Antimicrobial Peptides of Lactic Acid Bacteria: Mode of Action, Genetics and Biosynthesis

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A survey is given of the main classes of bacteriocins, produced by lactic acid bacteria: I. lantibiotics II. small heat-stable non-lanthionine containing membrane-active peptides and III. large heat-labile proteins. First, their mode of action is detailed, with emphasis on pore formation in the cytoplasmatic membrane. Subsequently, the molecular genetics of several classes of bacteriocins are described in detail, with special attention to nisin as the most prominent example of the lantibiotic-class. Of the small non-lanthionine bacteriocin class, the *Lactococcus* lactococcins, and the *Lactobacillus* sakacin A and plantaricin A-bacteriocins are discussed. The principles and mechanisms of immunity and resistance towards bacteriocins are also briefly reported. The biosynthesis of bacteriocins is treated in depth with emphasis on response regulation, post-translational modification, secretion and proteolytic activation of bacteriocin precursors. To conclude, the role of the leader peptides is outlined and a conceptual model for bacteriocin maturation is proposed.

Keywords. Antimicrobial peptides, Bacteriocins, Biosynthesis, Genetics, Immunity, Lactic acid bacteria, Lantibiotics

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1 Lactic Acid Bacteria and Their Bacteriocins

1.1 Lactic Acid Bacteria

Lactic acid bacteria are Gram-positive, catalase-negative, oxidase negative, non-sporulating microaerophilic bacteria whose main fermentation product from carbohydrates is lactate. The lactic acid bacteria comprise both cocci (e.g. *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Tetragenococcus*, *Streptococcus*, *Enterococcus*) and rods (*Lactobacillus*, *Carnobacterium*, *Bifidobacterium*). Many of these lactic acid bacteria are generally recognized for their contribution to flavor and aroma development and to spoilage retardation [1]. Therefore, the traditional use of these microorganisms in the fermentation of foods and beverages has resulted in their application in many starter cultures currently involved in the fermentation of a wide variety of agricultural raw materials such as milk, meat, fruit, vegetables, cereals, etc. [2–7]. The lactic acid bacterial strains present in these starter cultures contribute to the organoleptic properties and the preservation of the fermented products by in situ production of antimicrobial substances such as lactic acid and acetic acid, hydrogen peroxide, bacteriocins, etc. [8–11]. Because of the general tendency to decrease the use of chemical additives, such natural inhibitors could replace the use of chemical preservatives such as sulfur dioxide, benzoic acid, sorbic acid, nitrate, nitrite, etc. [12]. For this reason, bacteriocins produced by lactic acid bacteria may be very promising as biological food preservatives in future food preservation [13]. Furthermore, certain lactic acid bacteria, especially some lactobacilli and bifidobacteria, are believed to play a beneficial role in the gastro-

intestinal tract [14]. Lactobacilli are also potentially useful as carriers for oral immunization, since orally administered lactobacilli trigger both a mucosal and systemic immune reaction against epitopes associated with these organisms [15, 16].

1.2 Bacteriocins

Bacteriocins are proteinaceous compounds produced by bacteria, both Gram-positive and Gram-negative, and they are active chiefly against closely related bacteria [17]. The discovery of bacteriocins dates back to 1925, when *E. coli* V was shown to produce an antimicrobial compound active against *E. coli* Φ [18]. These antimicrobial substances by *E. coli* were named colicins and 17 different types, based on their adsorption, were later reported [19]. Like the colicins (25–90 kDa, produced by *E. coli* and active against other *Enterobacteriaceae*) and microcins (<10 kDa, produced by *Enterobacteriaceae* and active against other Gram-negative bacteria), the bacteriocins produced by Gram-positive bacteria were defined as proteinaceous compounds that kill only closely related species [17, 20]. Although true for the majority of compounds, it is now evident that bacteriocins produced by lactic acid bacteria display bactericidal activity beyond species that are closely related [21]. Except for the colicins and the microcins, many other bacteriocins produced by non-lactic acid bacteria such as *Bacillus*, *Staphylococcus*, *Streptomyces*, *Streptoverticillium*, etc. have been reported [1, 22, 23].

The first report of the production of a bacteriocin produced by lactic acid bacteria was made in 1928 [24]. The substance was determined as a polypeptide [25] and subsequently named nisin [26, 27]. Since that time the bacteriocin field has expanded exponentially, and now bacteriocins produced by all genera of the lactic acid bacteria have been reported [1, 21].

The majority of bacteriocins from lactic acid bacteria have been characterized according to the early definition of a proteinaceous inhibitor, estimation of their molecular mass, and determination of their inhibition spectrum [1, 21]. Recent developments in the biochemical and molecular biological characterization of many of these compounds have elucidated their genetic organization, structures and mode of action. Despite their heterogeneity, bacteriocins produced by lactic acid bacteria were subdivided into three distinct classes based on these genetic and biochemical resemblances [28].

1.2.1 *Lantibiotics (Class I)*

Lantibiotics are small, membrane-active peptides (<5 kDa) containing the unusual amino acids lanthionine, β-methyl-lanthionine, and the dehydrated residues dehydroalanine and dehydrobutyrine; e.g. nisin, lacticin 481, carnocin U-149, lactocin S, sublancin 168 [29–38]. The intrachain positioning of these polycyclic structures of the lantibiotics has been used to group them into linear (Group IA) or circular (Group IB) lantibiotics [39]. Based on similarities in the

size, net charge and sequence of the leaders, the group IA lantibiotics can be further classified into two main groups, i.e. class IA_I (nisin) and class IA_{II} (lacticin 481). The lactocin S *N*-terminal extension displays no homology with the class IA_I or class IA_{II} leader peptides and may therefore represent a new class [40].

1.2.2
Small, Heat-Stable, Non-Lanthionine Containing, Membrane-Active Peptides (Class II)

These are less than 10 kDa in size and are characterized by a Gly-Gly$^{-2/-1}$ Xaa processing site in the bacteriocin precursor. This site is not restricted to class II bacteriocins, as it is also present in some lantibiotics [41]. The mature bacteriocins are predicted to form amphiphilic helices with varying amounts of hydrophobicity, β-sheet structure, and moderate (100 °C) to high (121 °C) heat stability; e.g. pediocin PA-1, lactococcin A, B, and M, leucocin A, sakacin A (= curvacin A), sakacin P, and lactacin F. Protein engineering of lactococcin B indicated that its cysteine residue was not necessary for activity [28]. Subgroups that can be defined within the class II bacteriocins are:

Class (IIA) Listeria-active peptides. They have a consensus sequence in the *N*-terminus of-T-G-N-G-V-X-C-; represented by pediocin PA-1. Other examples are sakacin A, sakacin P, leucocin A, mesentericin Y105 [42–45].

Class (IIB) Poration complexes consisting of two proteinaceous peptides. These two peptides are necessary for full activity; examples are lactococcin G, lactococcin M, lactacin F and two-component peptide systems found in the operon located in the plantaricin A gene cluster [46–49].

Class (IIC) Small, heat-stable, and non-modified bacteriocins translated with *sec*-dependent leaders. Only two reports have been made up to now; divergicin A and acidocin B [50, 51].

1.2.3
Large Heat-Labile Proteins (Class III)

These are greater than 30 kDa in size; examples are helveticin J, helveticin V, acidophilicin A, lactacins A and B [52–56].

A fourth class, proposed by Klaenhammer [21] is rather questionable. This class comprised the complex bacteriocins, composed of protein plus one or more chemical moieties (lipid, carbohydrate) required for activity; plantaricin S, leuconocin S, lactocin 27, pediocin SJ-1 [57–61]. The existence of this fourth class was supported by the observation that some bacteriocin activities were destroyed by glycolytic and lipolytic enzymes [60]. However, such bacteriocins have not yet been characterized adequately at the biochemical level and the recognition of this class therefore seems to be premature. The class IIC of the Klaenhammer [21] classification has recently been shown not to exist.

2 Mode of Action

The class I bacteriocin nisin and some of the class II bacteriocins have been shown to be membrane-active peptides that destroy the integrity of the cytoplasmic membrane via the formation of membrane channels (Fig. 1). In doing so, they alter the membrane permeability and therefore cause leakage of low molecular mass metabolites or dissipate the proton motive force, thereby inhibiting energy production and biosynthesis of proteins or nucleic acids [1, 62]. Most bacteriocins produced by lactic acid bacteria display a bactericidal effect on the sensitive cells, all or not resulting in cell lysis [63–67]. On the other hand, other bacteriocins, such as lactocin 27 [68], leucocin A [69] and leuconocin S [59] have been reported to act bacteriostatically. However, the designation of lethal versus static effect can be dependent upon aspects of the assay system, including the number of arbitrary units, the buffer or broth, the purity of the inhibitor, and the indicator species and cell density used [1]. The mode of action of numerous bacteriocins has been reported and, therefore, only a few of them, representing the different classes are described in this section.

The class IA_1 lantibiotic nisin was shown to form ion-permeable channels in the cytoplasmic membrane of susceptible cells, resulting in an increase in the membrane permeability, disturbing the membrane potential and causing an efflux of ATP, amino acids, and essential ions such as potassium and magnesium. Ultimately, the biosynthesis of macromolecules and energy production are inhibited resulting in cell death. Nisin does not require a membrane receptor but requires an energized membrane for its activity, which appeared to be dependent on the phospholipid composition of the membrane [67].

Lactococcin A can alter the permeability of the *L. lactis* cytoplasmic membrane leading to the loss of proton motive force and leakage of intracellular ions and constituents [65, 70]. LcnA acts in a voltage independent manner on intact cells or membrane vesicles, but not on liposomes suggesting that a specific membrane receptor is required for LcnA recognition and action [65, 70].

Analogously, the antimicrobial activity of Las5 was not dependent on an energized membrane, but required a trypsin-sensitive protein receptor to elicit bactericidal action on protoplasted cells [64, 70].

The voltage independent activity of lactococcin B, similar to thiol-activated toxins, was proposed to be dependent on the reduced state of its unique cysteine residue on position 24 [71]. Recently, it was shown by means of protein engineering that the Cys-24 residue was not necessary for activity of lactococcin B [28]. Lactococcin G is a novel lactococcal class IIB bacteriocin whose activity depends on the action of two peptides [47]. The combination of the α and β peptide dissipated the membrane potential, induced a dramatic decrease in the cellular ATP level, and resulted in a rapid efflux of potassium [72].

