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Lantibiotics: structure, biosynthesis and mode of action

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Abstract

The lantibiotics are a group of ribosomally synthesised, post-translationally modified peptides containing unusual amino acids, such as dehydrated and lanthionine residues. This group of bacteriocins has attracted much attention in recent years due to the success of the well characterised lantibiotic, nisin, as a food preservative. Numerous other lantibiotics have since been identified and can be divided into two groups on the basis of their structures, designated type-A and type-B. To date, many of these lantibiotics have undergone extensive characterisation resulting in an advanced understanding of them at both the structural and mechanistic level. This review outlines some of the more recent developments in the biochemistry, genetics and mechanism of action of these peptides.

Keywords: Bacteriocin; Lantibiotic; Nisin; Lacticin 3147; Mode of action

Contents

1. Introduction

Bacteriocins are one of a number of antimicrobial substances produced by lactic acid bacteria (LAB), including organic acids, hydrogen peroxide, diacetyl and inhibitory enzymes [1,2]. The LAB have been used for centuries in the fermentation of food, not only for flavour and texture, but also due to the ability of starter-derived inhibitors to prevent the growth of spoilage and pathogenic microorganisms [3,4]. The prototype LAB bacteriocin, nisin, was first discovered in 1928, when Rogers [5] observed metabolites of *Streptococcus lactis* (now reclassified as *Lactococcus lactis*) which were inhibitory to other LAB. The commercial application of nisin in the preservation of a

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number of processed foods, and the award of FDA approval in 1988 for its use as a biopreservative elicited considerable interest in other bacteriocins from GRAS (generally regarded as safe) organisms. These bacteriocins also have potential practical applications, and a great number of diverse bacteriocins have been identified and characterised in recent years.

Tagg et al. [6] defined bacteriocins as 'proteinaceous compounds which kill closely related bacteria', with a bactericidal mode of action. Even though many characterised bacteriocins concur with this definition, it has become apparent that some have a broad host-range, inhibiting many different species. In 1993, Klaenhammer [7] defined a number of distinct classes of LAB bacteriocins; the Class I bacteriocins (lantibiotics) are small (<5 kDa) peptides containing the unusual amino acids lanthionine (Lan), β-methyllanthionine (MeLan) and a number of dehydrated amino acids [8,9]. Examples include nisin [10], lacticin 481 [11], and the two-component lantibiotics such as cytolysin produced by *Enterococcus faecalis* [12], lacticin 3147 produced by *L. lactis* [13], and staphylococcin C55 produced by *Staphylococcus aureus* [14]. Class II bacteriocins are small (<5 kDa) heat-stable, non-Lan-containing, membrane-active peptides; this class is subdivided into *Listeria*-active peptides with the N-terminal consensus sequence YGNGV (Subclass IIa), e.g. pediocin PA-1 [15], sakacin A [16], and enterocin A [17]; bacteriocins requiring two components for activity (Subclass IIb), e.g. lactococcin G [18], lacticin F [19,20] and the *sec*-dependent secreted bacteriocins (Subclass IIc), e.g. acidocin B [21]. Members of Class III are large (>30 kDa) heat-labile proteins, e.g. helveticin J [22]. A fourth class, the 'complex bacteriocins' has also been suggested, which require non-proteinaceous moieties for activity. This class, however, has not been studied sufficiently at the biochemical level. Indeed, experimental evidence suggests that the activities responsible for the antagonistic effects observed may be artefacts resulting from interactions between constituents from the cells and the growth medium [23].

Studies on the genetics and biochemistry of bacteriocins have principally focused on members of Class I and II, due to the abundance of these peptides and their potential commercial applications. Class II bacteriocins have recently been comprehensively reviewed by others [24–27]. This review focuses on Class I bacteriocins known as lantibiotics, and particularly those that act by disrupting membrane integrity, i.e. the type-A lantibiotics. This unique group of peptides are produced by a number of Gram-positive bacteria, and a number have been characterised at the genetic and biochemical level.

