of their biogenesis.

4.5.3 Uberolysin

S. uberis, one of the causative agents of bovine mastitis, is a prolific producer of bacteriocins. Individual strains produce different combinations of various classes of bacteriocins, including the lantibiotic nisin U (Sect. 4.2.1), the pediocin-like ubericin A (Sect. 4.3.1), and a novel circular bacteriocin called uberolysin (R.E. Wirawan et al., submitted). In contrast to cyclic peptides such as AS-48, uberolysin is a ca. 7-kDa heat-labile cyclic bacteriocin that lyses only actively growing cells. The uberolysin biosynthetic locus, designated *ubl*, has been completely sequenced and contains five genes, two of which (*ublB* and *ublD*) display limited homology to the putative maturation (*cirB*) and transport (*cirD*) genes of the circularin A locus, respectively

(Kemperman et al. 2003b). The 76-aa uberolysin precursor peptide is deduced to possess an atypical 6-aa leader peptide, and circularization is predicted to occur between Leu^{+1} and Trp^{+70}. It is envisaged that a cocktail of various *S. uberis*-derived bacteriocins could be developed as an effective preventative agent against bovine mastitis.

4.6 Concluding Remarks

And so as researchers, advantaged by increasingly sophisticated genetic and protein technologies, continue to delve both deeper and more expansively within the amazing repertoire of antimicrobials invented by microbes to advantage their own survival, we can eagerly anticipate further big surprises. It is important to appreciate that bacteriocins, defined as such for our own convenience, represent only one facet of a probably seamless continuum of bacterial antimicrobial activities. By necessity, the modified scheme for the classification of bacteriocins of Gram-positive bacteria that we have proposed here will inevitably continue to evolve. We have attempted to present the reader with some views (albeit colored by our own experiences) of a knowledge slice from the now vast, but still mounting, literature in this field. It is difficult to predict what might be the highlights or even the trends of a review of this field a decade from now. Nevertheless, we are tempted to anticipate more interest in the larger (class III) bacteriocins and further knowledge of the factors, especially of the molecular mechanisms, influencing expression of bacteriocins in natural ecosystems. Perhaps we may also see further developments and successes in the Pasteurian application of bacteriocin-producing bacteria to infection control – the great man would have been well-pleased by that!

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