The class IIA pediocins PA-1/AcH and JD were reported to exhibit their bactericidal action at the cytoplasmic membrane and to cause a collapse of the pH gradient and proton motive force [66, 73]. Furthermore, a leakage of K^+, UV-adsorbing materials, permeability to ONPG, and in some cases cell lysis, although not attributed to the primary pediocin AcH action were observed [66,

74]. Pediocin PA-1 was shown to dissipate the proton motive force and inhibit the amino acid transport in sensitive cells [75]. Lipoteichoic acid is essential for non-specific pediocin AcH binding, and sensitive cells present a specific receptor that potentiates contact with the membrane [17, 66]. Pediocin PA-1 displays an important *N*-terminal -Y-G-N-G-V-X-C- consensus common with other *anti-Listeria* bacteriocins such as sakacin A (= curvacin A) and P, and leucocin A. This finding suggests an important role of the *N*-terminus in either the recognition and/or activity of the pediocin-like bacteriocins.

The mechanism of action of the class III bacteriocins remains to be elucidated [21].

In general, the secondary structures of membrane-active peptides play a significant role in their biological activity [76]. For several of the membrane active bacteriocins, the presence of amphiphilic α-helices or β-sheets which form a hydrophobic and a hydrophilic face has been predicted [43, 47, 70]. These features suggest that lateral oligomerization of peptide monomers occurs in the membrane according to the so called barrel-stave mechanism with the hydrophobic side facing the membrane and the hydrophilic side forming the pore of the channel (Fig. 1) [21]. In case of a class IIB bacteriocin (lactococcin M, G, plantaricin S, lactacin F), of which the activity depends on the complementation of two molecules, a two-component poration complex is predicted [21, 47, 65, 77].

The need of a receptor, present in the target membranes of bacteriocin susceptible organisms has been extensively studied for microcin 25, produced

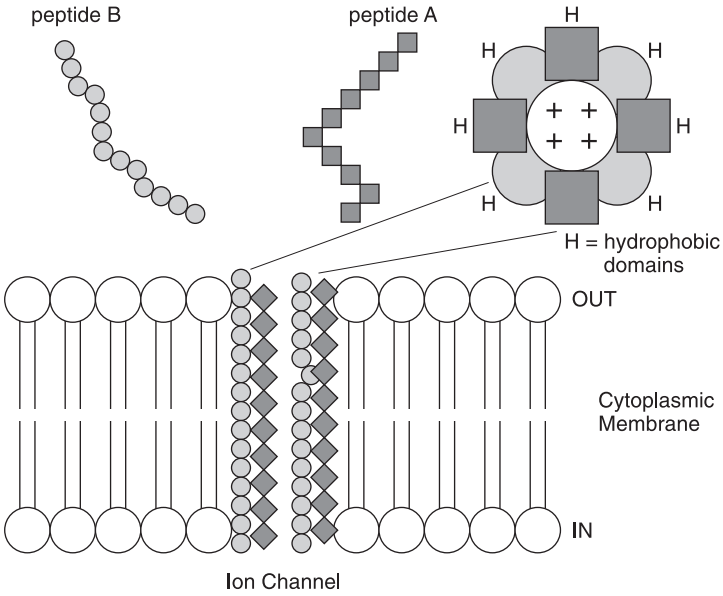

Fig. 1. Barrel-stave poration complexes proposed for class II bacteriocins. Complexes may be formed between one or two amphiphilic peptides which oligomerize and form membrane pores and ion channels [21]

by members of the *Enterobacteriaceae* [78]. Selection of spontaneous mutants for insensitivity to the peptide antibiotic microcin 25 led to the isolation of five categories of mutations, located in the *fhuA*, *exb*, *tonB* and *sbmA* genes [79]. The latter three are all proteins of the cytoplasmic membrane, whereas FhuA is a multifunctional protein of the outer membrane. [78, 79]. The region of FhuA, which is important of microcin 25 interaction has subsequently been mapped [80]. Several of these mutants showed an additional resistance to colicin M, colicin B, and to bacteriophages T1 and Φ80 [79]. These results indicate that microcin 25 interacts with an extracellular domain of the multifunctional receptor FhuA, and is imported through the TonB pathway and the SbmA protein [79].

In conclusion, pore formation in the cytoplasmic membrane seems to be a common mode of action of those LAB bacteriocins for which the mode of action has been determined. Some of the class II bacteriocins (lactococcin A, B, G and lactacin F) require a specific receptor molecule for adsorption, whereas nisin also acts on liposomes and exerts a receptor-independent action. Differences between narrow or wide host-range bacteriocins seem to be correlated with this aspect of a specific receptor, needed for activity. However, which bacteriocin domains confer binding specificities to lipid, protein, or reactive groups remain to be elucidated.

3
Genetics of Bacteriocins Produced by Lactic Acid Bacteria

3.1
Nisin, the Most Prominent Member of the Class IA_I Lantibiotics

The class I bacteriocins, the so-called lantibiotics, contain the posttranslationally modified amino acids lanthionine and methyl-lanthionine and their precursors dehydroalanine and dehydrobutyrine [39, 81, 82]. Nisin is a pentacyclic class IA_I lantibiotic consisting of 34 L-amino acids, including two dehydroalanine residues (positions 5 and 33), a dehydrobutyrine residue (position 2) and five intramolecular thio-ether lanthionine (residues 3–7) and methyl-lanthionine (residues 8–11, 13–19, 23–26, 25–28) bridges (Fig. 2). Two different forms, nisin A and nisin Z were shown to differ in only one amino acid residue [83]. During maturation, a 23-residue leader peptide is cleaved from a 57-residue precursor molecule to result in the mature bactericidal peptide of 34 amino acid residues. Many of these lantibiotics are produced by non-lactic acid bacteria, such as *Staphylococcus*, *Bacillus*, *Streptococcus*, *Actinoplanes*, *Streptomyces*, *Streptoverticillium* [1, 22]. Some of them, for instance subtilin, Pep5, and epidermin have been genetically studied in detail [29, 84–89]. The organization of the genetic determinants is comparable to that of nisin, produced by *Lactococcus lactis* subsp. *lactis* [22, 29, 84, 86–88, 90–93].

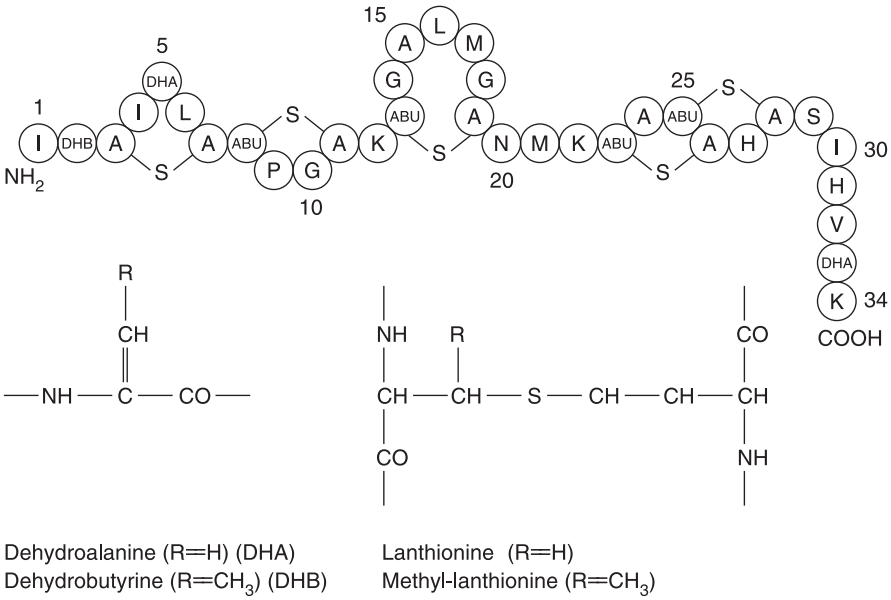

Fig. 2. The primary structure of nisin, a representative of the class IA_I lantibiotics

3.2 Conjugative Transposition of the Sucrose-Nisin Gene Cluster

A genetic linkage between nisin production, nisin immunity and the ability to use sucrose as a carbon source, was corroborated by the observation that these properties were transferred in a conjugation-like process [94]. It appeared that nisin and sucrose genes were clustered on chromosomal elements that were conjugative transposons [32, 37, 95, 96].

The best characterized conjugative sucrose-nisin transposons are the 70-kb *Tn5276* and *Tn5301* [95, 97]. The conjugative transposon *Tn5276* and Tn5301 [95, 97] has been found to display a RecA-independent insertion in at least five different chromosomal sites in derivatives of the *L. lactis* strain MG1363, but a single insertion site was preferred and integration of *Tn5276* occurred in a single orientation [97]. The organization of the *Tn5276* is given in Fig. 3. The insertion sequence *IS1068* at the left end of *Tn5276* was described as an *isoIS904* element because of its similarities with *IS904*, present at the same location in an other sucrose-nisin conjugative element, *Tn5301* [32, 37]. Sucrose-nisin conjugative elements lacking this *IS1068* still showed efficient conjugative transposition [37]. It is more likely that the *xis*/*int* genes found at the right end of *Tn5276* and shown to be required for the recA-independent excision of *Tn5276* ends in *E. coli*, are involved in site-specific insertion of *Tn5276* in *Lactococcus* [37, 98]. The *int* gene could encode a protein of 379 amino acids with homology to various integrases [98]. The *xis* gene encodes a small basic protein that enhances the excision process of *Tn5276* [98]. Similar genes are located at the ends of the conjugative transposons *Tn1545*/*Tn916* [99].

3.3
Genetic Organization of the Sucrose-Nisin Transposon TN5276

The nisin gene cluster *nisABTCIPRKFEG* of approximately 15 kb includes eleven different genes (Fig. 3) [40, 91, 92, 95, 96, 100–103]. Except for *nisK*, all other genes were essential for nisin production or immunity [92, 101, 103, 104]. The structural *nisA* and the *nisI* gene were shown to be both necessary for producer strain immunity [92]. The *nisP* gene encodes a subtilisin-like serine protease [101], whereas the *nisB* and *nisC* genes are very conserved in other lantibiotic operons and therefore very likely to encode proteins involved in the post-translational modification reactions of lantibiotics [40]. *NisT* belongs to the ABC family of exporter proteins, involved in ATP-dependent secretion [105, 106]. The proteins corresponding to the *nisR* and *nisK* genes, display homology to the well-known two-component signal transduction regulator and sensor-kinase proteins [101, 107, 108].