2. Molecular analysis of lantibiotics

The production of ribosomally synthesised linear antimicrobial peptides is well conserved in nature, and almost all groups of organisms have been shown to produce such peptides [28,29]. The lantibiotics (from '*lan*thionine-containing an*tibiotic*') are unique in that they are produced on the ribosome as a prepeptide which undergoes extensive post-translational modification to form the biologically active peptide [30–32]. The term is used to encompass peptides containing unusual amino acids normally not found in nature, e.g. the thioether amino acids Lan and/or MeLan, in addition to a number of modified residues, such as 2,3-didehydroalanine (Dha) and 2,3-didehydrobu-

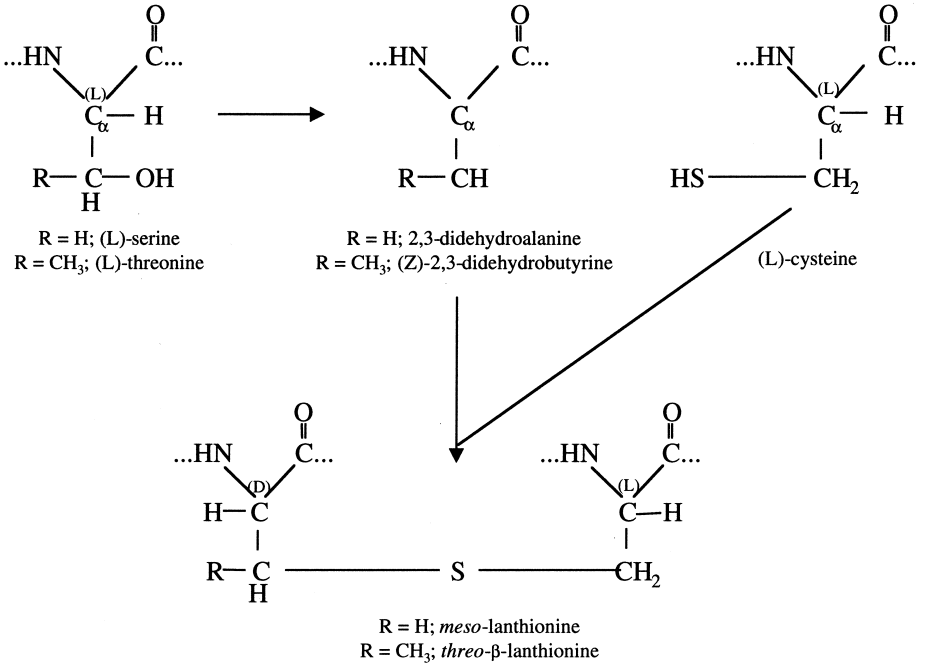

Fig. 1. General mechanism for the formation of the thioether Lan during lantibiotic maturation. Adapted from [33].

Table 1
Examples of lantibiotics characterised to date

Lantibiotic	Producing strain(s)	Reference
Type-A Lantibiotics[a]		
Type-A(I)		
Nisin A	*L. lactis* NIZOR5, 6F3, NCFB894, ATCC11454	[10]
Nisin Z	*L. lactis* N8, NIZO22186	[34,35]
Subtilin	*B. subtilis* ATCC6633	[36]
Epidermin	*Staphylococcus epidermidis* Tu3298	[37]
Gallidermin	*Staphylococcus gallinarum* Tu3928	[38]
Mutacin B-Ny266	*S. mutans*	[39]
Mutacin 1140	*S. mutans* JH1000	[40]
Pep5	*S. epidermidis* 5	[41]
Epicidin 280	*S. epidermidis* BN280	[42]
Epilancin K7	*S. epidermidis* K7	[43]
Type-A(II)		
Lacticin 481	*L. lactis* CNRZ481, ADRIA85LO30	[11,44]
Cytolysin	*E. faecalis* DS16	[45]
Lacticin 3147	*L. lactis* DPC3147	[13]
Staphylococcin C55	*S. aureus* C55	[14]
Salvaricin A	*Streptococcus salvarius* 20P3	[46]
Lactocin S	*L. sake* L45	[47]
Streptococcin A-FF2	*Streptococcus pyogenes* FF22	[48]
Sublancin 168	*B. subtilis* 168	[49]
Carnocin U149	*C. pisicola*	[50,51]
Variacin 8	*Micrococcus varians* MCV8	[52]
Cypemycin	*Streptomyces* ssp.	[53]
Type-B lantibiotics		
Cinnamycin	*Streptomyces cinnamoneus*	[54]
Duramycin B	*Streptoverticillium* ssp.	[55]
Duramycin C	*Streptomyces griseoluteus*	[55]
Ancovenin	*Streptomyces* ssp.	[56]
Mersacidin	*B. subtilis* HIL Y-85, 54728	[57]
Actagardine	*Actinoplanes*	[58]

[a]Separated into two groups based on leader sequences and gene cluster composition.

tyrine (Dhb) [33]. The presence and influence of these residues on the structure and activity of lantibiotics, and the novel enzymes found in their biosynthetic pathways responsible for the specific amino acid modifications has attracted significant research interest.