The *nisFEG* gene cluster downstream from *nisABTCIPRK* appeared to be involved in immunity to nisin besides the *nisA* and *nisI* gene products [104]. *NisI-NisF* is homologous to an ABC transporter of *Bacillus subtilis* and the MbcF-MbcE transporter of *E. coli*, which are involved in subtilin and microcin B17 immunity, respectively [104, 109, 110]. *NisG* displays homology with pre-

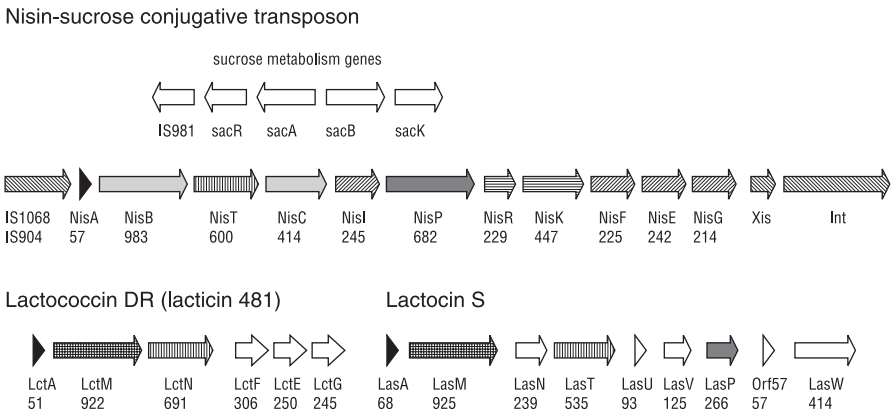

Fig. 3. Representative lantibiotic gene clusters. A representative of the class IA_I leader peptides included in this figure is nisin [40, 92, 103, 104, 107]. A representative of the class IA_{II} lantibiotics included in this figure is lactococcin DR (lacticin 481) [118]. The lactocin S leader does not follow the class IA_I or the class IA_{II} rules and might therefore represent a new maturation pathway [40, 82]. The structural genes are highlighted as black arrowheads. Genes with similar proposed function or substantial sequence similarity are highlighted in the same manner. The number of amino acids encoded by each gene is indicated below the arrowheads, which indicate the direction of transcription. The function of several genes in the lactocin S gene cluster is unknown (*lasN*, *lasU*, *lasV*, *lasW* and *orf57*). The suffix A is used to indicate the structural genes, the suffix T is used for ABC transporters, and the suffixes B, C and M indicate the potential modification enzymes. *NisI* is the nisin immunity protein, MsR and NisK constitute the two-component signal transduction system, and NisFEG forms a heteromer involved in nisin immunity as an ABC exporter

dominantly hydrophobic proteins with three or four potential transmembrane domains, described to play a role in immunity to colicins [111, 112]. Approximately 1 kb downstream from nisG, three more open reading frames with an opposite orientation were observed. The largest protein of 318 amino acids shows similarities with the helix-loop-helix type of DNA binding proteins, and its *N*-terminus of 27 amino acids was identical to the SacR regulatory protein [104]. The two further open reading frames showed homology to the lactococcal insertion element, IS981 [104].

Based on DNA homology, five different promoter regions were identified in the nisin gene cluster in front of *nisB*, *nisT*, *nisC*, *nisR* and *nisF* and two potential transcription terminators downstream from *nisB* and *nisK* [100, 104, 113, 114]. The promoter preceding *nisA* and the promoter upstream from *nisR* [103] both display characteristics of positively regulated promoters [114]. The intergenic region between *nisA* and *nisB* contains an inverted repeat that could act as a transcription terminator [91], transcription attenuator [103], or a signal for internal processing between the *nisA*/Z and *nisB* gene [115]. Recent studies showed that the *nisZBTCIPRKFEG* gene cluster consists of at least two operons resulting in a *nisZBTCIPRK* and a *nisFEG* transcript [115, 116]. The *nisZBTCIPRK* transcript is processed downstream from the structural *nisZ* gene [115]. Both promoters were inducible by raising of extracellular nisin concentrations, suggesting that nisin biosynthesis and immunity were autoregulated [115].

The *sac* genes, encoded by the *Tn5276* conjugative transposon in the opposite orientation of the nisin gene cluster are involved in sucrose metabolism and organized in two divergent operons with a back-to-back configuration [97]. Both operons are controlled by a sucrose-inducible promoter [97] and result in a rightward transcript, containing the *sacBK* (sucrose-specific PTS enzyme II and a putative fructose kinase) genes and a leftward transcript, containing the *sacAR* (sucrose-6-phosphate hydrolase and a putative regulator) genes [103].

3.4 The Class IA_{II} Lantibiotics Lactococcin DR and Lactocin S

Besides nisin, three other lantibiotics produced by LAB, namely lactococcin DR (= lacticin 481) [30, 117–119], lactocin S [31, 33, 82] and carnocin U-I49 [35, 36, 120] have been reported, but only the first two were genetically studied in more detail (Fig. 3) [82, 118–121]. Both lactocin S and lactococcin DR are members of the class IA_{II} lantibiotics. Lantibiotics of this class have a divergent leader peptide compared with the class IA_I lantibiotics represented by nisin, but also their genetical organization differs significantly from that of the class IA_I lantibiotics [40, 82].

Downstream from the structural lacticin 481 (= lactococcin DR) gene, *lctA* (= *lcnDR1*), *lctM* (= *lcnDR2*) encoded a protein of 922 amino acids [82, 118]. A 925-residue protein (LasM) with striking homology to LctM, was encoded downstream from the structural lactocin S gene [82], and cylM, a 993-amino acid residue protein was shown for the non-lactic acid bacteria lantibiotic, cytolysin [122]. This protein family is typical for the class IA_{II} lantibiotics, and

no homologues were found yet in any other bacteriocin operon [40]. The *C*-terminus of LctM was shown to display striking homology with NisC, proposed as a post-translational modification enzyme for nisin [118, 123]. Both the lactococcin DR and the lactocin S operons contain coding information for an ABC transporter protein (LctT (= LcnDR3), LasT) [82, 118]. For lactocin S, the structural *lasP* gene encodes a proteinase of 266 amino acid residues [82]. The lactocin S gene cluster contains several other genes (*lasN*, *U*, *V*, *W* and *orf57*) without putative function or homologous counterparts in other bacteriocin operons [82].A similar cluster was recently identified for lacticin 481 [119]. The proteins encoded by IctF, IctE and IctG were proposed to form an ABC transporter and should play some role in the immunity against lacticin 481.

3.5
Class II Non-Lantibiotic Bacteriocins

3.5.1
Introduction

The class II non-lantibiotic bacteriocins consist of a large heterologous group produced by different species of the lactic acid bacteria [21]. Despite this heterogeneity, all class II bacteriocins display a very conserved *N*-terminal leader peptide and a characteristic double-glycine-type ($Gly^{-2}Gly^{-1}Xaa$) proteolytic processing site [21]. The conserved mechanism of secretion and processing suggested by these findings is reflected in the organization of the operon structures encoding these bacteriocins (Fig. 4). The genetic determinants involved in the production of several class II bacteriocins have been genetically studied in detail (Fig. 4): lactacin F produced by *Lactobacillus johnsonii* [48, 124], lactococcin G, and the lactococcin A, B and M gene cluster of *Lactococcus lactis* [125–128], leucocin *A-UAL* produced by *Leuconostoc gelidum* [42, 129] and mesentericin Y105 produced by *Leuconostoc mesenteroides* [124], the identical bacteriocins pediocin PA-1.0 and pediocin AcH produced by *Pediococcus acidilactici* [130–132], sakacin A produced by *L. sake* [133] and plantaricin A, produced by *L. plantarum* [49, 134] (Fig. 4).

3.5.2
The Lactococcal Bacteriocins, Lactococcin A, B and M

The lactococcin A, B, and M operon cluster on the 60-kb conjugal plasmid p9B4–6, was the first class II bacteriocin genetic determinant to be analyzed. Two fragments, conferring bacteriocin production and immunity, were cloned [46, 135]. The lactococcin A operon encoded one bacteriocin (LcnA) and an immunity protein (LciA), and expressed a high antagonistic activity against *L. lactis* indicator strains.

In the lactococcin M operon, two bacteriocins (LcnM and LcnN) and one immunity protein (*LciM*) were found, displaying a low antagonistic activity against *L. lactis* indicator strains [46, 135]. In the lactococcin M operon, disruption of both *lcnM* and *lcnN* resulted in a non-producer phenotype. Therefore,

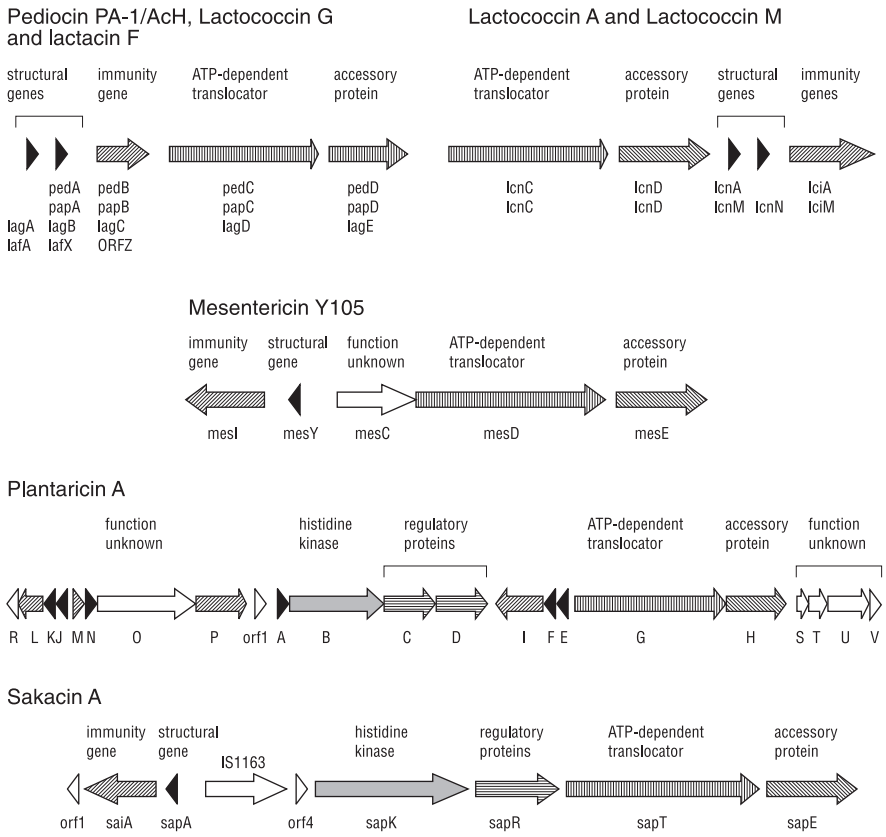

Fig. 4. Representative class II non-lantibiotic bacteriocin operons. The class II non-lantibiotic bacteriocins include lactococcin A, B, M and G [46, 125, 126, 128, 135, 136], lactacin F [77], pediocin PA-1 and AcH [62, 130, 131], mesentericin Y105 [124], sakacin A [133] and plantaricin A [49, 134]. The structural genes are high1ighted as black arrowheads. Genes with similar proposed function or substantial sequence similarity are highlighted in the same manner. The arrowheads indicate the direction of transcription. The function of MesC, and SapOSTUV of the mesentericin Y150 and plantaricin A operons is unknown

two gene products are required for activity, and lactococcin M was classified as a class IIB bacteriocin according to the classification of Nes et al. [128] (1995). Cloning and sequence analysis of the region downstream from lactococcin A identified a third lactococcin operon, designated as *LcnB* and its immunity protein *LciB* [136]. Site-directed mutagenesis showed that *LciA* and *LciM* were essential for producer strain immunity but did not cross-protect against the other bacteriocins [46]. Lactococcin A, produced by *L. lactis* subsp. *cremoris* LMG2130 was independently purified and sequenced [125]. The structural gene was mapped to a 55-kb conjugal plasmid of the producer strain and DNA sequencing revealed that this lactococcin was identical to the high activity lactococcin *A*, cloned and sequenced by van Belkum et al. [135].

Completion of the current genetic view of the lactococcin system was provided by Stoddard et al. [126]. The production of a bacteriocin of *L. lactis* subsp. *lactis* biovar *diacetylactis* WM4 was linked to a 131-kb plasmid *pNP2* [137]. DNA sequence of a 5.6-kb AvaII fragment of *pNP2* revealed two large open reading frames upstream from the *lcnA* and *lciA* genes, designated as *lcnD* (716 amino acids) and *lcnC* (474 amino acids) [126]. *Tn5* insertional mutagenesis of both *lcnD* and *lcnC* disrupted lactococcin *A* production without affecting immunity [126]. LcnC displayed highest homology with the HlyB-like family of ATP-dependent membrane translocators (Stoddard et al., 1992). It contains a highly conserved ATP-binding site and six *N*-terminal hydrophobic domains, which could promote integration in the cytoplasmic membrane. LcnD showed structural similarities to proteins of the HlyD and PrtE secretion systems of *E. coli* [126]. The carboxy-terminal part of LcnD was also encoded by the partial open reading frames upstream from the structural *IcnA* and *IcnM* genes of the 60-kb plasmid p9B4–6 [127]. The three lactococcin operons were preceded by conserved and functional promoter regions. The promoter upstream from *lcnA*, overlapped with a 19-bp inverted repeat. This palindromic sequence with a $\Delta G = -9.9$ kcal/mol could form a hairpin and therefore may resemble an SOS box for binding of the *Escherichia coli* RecA-sensitive *LexA* repressor [125]. However, *lcnA* has not been found to be inducible [125]. Stoddard et al. [126] noted that there were no obvious transcription terminators positioned between lcnC and lcnA, suggesting a possible read through in the *lcnA*/*lciA* operon, and speculated that besides a large transcript covering all four genes, a smaller transcript spanning *lcnA* and *lciA* was produced.

The transporter proteins LcnD and LcnC were shown to be essential for lactococcin A production but not for immunity [126]. Secretion systems based on such ATP-binding exporters have been reported for both Gram-negative and Gram-positive bacteria for export of extracellular proteins whose secretion does not depend on the general signal peptide-dependent export pathway [111, 138]. Such examples for Gram-negative bacteria include the haemolysin and colicin V proteins of *E. coli* [139, 140] cydolysin produced by *Bordetella pertussis* [141], leucotoxin produced by *Pasteurella haemolytica* [142], and metalloproteases B and C of *Erwinia chrysanthemi* [143]. For Gram-positive bacteria, these proteins have been described for ATP-dependent membrane translocation, for instance required for competence in *Streptococcus pneumoniae* [144]. It was therefore likely that a universal export apparatus, involved in class II bacteriocin secretion could consist of these two exporter proteins. Genetic analysis revealed that in the case of the identical *Pediococcus* bacteriocins, pediocin PA-1.0/AcH, the *Lactococcus* bacteriocin lactococcin G, the *Leuconostoc* bacteriocin mesentericin Y105, and the *Lactobacillus* bacteriocins sakacin A and plantaricin A an ATP-dependent ABC exporter apparatus was encoded adjacent to the bacteriocin operon [124, 127, 128, 130–132, 136, 145, 146] (Fig. 4).

3.5.3
The Class IIA Bacteriocins Pediocin PA-1/AcH and Mesentericin Y105

Although the class IIA, anti-listerial bacteriocins pediocin PA-1/AcH and mesentericin Y105, are produced by *Pediococcus* and *Leuconostoc* sp., respectively, they are nevertheless genetically organized in a way almost identical to the lactococcal bacteriocins (Fig. 4) [124, 130, 131]. MesC, a 137-amino acid protein upstream mesD in the mesentericin Y105 gene cluster shows no homology with known protein [124].

3.5.4
The Lactobacillus Bacteriocins Sakacin A and Plantaricin A

Axelsson et al. [147] (1993) shotgun cloned the plasmid fraction of *L. sake* Lb706 directly in a sakacin A non-producing and sensitive variant *L. sake* Lb706-B. One of the two clones, necessary for the restoration of immunity encoded a 430-amino acid residue protein designated as *SakB* [147]. Hybridization and sequence analysis revealed that *sakB* complemented a mutated copy of *sakB* present in *L. sake* Lb706-B. The gene mapped 1.6 kb from the structural sakacin A gene on the 60-kb plasmid. Further investigation showed that SakB was part of a two-component, bacterial signal transduction system, adjacent to the sakacin A operon [133]. SakB was renamed SapK, and showed striking homology to the *Staphylococcus aureus* AgrC histidine protein kinase (HPR). A second member of the two-component signal transduction apparatus, SapR, was encoded downstream from SapK. The SapR protein has homology to AgrA, a member of the response regulator (RR) family [108, 133, 148].

A comparable signal transduction system was found to be encoded in the same operon as the structural plantaricin A gene (*plnA*), and was transcribed as a 3.3 kb *plnABCD* messenger [134]. PlnB, PlnC, and PlnD showed highest homology to their counterparts in the *agr* (accessory gene regulatory) two-component regulatory system of *Staphylococcus aureus* [134, 149, 150]. PlnB showed highest homology to the histidine protein kinase family and is predicted as an integral protein of the cytoplasmic membrane with six transmembrane domains located in its *N*-terminus [134]. *PlnC* and *PlnD* are very homologous and corresponded to the response regulator family protein of the *Staphylococcus aureus agr* locus [134, 149, 150]. Additionally, recent findings suggest that two bacteriocins of the two-peptide type (PlnJ and PlnK of the *plnJKLR* operon and *PlnE* and *PlnF* of the *plnEFI* operon) and a bacteriocin of the one-peptide type (*PlnM* of the *plnM*NOP operon) were located adjacent to the *plnABCD* cluster and could hence be responsible for bacteriocin activity [49]. *PlnI* (257 amino acids), *PlnP* (247 amino acids), and *PlnL* (138 amino acids) encode hydrophobic proteins with three (*PlnL*) and seven (*PlnI* and *PlnP*) transmembrane domains, respectively. In the case of *PlnI* and *PlnL*, these proteins are encoded in the 3′ end immediately downstream from the bacteriocin determinants, following the conserved genetic organization of all two-component bacteriocins described up to now [49, 77, 128, 136]. PlnP however, is separated from the bacteriocin genes by *plnO*, an open reading frame of 399

amino acids [49]. However, based on its striking homology with PlnI, it could also be considered as an immunity protein for PlnN [49]. Upstream from *plnN*, a 66 amino acid protein, PlnM with one putative transmembrane helix was found. Besides PlnP, PlnM could hence be considered as a second valid candidate for PlnN immunity, although located in the 5′ end of the operon. Two proteins, PlnG (ABC transporter) and plnH (accessory protein), shown to constitute an ATP-dependent transport apparatus, were located downstream of the *plnABCD* operon [49]. The homologous counterparts in the sakacin A operon were named SapT and SapE, respectively [133]. The region encoding plantaricin A activity has proven to be a multiple gene locus consisting of not less than 22 different open reading frames in the same or opposite orientation to the previously described *plnABCD* operon [49, 134]. Besides the above described proteins, PlnROSTUV and orf1 display no homology with other protein sequences, and their function in plantaricin A activity has not been elucidated [49].

The sakacin A region was transcribed as two operons: the first one encompassed the structural sakacin A gene *sakA*, and its immunity factor SaiA, and the second covered the *sapK*, *sapR*, *sapT* and *sapE* genes involved in transcription regulation and sakacin A export [133]. Northern blot analysis revealed that the putative SapR/SapK system probably acted as a transcription activator [133]. A 35-bp region, upstream from the putative *sapA* promoter, and a similar sequence upstream from *sapK* were necessary for proper expression and could be possible targets for transcriptional activation [133]. Five promoters (upstream from *plnA*, *plnE*, *plnJ*, *plnM* and *plnG*) and six rho-independent transcription terminators (downstream from the operons *plnABCD*, *plnJKLR*, *plnMNOP*, *plnEFI* and the ORFs *plnF* and *plnN*) have been mapped in the plantaricin A cluster, resulting in a complex expression pattern [49, 134]. The −10 consensus sequences were located 6 to 7 bp upstream from the transcription start site, but the −35 consensus was more difficult to identify [49]. Just upstream from the putative − 35 region, all promoters were seen to harbor two direct repeats spaced by an A + T-rich strand of 12 bp [49].

3.5.5
Class IIB Bacteriocins

Two bacteriocins and an immunity protein, organized in one operon, comparable with the lactococcin M operon, were detected in the case of lactacin F, lactococcin *G*, and in two operons (*plnEFI* and *plnJKLR*) of the plantaricin A gene cluster. All four depend on complementation of two bacteriocin peptides for highest activity and therefore belong to the class IIB bacteriocins [46, 49, 124, 128].

Purification of lactococcin G identified two peptides, α and β, that individually exhibited marginal levels of activity. Upon complementation of the two peptides in a 7α:1β ratio, a seven-fold increase in activity was noted [47]. Allison et al. [48] proved that lactacin F activity and host range were expanded upon the complementation of two heterologously expressed peptides of the lactacin F operon, although initially only one bioactive peptide (LafA) was purified

from the *L. johnsonii* VPI11088 fermentation supernatant using *L. helveticus* NCK338 as an indicator organism [151]. Therefore, the lafA gene product, LafA, is a bacteriocin that kills *L. helveticus* NCK338. Expansion of the host range to include *L. delbrueckii* and *Enterococcus faecalis* occurs only after the interaction of LafA and LafX. The need of complementation of two bacteriocins for optimal activity is reflected in the presence of two bacteriocins encoded in the same operon and, together with lactococcin M [46], lactacin F [77], plantaricin A [49] and lactococcin G [128] they are the only class IIB bacteriocins determined on the genetic level.

Plantaricin A was shown to be dependent on complementation of two almost identical peptides which differed only in one *N*-terminal alanine residue [152]. Therefore, plantaricin A was the first known class IIB bacteriocin not to be encoded by two different adjacent structural bacteriocin genes [134]. When the plantaricin A gene cluster was genetically analyzed only one structural gene encoding plantaricin A was detected [134]. Detailed analysis of the plantaricin A genetic determinants revealed that plantaricin A acts as an inducer peptide of an agr-like signal transduction system and does not possess any bacteriocinogenic activity [49, 134, 145]. Recent findings confirmed that bacteriocin activity is most likely encoded by two two-peptide type and one one-peptide type bacteriocins adjacent to the *plnA*BCD operon [49, 134]. The proteins that constitute the production and maturation machinery of class IIB bacteriocins do not differ significantly from the other class II bacteriocins, as deduced from the lactococcin M and G, and plantaricin A operons [127, 128, 134, 145].

4 Immunity and Resistance Towards Bacteriocins

Three important phenotypes can confer non-sensitivity to bacteriocins: (i) immunity is genetically linked with bacteriocin production and exerts the strongest level of non-sensitivity, (ii) resistance can occur as the appearance of spontaneous mutants following selection on the bacteriocin; and (iii) resistance conferred by a gene that is not genetically linked with bacteriocin production. These three categories of resistance are likely to be similar for any bacteriocin [21].

The genetic determinant for nisin immunity has been defined as *nisI*, the fifth gene encoded in the nisin operon [92, 101, 153]. The entire NisI protein showed no significant similarities to other proteins, but its *N*-terminus strongly resembles that of signal peptide sequences of lipoproteins from Gram-negative *E. coli* and Gram-positive *Bacillus* and *L. lactis* [92, 107, 154]. Bacterial lipoproteins are a group of exported proteins that are anchored to the cellular or outer membrane by lipid moieties. The lipids are covalently linked to the cysteine residue located at the *N*-terminus of the secreted protein [92]. Furthermore, the typical consensus sequence of the cleavage site and the tripartite structure of signal peptides is also found in the *N*-terminus of NisI [92, 107]. NisI therefore is a membrane-bound lipoprotein located on the outside of the cell membrane [92, 107, 155]. Similar results have been described for the only other immunity protein reported thus far for a lantibiotic, i. e. PepI, which

is encoded by the *pep5* operon [85, 156, 157]. The mechanism of immunity conferred by the NisI protein remains very speculative. The lipoprotein NisI could, when attached to the exterior of the cellular membrane by lipid moieties, confer immunity by direct interaction with extracellular nisin or by disturbing the association of nisin aggregates, thus preventing channel formation [107].

For all class II bacteriocins genetically studied until now, a protein conferring immunity to the producer organism was encoded in the 3′ end of the bacteriocin operon, for example lactococcin A [46], lactococcin B [136], lactococcin M [46], lactococcin G [128], pediocin PA-1 and AcH [130, 131], mesentericin Y105 [124], carnobacteriocin B2 and BM1 [158], leucocin UAL-187 [42], plantaricin A [49] and sakacin A [133]. These immunity proteins have a high pI [49]. Furthermore, those associated with two-peptide bacteriocins consist of 110 to 154 amino acids containing several transmembrane domains [65, 77, 128], while those of the one-peptide bacteriocins are generally smaller (51 to 113 residues) and contain few (one or two) or no putative transmembrane helices [65, 130, 133, 136, 158, 159]. Recently, a new class of immunity proteins was reported consisting of 247 to 257 residues spanning the cytoplasmic membrane seven times [49].

Based on these findings, it seems that an important group of the immunity proteins exert their activity at the cytoplasmic membrane, although lciA is the only immunity protein studied in detail. The lactococcin A immunity factor was purified and shown to interact with the cell membrane, whereas the presence of free intracellular lciA is considered as a reservoir of immunity factor protein [161]. LciA may span the membrane once by virtue of an α-amphiphilic helix between residues 29 and 47 [162]. Topological studies showed that the carboxy-terminus of LciA was orientated at the outside of the cytoplasmic membrane [162]. LcnA acts on intact cells or membrane vesicles, but not on liposomes suggesting that a specific membrane receptor is required for LcnA recognition and action [65, 162]. Membrane vesicles are protected from LcnA action if they are derived from cells expressing LcnA immunity. Exposing lactococcin A-sensitive cells to excess of the immunity protein did not affect the LcnA-induced killing of the cells, indicating that the immunity protein does not protect cells by simply binding to lactococcin A, or to externally exposed domains of the cell surface [161]. Comparable results were reported for carnobacteriocin immunity factors [158]. This suggests that LcnA immunity occurs at the cytoplasmic membrane via a mechanism that either blocks a receptor, prevents LcnA channel formation, or inactivates the bacteriocin [62, 65]. The cell localization and mode of action of immunity proteins without apparent potential membrane-spanning helices is not yet known, although membrane association of such proteins can not be excluded. Interestingly, two such proteins MesI and PedB display an almost identical hydrophobicity plot, suggesting a common mode of action.

Recently, three additional open reading frames, *nisF*, *nisE* and *nisG*, were revealed adjacent to *nisK* [104]. A comparable gene cluster, *epiFEG* has been described for the lantibiotic epidermin, produced by *Staphylococcus epidermidis* [163]. The NisE/EpiE and NisG/EpiG proteins are both predominantly hydrophobic with six transmembrane domains [104, 163]. The NisF/EpiF com-

ponent contains two potential ATP-binding consensus sites [104]. The proteins encoded by these operons resemble the *E. coli* MalFGK2 and HisMQP2 transporters [163–165] and the SpaFG and McbFE proteins, which are involved in immunity against subtilin [110] and microcin B17 [109], respectively. The hydrophobicity plot of NisF and NisE together resembles that of the complete SpaF protein [104]. It was therefore proposed that NisF and NisE constitute the transmembrane and ATP-binding domains of an ATP-dependent translocator [104]. Based on homologies with colicin immunity proteins, NisG was predicted to have a similar function in nisin immunity [104]. In the case of epidermin, EpiE and EpiG were, based on mutual homology, both predicted as ABC transporter membrane components with six potential membrane-spanning helices, a common feature of these transporter systems [163]. EpiEGF2 were therefore thought to act as a hetero-tetrameric complex, including EpiG, in comparison with the well-characterized MalFGK2 and HisMQP2 transporters [163–165] but in contradiction with the postulated function for NisG [104]. Immunity conferred by this ABC secretory system could be mediated by active extrusion or by their uptake and intracellular degradation [104, 163].

A gene, *nsr*, conferring resistance against nisin has been isolated from *L. lactis* subsp. *lactis* biovar *diacetylactis* DRC5, which is a nisin-nonproducer [166, 167]. Nsr is a 318-amino acid residue protein with a hydrophobic *N*-terminus, resulting in membrane association. The level of resistance conferred by Nsr was only 10% of the immunity of the nisin producer strain [107]. The nsr gene did not hybridize with genomic DNA of the nisin producer strain *L. lactis* subsp. *lactis* ATCC 11454, demonstrating that the genetic determinants for immunity and resistance are different, as are their expected mechanisms of action [167]. Although nisin resistance has been reported among a variety of Gram-positive bacteria [34], in the only case studied up to now, *B. cereus* produced a nisin reductase that presumably inactivated one or more of the dehydroresidues required for nisin activity [168, 169].

5
Biosynthesis of Bacteriocins Produced by Lactic Acid Bacteria

5.1
Response Regulation

Many of the bacterial metabolic pathways are induced by various extracellular stimuli. Those environmental conditions are sensed and signaled through, by means of signal transduction systems. Many of these systems consist of two components, a sensor, often located in the cytoplasmic membrane and a cytoplasmic response regulator [108, 170–172]. They are therefore generally called two-component systems. The environmental sensor acts as a histidine protein kinase (HPK) and modifies the response regulator (RR) protein, which in turn triggers an adapting response, in most cases by gene regulation. Most histidine protein kinases consist of an *N*-terminal sensory domain and a cytoplasmic *C*-terminal transmitter. The latter contains an autokinase domain and a conserved histidine residue as a site for phosphorylation. Both domains

are linked by membrane-spanning segments [28]. Most response regulator proteins contain an *N*-terminal aspartic residue as a site for phosphorylation and a *C*-terminal output domain involved in mediating an adaptive response [28]. Response regulators bind as dimers to a specific site (mostly direct or inverted repeats) present near the promoter, thereby stimulating or inhibiting binding of the RNA polymerase to the promoter region [173–177]. Interestingly, direct repeats referred to as potential binding sites for response regulator dimers have been reported upstream from the promoters of the different operons involved in the production of several inducible bacteriocin promoters, suggesting a common positive mechanism of regulation for bacteriocin production [49].

Such a regulatory operon, encoding an inducer peptide (plantaricin A), a histidine protein kinase with six transmembrane domains (PlnB) and two regulatory proteins (PlnC and PlnD) has been reported for plantaricin A [49, 134]. Genes for a histidine protein kinase (*nisK*, *sakK*) and a response regulator (*nisR*, *sakR*) were also found in the locus encoding sakacin A, sakacin P, carnobacteriocin A and nisin production [28, 92, 101, 103, 107, 133].

PlnB/SakK, PlnC/SakR and PlnD show highest homology with their counterparts in the *agr* (accessory gene regulatory) system of *Staphylococcus aureus* [134, 149, 150]. The biosynthesis of extracellular proteins which are subject to growth phase-dependent control and play an important role in staphylococcal infection are regulated by the *agr* locus, which consists of two divergent operons [134, 148, 178–180]. The first transcription unit encoded AgrA (RR), AgrC (HPK) and AgrD. An octapeptide processed from AgrD, is involved in activation of the *agr* locus [49, 181]. Activation of the *agr* operon also results in a higher transcription level of *hld*, which in turn is responsible for the *agr*-dependent regulation of the above mentioned extracellular toxins and enzymes [134, 180]. Although initially purified, and characterized as a bacteriocin depending on the complementation of two almost identical peptides, it is now believed that plantaricin A is not a bacteriocin but acts as an *agr*-dependent inducer molecule [47, 49, 134, 145]. Extracellular addition of plantaricin A to a Bac^- mutant restored transcription of the different units invo1ved in bacteriocin production as well as antagonistic activity, indicating a role as induction factor for plantaricin A [49]. In general, these induction factors (IF) involved in bacteriocin production are (i) bacteriocin-like peptides with a double-glycine leader peptide, (ii) their mature form is shorter than a regular bacteriocin and, (iii) the genes encoding IF are located upstream from the histidine kinase gene of the two component system [28]. Small peptides preceding the histidine proteinase kinase, response regulator tandem have also been reported for sakacin A (orf4), sakacin P (orf Y) and carnobacteriocins A, B 1 and BM2 (orf6) [28, 49] (Fig. 5). The role of orf4 in induction of the sakacin P production has already been established [49].

Analogously, it has been shown that NisK and NisP constitute the histidine proteinase kinase and response regulator components of the nisin signal transduction system [92, 101, 107]. NisK is a 447-residue, membrane-integrated protein with two potential *N*-terminal membrane anchors and a cytoplasmic carboxy-terminus [107]. The carboxy-terminus contains a His-238 residue for

Bacteriocin	Peptide sequence
Plantaricin A:	KSSAYSLQMGATAIKQVKKLFKKWGW
Sakacin P:	MAGNSSNFIHKIKQIFTHR
Sakacin A:	TNRNYGKPNKDIGTCIWSGFRHC
Carnobacteriocin A:	SKNSQIGKSTSSISKCVFSFFKKC

Fig. 5. The amino acid sequence of the putative induction factors of class II non-lantibiotic bacteriocins plantaricin A, sakacin A and P and carnobacteriocin A [28]

autophosphorylation and might be the signal-transducing domain with kinase activity [107]. The region between the membrane anchors is hydrophilic and may correspond to the extracellular sensor domain [107]. NisR is a 229-residue protein of the cytoplasm. The *N*-terminus, which forms the part with highest similarity among regulatory proteins contains a very conserved Asp-53 residue where phosphorylation takes place [182, 183]. The exact role of NisK in NisR phosphorylation must still be determined, since inactivation of NisK did not affect nisin production in a plasmid-based complementation system [101]. Mature nisin acts as an inducer of both the *nisA*BTCIKR and nisFEG operon [115]. Extracellular administered nisin complements for the *nisZ*B anti-sense and the *nisT* knock-out mutation, and results in the restoration of transcription of both nisin operons [115]. Nisin induction also resulted in a higher amount of *NisI* gene and an increased level of immunity [115]. The requirement of the structural *nisA* gene for full immunity of the nisin producer had also been recognized by Kuipers et al. [92]. In contradiction to the class II non-lantibiotic inducible bacteriocins, nisin serves a dual function of being a bacteriocin and an induction factor involved in autoregulation [92].

In conclusion, the large similarity among the different systems suggests that a two-component signal transduction mechanism, including a histidine protein kinase and a regulatory protein is a common feature in the regulation of bacteriocin production. The external stimuli triggering the induction or autoinduction system which induces bacteriocin production remain to be elucidated.

5.2 Post-Translational Modifications

The lantibiotics differ extensively from the class II bacteriocins in that they contain post-translationally modified amino acids, as for example dehydrated amino acids and lanthionine residues, forming intramolecular thioether bridges [39, 184]. The chemical modification reactions leading to the typical lanthionines were first proposed by Ingram [185] and are assumed to be catalyzed by specific enzymes encoded in the lantibiotic gene cluster. In the lantibiotic lactocin S, D-alanine residues were discovered, probably by conversion of dehydrated serine residues via a dehydrogenation reaction [82]. In some

lantibiotics produced by non-lactic acid bacteria, such as Pep5, lactocin S and epilancin K7, the *N*-terminal threonine and serine residues are modified into 2-oxy-butyryl, 2-oxy-propionyl and 2-hydroxy-pyruvyl residues, respectively [82, 186, 187]. The *N*-terminal deaminations of dehydroamino acids are considered to occur spontaneously [82]. In the case of epilancin, the 2-oxy-pyruvyl group may be enzymatically reduced to a 2-hydroxy-pyruvyl residue which then would be the very last biosynthetic reaction [82]. In addition, the *C*-terminal cysteine residues of epidermin and the related gallidermin are modified into *S*-[(*Z*)-2-aminovinyl]-D-cysteine [90, 188, 189]. In contrast to earlier reports where *C*-terminal modifications were claimed to occur after lanthionine bridge formation [90], it has recently been shown that the *C*-terminal modification reaction, catalyzed by EpiD, may take place intracellularly as the first step in the post-translational modification of epidermin [184, 189]. This modification reaction and its corresponding enzyme are unique for epidermin and have not been found in any other lantibiotic. Detection of sixfold, fivefold, fourfold, etc. dehydrated pre-Pep5 molecules in the cytoplasmic fraction of the PepS producer showed that (i) dehydration and ring formation are separate steps and (ii) ring formation happens after dehydration [82].

The presence of two genes, nisB and nisC, encoding 993- and 414-residue proteins without significant homology to other known proteins, but conserved in several lantibiotic operons, has made them strong candidates for post-translational modifications in the maturation pathway of lantibiotics [40]. Limited similarity between NisB and *E. coli* IlvA, a threonine dehydratase, was reported and hence a dehydratase function for NisB was suggested [40]. Mutation studies of NisB, NisC, EpiB, EpiC, and SpaB indicated that these proteins were essential for nisin, epidermin and subtilin biosynthesis, respectively [40, 86, 87, 190]. As no precursors have been identified and characterized in these mutants, conclusions about the reaction that is catalyzed by these proteins remain speculative [40]. Secondary-structure predictions and experimental evidence confirmed that NisB and SpaB are both membrane-bound [100].

Some lantibiotic operons, such as lactocin S, lactococcin DR (lacticin 481) or cytolysin contain no homologues of the *nisB* and *nisC* genes [82, 118, 122]. In these operons, the homologous genes *lasM*, *lctM*, and cylM were found [82, 118, 122]. Since CylM and LctM contain a *C*-terminal domain with striking homology to NisC, it could be hypothesized that they combine the function of a dehydratase and the enzymatic reaction leading to lanthionin ring formation [40, 122, 123]. There is a striking correlation between this grouping and the classification of lantibiotics based on their leader sequences [22, 40]. It is therefore tempting to assume that NisB/NisC and its homologues interact with class IA_I leader peptides, whereas an interaction between the larger proteins of the CylM family and the class IA_{II} leader peptides occurs [40]. The post-translational modification reactions of a pre-lantibiotic leads to the formation of an inactive precursor molecule, consisting of the completely matured lantibiotic, still attached to its leader peptide [101, 102]. The *N*-terminal modification of the lantibiotics Pep5, lactocin S and epilancin K7, occurs after proteolytic cleavage of the precursor and is therefore an exception to this rule [82].

5.3
Secretion and Proteolytic Activation of Bacteriocin Precursors

5.3.1
ATP-Dependent Translocation and Processing

The NisP protein product encoded by the *nisP* gene upstream from *nisR* in the nisin gene cluster showed an *N*-terminal signal sequence, a catalytic domain with a high degree of similarity to that of the subtilisin-like serine proteases, and a carboxyterminal membrane anchor [101]. Those features of its primary structure are indicative of secretion followed by membrane anchoring resulting in an extracellularly located catalytic *N*-terminal tail [40, 101]. Precursor nisin devoid of biological activity could be activated by incubation with cell membranes from a *nisP*-expressing strain, while mutation of NisP resulted in the secretion of a fully modified but unprocessed nisin precursor, indicating that the activating NisP protease is membrane located and involved in maturation of nisin [101].

The *nisT* gene encodes a 600-residue protein with strong homology to ABC exporters [92, 100]. These ABC transporters share two main regions of homology, i. e. an ATP-binding motif in the *C*-terminal half and six transmembrane domains located in the *N*-terminal half of the protein [105]. These data indicate that proteolytic cleavage is a process that occurs at the extracellular face of the cytoplasmic membrane following secretion [101]. The counterparts of NisP in the epidermin (EpiP) and cytolysin (CylP) operons contain a signal sequence but lack a membrane-spanning domain, suggesting that they are attached in another way or are not membrane associated [88, 122, 190]. PepP, LasP and ElkP involved in respectively Pep5, lactocin S and epilancin K7 proteolytic processing all lack a signal sequence, and may therefore function intracellularly, which is in agreement with their *N*-terminal modification [40, 186, 187]. The subtilin gene cluster did not contain a peptidase-like protein [87, 89, 110]. However, the subtilin producer *B. subtilis* is known to contain a variety of secreted proteases that could be involved in proteolytic activation [40].

ABC transporters encoded in the same operon or an operon adjacent to the structural bacteriocin gene have been reported for all class II bacteriocins genetically studied in detail [49, 62, 118, 119, 127, 130, 131, 136, 146]. Those proteins are characterized by six transmembrane domains, a carboxy-terminal ATP-binding cassette and an *N*-terminal proteolytic domain, both located in the cytoplasm [146] (Fig. 6.). The energy needed for the translocation process is provided by hydrolysis of ATP [146, 191, 192]. It was remarkably that the ABC transporters involved in the secretion of the lantibiotics nisin, epidermin and subtilin are devoid of a conserved *N*-terminal extension of about 150 amino acids that is only present in the larger transporters and involved in proteolytic processing of the prebacteriocin [146]. Among the larger ABC transporter proteins, two conserved amino acid stretches were shown to be exclusively found in *N*-terminal extensions of bacteriocin transporters and not in exporters of substrates that are not proteolytically processed during transport, such as the haemolysin transporters AppB [193], HlyB [194], LktB [195], and

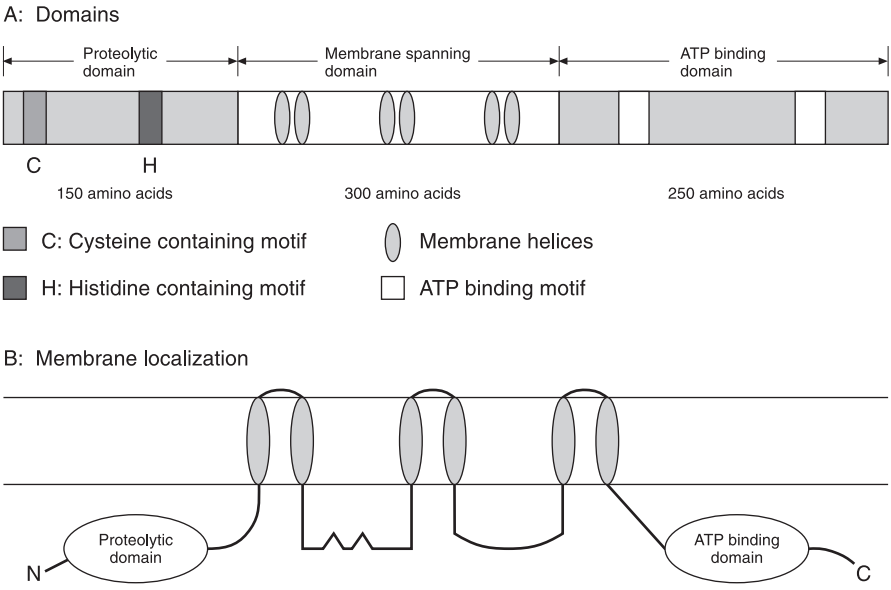

Fig. 6. The organization of the domains of ABC transporters of class II bacteriocins with double-glycine leader peptides and the presumed localization of the domains in relation with the cytoplasmic membrane

CyaB [146, 196–198]. Since it was shown that the 150 *N*-terminal amino acids of the lactococcin G and pediocin PA-1.0 ABC transporters were capable of cleaving the corresponding precursor bacteriocins at the correct consensus processing site, its role for class II leader peptide processing was accepted [132, 146]. Replacement of the cysteine residue on position 13 with an alanine residue resulted in a complete loss of proteolytic activity, demonstrating that this residue is part of the active site 146]. It was therefore concluded that the new class of proteolytic ABC transporters belonged to the cysteine proteases [146]. The absence of this proteolytic domain in ABC transporters of class Al lantibiotics is complemented by an individually encoded subtilisin-like protease in these operons, and thus processing and translocation of the class IA_I lantibiotics depends on the action of two divergent proteins [88, 101, 122, 190].

As mentioned before, NisB, NisC and their homologues are likely to interact with class IA_I leader peptides, whereas an interaction between the large proteins of the CylM family and the class IA_{II} leader peptides was proposed [40]. Leader peptides of the class IA_{II} lantibiotics such as lactococcin DR [118], salivaricin A, streptococcin A-FF22 [199] and cytolysin [122] do not contain the -F-N-L-D-V- box typical for class IA_I lantibiotics [40]. Unexpectedly, these class IA_{II} leader peptides showed considerable similarities with leaders of the class II non-lantibiotic bacteriocins [21, 40, 146]. Furthermore, they also contained a proteolytic processing site, identical or comparable to the double-glycine cleavage site of class II non-lantibiotic peptides [21, 40, 146]. It is therefore likely that the processing and secretion mechanism of the class IA_{II} lantibiotics more

closely resembles that of the non-lantibiotics. In agreement with this model, an ABC transporter including the *N*-terminal proteolytic domain was encoded in the lactococcin DR (LctT) and cytolysin (CylT) operons, indicating that they share a common mechanism of export with the non-lantibiotic, double-glycine type leader peptide bacteriocins [118, 122]. However, in contradiction to the class II non-lantibiotic bacteriocins, downstream from the 714-amino acid residue translocator CylT, a 412 amino acid residue protease is encoded [122]. Functional analysis studies and newly obtained information residing from other class IA_{II} bacteriocin operons will have to resolve this apparently conflicting data.

Recently, a new lantibiotic, lactocin S has been characterized. Its leader peptide is typical for the class IA_{II} lantibiotics but lacks a double-glycine processing site, suggesting an alternative proteolytic cleavage [82, 121]. This hypothesis is reflected in the lactocin S operon which encodes an ABC transporter of 535 amino acids, a protease of 266 residues as well as several open reading frames with unknown functions [82, 121].

5.3.2
Accessory Proteins of the Class II Non-Lantibiotic Bacteriocins

Additionally, all non-lantibiotic class II bacteriocin operons, studied so far, were shown to encode the homologous counterpart of HlyD, the accessory protein of the haemo1ysin A secretion apparatus of *E. coli* [49, 124, 127, 128, 130–133]. These so-called accessory proteins are encoded adjacent to the structural ABC exporter gene and characterized by a unique, *N*-terminal transmembrane domain [49, 124, 127, 128, 130–133]. They are predicted as integral proteins of the cytoplasmic membrane speculated to facilitate signal sequence independent secretion. It has been shown that the biosynthesis of non-lantibiotic bacteriocins requires both the ABC transporter and the accessory factor [105, 126, 133]. These accessory proteins belong to a recently identified, novel class of export proteins designated as the membrane fusion protein (MPF) family [200]. Proteins of the MPF family have been hypothesized to cause local interaction of the bacterial membranes, allowing direct exchange of substrates between the two membranes of the Gram-negative envelope [201]. For some members of the HlyD protein family, including HlyD, evidence has been presented that ten residues of their periplasmic carboxy-terminus mediate association with the outer membrane [202]. Based on these data, the long carboxy-terminal tail of the accessory proteins for the secretion of non-lantibiotic class II bacteriocins of Gram-positive bacteria could hence be expected at the extracellular face of the cytoplasmic membrane since Gram-positive bacteria do not have an outer membrane. The function of such MPF in the cytoplasmic membrane of Gram-positive bacteria, however, is not clear. Counterparts of these accessory proteins in either class IA_I or IA_{II} lantibiotic operons have not yet been reported.

5.3.3
Conclusion

In conclusion, these data indicate striking differences in the processing and secretion mechanism of lantibiotic class IA_I and class IA_{II} and non-lantibiotic class II bacteriocins. Secretion and processing of class IA_I lantibiotics is mediated by two different proteins. Processing occurs extracellularly, except for class IA_I lantibiotics that are *N*-terminally modified prior to export. Class IA_{II} lantibiotics and class II non-lantibiotic bacteriocins are both characterized by an ABC transporter containing a conserved proteolytic processing domain in its *N*-terminal tail. Processing occurs at the cytoplasmic side of the cellular membrane followed by secretion of the mature bacteriocin. Although its function and occurrence in other class IA_{II} lantibiotics remains to be investigated, an additional proteolytic enzyme was detected in the cytolysin operon. The accessory factor protein, also conserved in Gram-negative secretion systems, was characterized as an unique feature of non-lantibiotic class II bacteriocin secretion systems. Based on its divergent leader peptide, containing an ELS box but missing a double-glycine type processing site, an alternative model is expected to emerge for lactocin S.

6
Role of the Leader Peptide

The bacteriocin leader peptides differ from the *N*-terminal signal sequences for export of proteins secreted by the *sec*-dependent pathway in that they lack a hydrophobic membrane-spanning stretch of 10 or more residues as well as the typical proteolytic processing site [82, 120]. Class IA_I leaders are slightly positively or negatively charged and contain conserved residues such as a proline residue at position –2, a serine residue at position –6 and the so-called -F-N-L-D-V-box (Fig. 7 [40]). Site-directed mutagenesis has shown that the very conserved proline residue is not essential for processing, but that a positive charge at position –1 and a small hydrophobic residue at position –4 are essential [102]. Mutation of the phenylalanine, leucine or aspartate residue in the -F-N-L-D-V-box or the serine residue at position –6 prevented biosynthesis of nisin, not even the precursor could be detected [102]. The results so far obtained with site-directed mutagenesis clearly demonstrate the importance of particular residues in the leader peptide for biosynthesis. Meanwhile, there is evidence that processing is the last step in lantibiotic maturation, so that modification reactions are made at the prepeptide stage, and that the precursor molecule is inactive [102]. Therefore, the leader peptide might have an essential role in biosynthesis, either in that it could contain a specific recognition motif which would direct the precursor towards biosynthetic enzymes and/or in that the leader peptide may interact with the propeptide region to stabilize a conformation which is essential for correct modification [39]. In contrast, the class IA_{II} lantibiotics contain a double-glycine type processing site and a leader peptide that matches the consensus of the non-lantibiotic class II bacteriocins [21, 40] (Fig. 7). However, putative modifying enzymes for the class IA_{II}

Class IA_I antibiotics

Precursor	Processing	
MEAVKEKNDL**FNLDV**KVNAKE**S**N-**DS**G-AE**PR**	IASK	epidermin
MEAVKEKNEL**FDLDV**KVNAKE**S**N-**DS**G-AE**PR**	IASK	gallidermin
MKNNKNL**FDLE**IKK--ET**S**Q-**NT**DELE**PQ**	TAGP	pep5
MSKFDD**FDLDV**VK---V**S**KQ**DS**K-IT**PQ**	WKSE	subtilin
MSTKD**FNLD**LVS---V**S**KK**DS**G-AS**PR**	ITSI	nisin A
MSTKD**FNLD**LVS---V**S**KK**DS**G-AS**PR**	ITSI	nisin Z
MNNSL**FDL**NLNK-GVE**T**Q-**KS**D-LE**PQ**	SASV	epilancin K7
FNLDV **S** **DS** **PR**		consensus

Class IA_{II} antibiotics

Precursor	Processing	
VLNKENQENYYSNKLELVGPSFE**ELS**LE**EM**EA**I**Q**GS**	GDVQ	cytolysin L2
MNAMKNSKDILNNAIE**EVS**EK**EL**ME**V**A**GG**	KRGS	salivaricin A
MEKNNEVINSIQ**EVS**LE**EL**DQ**II**G**A**	GKNG	streptococcin FF-22
MENLSVVPSFE**ELS**VE**EM**EA**I**Q**GS**	GDVQ	cytolysin L1
MKEQNSFNLLQ**EVT**ES**EL**DL**IL**G**A**	KGGS	lacticin 481
EVS **EL** **I** **GA**		consensus

Class II non-lantibiotic bacteriocins

Precursor	Processing	
MMNMKPTFSYEQ**LD**NS**AL**EQVV**GG**	KYYG	leucocin A
MKNQLNFNI**VS**DE**EL**SEAN**GG**	KLTF	lactococcin A
MKNQLNFNI**VS**DE**EL**AEVN**GG**	SLQY	lactococcin B
MKNQLNFEI**LS**DE**EL**QGIN**GG**	IRGT	lactococcin M
MKKIEK**LT**EK**EM**ANII**GG**	KYYG	pediocin PA-1
MMMVKE**LS**MT**EL**QTIT**GG**	ARSY	sakacin A
MKQFNY**LS**HK**DL**AVVV**GG**	RNNW	lactacin F
LS **EL** **GG**		consensus

Fig. 7. Alignment of leader peptides of class IA_I and class IA_{II} lantibiotics and class II non-lantibiotic bacteriocins. Lantibiotics with class IA_I leaders include nisin A and nisin Z from different strains of *Lactococcus lactis* [83], subtilin from *Bacillus subtilis* [84], epidermin, gallidermin and Peps from various *Staphylococcus epidermidis* strains [29, 90, 188]. Lantibiotics with class IA_{II} leaders include lacticin 481 (lactococcin DR) from *L. lactis* [211], streptococcin A-FF22 from *Streptococcus* sp. [199], salivaricin A from *Streptococcus salivarius* and cytolysin L1 and L2 from *Enterococcus faecalis* [122]. Bacteriocins of the non-lantibiotic class II type include leucocin A from *Leuconostoc gelidum* [69], lactococcin A, B and M from *L. lactis* [46, 125, 126, 135, 136], pediocin PA-1 from *Pediococcus acidilactici* [130], and sakacin A and lactacin F from *Lactobacillus* sp. [43, 203]

lantibiotics differ significantly from those of the class IA_I lantibiotics [40, 82, 118, 122].

Lactacin F was the first non-lantibiotic bacteriocin characterized in lactic acid bacteria for which both DNA and protein sequences were available [77, 203]. This information demonstrated that lactacin F is translated as a 75-amino acid residue precursor which is posttranslationally processed by cleavage of a

18-residue *N*-terminal leader peptide at a very conserved double-glycine-residue processing site. Site-directed mutagenesis of the lactacin F (LafA) precursor was employed to modify the glycine residues at positions –1 and –2, and the valine (–3 position) and arginine (+1 position) residues [77]. Replacement of glycine at position –1 with valine, or replacement of the glycine residue at position –2 with arginine, or even the polarityneutral amino acid serine eliminated lactacin F expression. Replacement of valine at position –3 with a charged residue (Asp) or arginine at position +1 with another positively charged residue did not disrupt bacteriocin activity [77]. Based on these experiments and sequence alignments, a more conserved pattern for the bacteriocin leader peptide was proposed [77] including (i) two conserved glycines at positions –1, and –2, (ii) hydrophobic residues at positions 4, –7, –12, and –15, (iii) a core of charged amino acids at positions –8 and –10, and (iv) a serine at position –11. Heterologous expression of lactacin F peptides in *Carnobacterium piscicola* LV17, and *Leuconostoc gelidum* resulted in the production of mature, bioactive lactacin F [204, 205].

These results confirm the conservative nature of the processing and secretion apparatus involved in class II non-lantibiotic maturation, as it was capable of recognizing and properly processing heterologously expressed lactacin F [204, 205]. Heterologous expression of the lactococcin A operon in *Pediococcus* and expression of the pediocin PA-1 operon in *Lactococcus lactis* also resulted in fully matured bioactive peptides [206]. Recently, the new bacteriocin divergicin A was shown to be secreted by the *sec*-dependent pathway [51]. The *N*-terminal extension of divergicin A had a -A-S-A-(positions –3 to –1) cleavage site and acts as a signal peptide that accessed the general export system of the cell [51]. Production of divergicin A was demonstrated in heterologous hosts containing the two genes associated with the bacteriocin and immunity [51]. These data indicate that a fully functional bacteriocin molecule can be produced in the absence of the typical leader peptide consensus and the corresponding ABC transporter gene.

Comparison of the leader peptides and the ABC transporter systems of the class IA_{II} lantibiotics and the class II non-lantibiotic bacteriocins suggest that both are processed and secreted in the same manner [21, 40, 146]. A striking feature of the class IA_{II} lantibiotic leader peptides consists of a very conserved glutamate residue at position –13, missing in the class II leaders and resulting in the ELS consensus [40]. The class IA_{II} lantibiotics, in contradiction to the class II non-lantibiotic bacteriocins undergo post-translational modification and their leader peptide would hence not only be involved in proper processing of the precursor, but might fulfill a comparable role in conformational stabilization during post-translational modification, as the class IA_I leader peptides [21, 40, 82].

7 Conceptual Model for Bacteriocin Maturation

A combination of the above described findings could hence result in the following hypothetical model for bacteriocin biosynthesis (Figs. 8 and 9). Firstly,

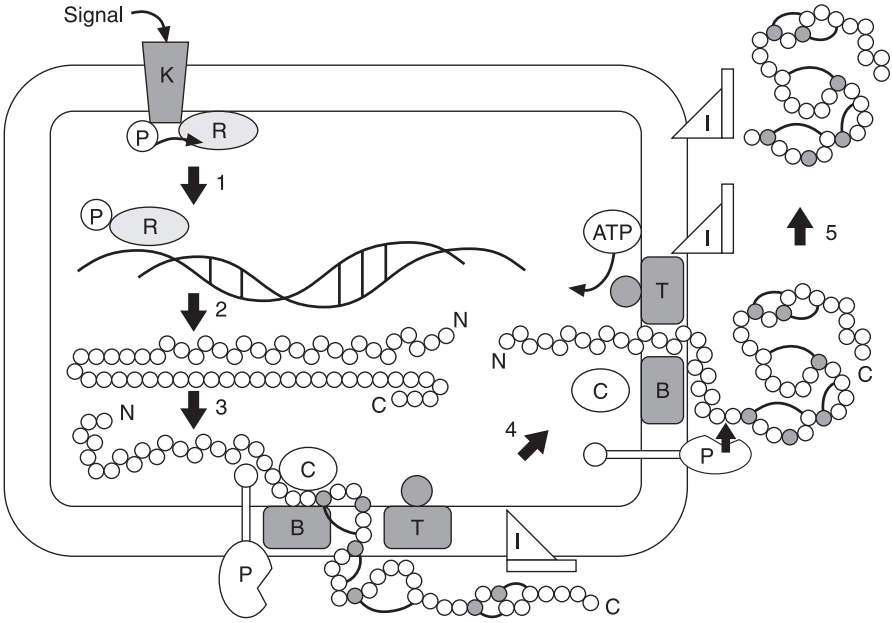

Fig. 8. A conceptual maturation pathway for nisin is given as a 5-step process [40]. A two-component signal transduction system induces transcription (*step 1*). Translation results in an inactive unmodified precursor peptide (*step 2*). The leader peptide is proposed to play a role in targeting of the precursor to a membrane-located modification complex (*step 3*). Dehydration and lanthionine and dehydro-lanthionine formation (*step 4*) is followed by extracellular processing and secretion (*step 5*)

an inducing signal activates, via the two-component signaling pathway, the promoters responsible for the expression of the operons involved in bacteriocin production. In the case of nisin, production and immunity have shown to be autoregulated. Expression of several inducible class II non-lantibiotic bacteriocins (e.g. sakacin A, sakacin P, plantaricin P) is controlled by a bacteriocin-like peptide, processed at a double-glycine consensus sequence site which lacks antagonistic activity. The two-component signal transduction system has not yet been shown for most of the class II non-lantibiotic bacteriocins, which may therefore display a constitutive expression. Transcription results in the concerted production of the proteins constituting the modification and secretion machinery, together with the inactive bacteriocin precursor molecule.

In case of the lantibiotics, this precursor contains free cysteines and no dehydrated residues. The lantibiotic precursor molecule is directed, presumably by virtue of the leader peptide, to a membrane-located complex containing the modifying enzymes NisB (possibly involved in dehydration) and *NisC* (conceivably involved in establishing the thioether bonds). Lantibiotics of the class IA_{II} type (lactococcin DR, cytolysin, lactocin S) lack genes of the nisB and nisC type in their corresponding operons [207]. It is assumed but not yet established that one protein belonging to the so called CylM family mediates a one-step for-

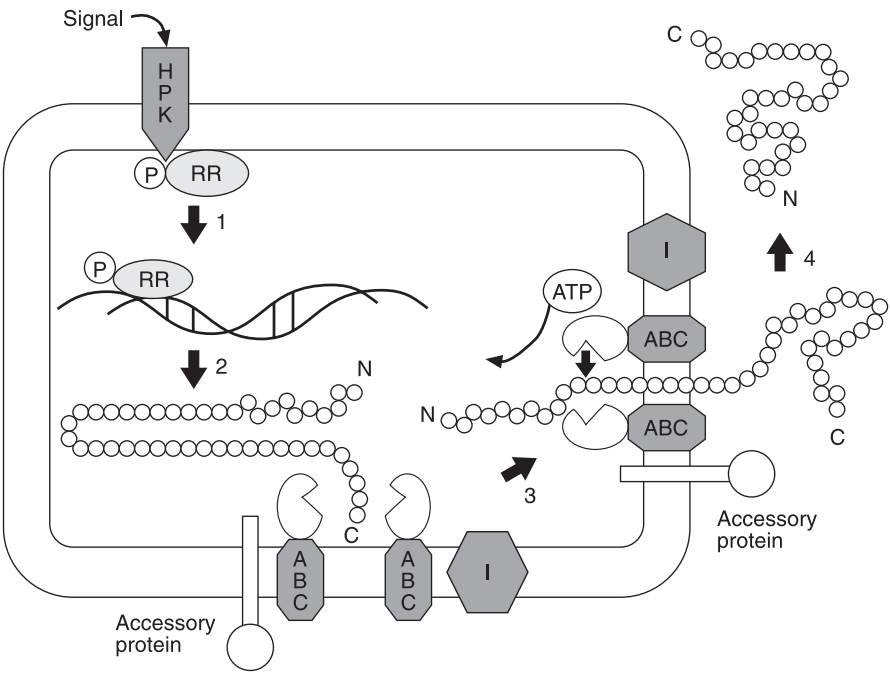

Fig. 9. Maturation pathway of a class II non-lantibiotic bacteriocin presented as a 4-step process. The HPK and RR constitute a protein complex involved in signal transduction of external stimuli (*step 1*), resulting in transcription and translation of the bacteriocin precursor (*step 2*). The inactive precursor peptide is targeted to a membrane anchored ATP dependent translocation complex and processing at the double-glycine consensus site occurs intracellularly (*step 3*) and is followed by secretion (*step 4*). The role of the accessory protein in this mechanism is not yet understood

mation of lanthionine and methyl-lanthionine bridges. This reaction presumably takes place in the cytoplasm of the cell. Bacteriocins of the non-lantibiotic type do not undergo such post-translational reactions. It is generally assumed that the modification reactions leading to lanthionine bridge formation are the first post-translational modification reactions following translation. Dehydration precedes lanthionine ring formation and both occur separated in time.

Conflicting reports have been made about the *C*-terminal modification of epidermin, mediated by the unique enzyme EpiD, but the most recent data suggest that this reaction could be expected as the first enzymatic modification involved in epidermin maturation. The modifications result in the formation of an inactive precursor molecule. At this point, the leader may help to maintain the peptide in an inactive form.

Subsequently, both the modified lantibiotic precursor and the unmodified non-lantibiotic bacteriocin precursor are secreted via an ABC transporter, at the expense of ATP hydrolysis. Processing of the class IA_{II} lantibiotics and the non-lantibiotic bacteriocins is associated with the intracellular *N*-terminal tail of the ABC exporter. Nisin, the representative of the lantibiotic class IA_{I} pre-

cursors is activated by the extracellular protease NisP, which is hooked to the cell membrane by means of a fatty acyl membrane anchor. Processing of *N*-terminally modified lantibiotics (Pep5, epilancin K7, lactocin S) occurs in the cytoplasm, prior to *N*-terminal modification. This latter modification is a spontaneous reaction, except; for the enzymatic reduction of epilancin K7, which would hence be the last step in the modification of this lantibiotic and secretion. The contribution of the leader peptide in this *sec*-independent process is not known, nor is its fate after processing or the polarity of the export process. Secretion results in the release of a bioactive bacteriocin.

While class II non-lantibiotic bacteriocins appear to be secreted by the *sec*-independent universal ABC transporter system, it has recently been shown that some bacteriocins do not possess a double-glycine leader peptide but are, instead, synthesized with a typical *N*-terminal leader peptide of the *sec-type*. So far four such *sec*-dependent bacteriocins have been reported divergicin A [51], acidocin B (50], bacteriocin 31 [208] and enterocin P [209]. Recently, two bacteriocins, enterocin L5OA and L5OB, were found to be secreted without an *N*-terminal leader sequence or signal peptide [210].

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