2.1. Structural aspects

Examples of lantibiotics described to date are listed in Table 1. According to a proposal by Jung in 1991 [59], lantibiotics are grouped into type-A and type-B peptides based on their structural and functional features. In general, type-A lantibiotics are elongated, cationic peptides up to 34 residues in length that show similarities in the arrangement of their Lan bridges. These peptides primarily act by disrupting the membrane integrity of target organisms, and include nisin, subtilin, and epidermin. Type-B peptides are globular, up to 19 residues in length, and act through disruption of enzyme function, e.g. inhibition of cell wall biosynthesis. Examples are the duramycins produced by *Streptomyces* species, mersacidin and actagardine [60]. A number of lantibiotics, however, do not fall into either category suggesting that as more are discovered, classification will undoubtedly become more complex.

2.1.1. Unusual amino acids

Lantibiotics are characterised by the presence of a high proportion of unusual amino acids, including the thioether amino acids Lan and MeLan and a number of dehydrated amino acids, such as the α,β-unsaturated amino acids Dha and Dhb [61]. This sequence-specific dehydration of serine (to Dha) and threonine (to Dhb) results in modified amino acids with electrophilic centres which can react with neighbouring nucleophilic groups. The thioether Lan is formed when the double bond in Dha is attacked by the thiol (–SH) group of a neighbouring cysteine residue [61]; MeLan results when the reaction partner is Dhb (Fig. 1). As a consequence of the presence of these intramolecular bridges, lantibiotics are polycyclic structures containing a number of Lan rings (Fig. 2).

In addition to those residues already mentioned, a number of other unusual amino acid-derived residues have been identified in lantibiotics. Epidermin is a tetracyclic peptide which contains unsaturated (*S*)-[(*Z*)-2-aminovinyl]-D-cysteine (AviCys) at the C-terminus of the mature

peptide, forming the fourth ring (Fig. 2; [62,63]). In Pep5, Dhb occupies the N-terminus when the propeptide is cleaved from the leader sequence [64]. However, dehydrated residues are not stable when N-terminally exposed, and spontaneous deamination occurs through the addition of a water molecule, resulting in the formation of 2-oxobutyryl (from Dhb) and 2-oxypropionyl (from Dha). 2-Oxopropionyl also arises in lactocin S, produced by *Lactobacillus sake*. In addition, an unusual feature of lactocin S is the presence of D-alanine at three defined positions where the gene encodes a serine. It was proposed by Skaugen et al. [65] that the in vivo conversion of L-serine to D-alanine occurs by way of Dha followed by enzymatic stereospecific hydrogenation, i.e. addition of H_2 to D-alanine. More recently, a serine to D-alanine conversion has been reported in both peptides of the two-component lantibiotic, lacticin 3147 [13]. This was a very significant finding given that this is only the second instance of D-alanine occurring in a ribosomally synthesised peptide, and the first instance of it in a two-component biologically active peptide. It has been proposed by Ryan et al. [13] that these residues may account for the broad antimicrobial inhibitory spectrum associated with lacticin 3147.

The presence of post-translationally modified residues in lantibiotics raises numerous questions concerning their function, and studies on the structure–function relationships of these peptides are becoming increasingly significant, improving our understanding of the mode of action of these unique compounds [66]. The ring conformations are thought to be essential for maintenance of peptide rigidity [67], insensitivity to proteolytic degradation, and resistance to thermal inactivation [68]. Also, D-amino acids are known to contribute to the activity/stability of compounds [69]. The function of the didehydroamino acids is, however, less well defined, although it has been suggested that they may contribute to antimicrobial activity by interacting with free sulfhydryl groups on the cell envelopes of target organisms [70]. Studies on nisin revealed that, while hydrolytic cleavage at Dha33 had negligible effects on biological activity, additional cleavage at Dha5 resulted in substantial loss of activity [71]. This is most likely due to the opening of ring A in the mature nisin peptide (Fig. 2); ring A is structurally well defined, and apparently vital for the biological activity of nisin [72]. Recently, van Kraaij et al. [73] have demonstrated the importance of ring C for the biological activity of nisin Z by replacing this Lan ring with a disulfide bond. Exchange of Ser^{5+} for a threonine codon in the nisin Z structural gene resulted in a nisin Z mutant harbouring a Dhb in place of Dha. The resulting modified peptide had a bactericidal activity that

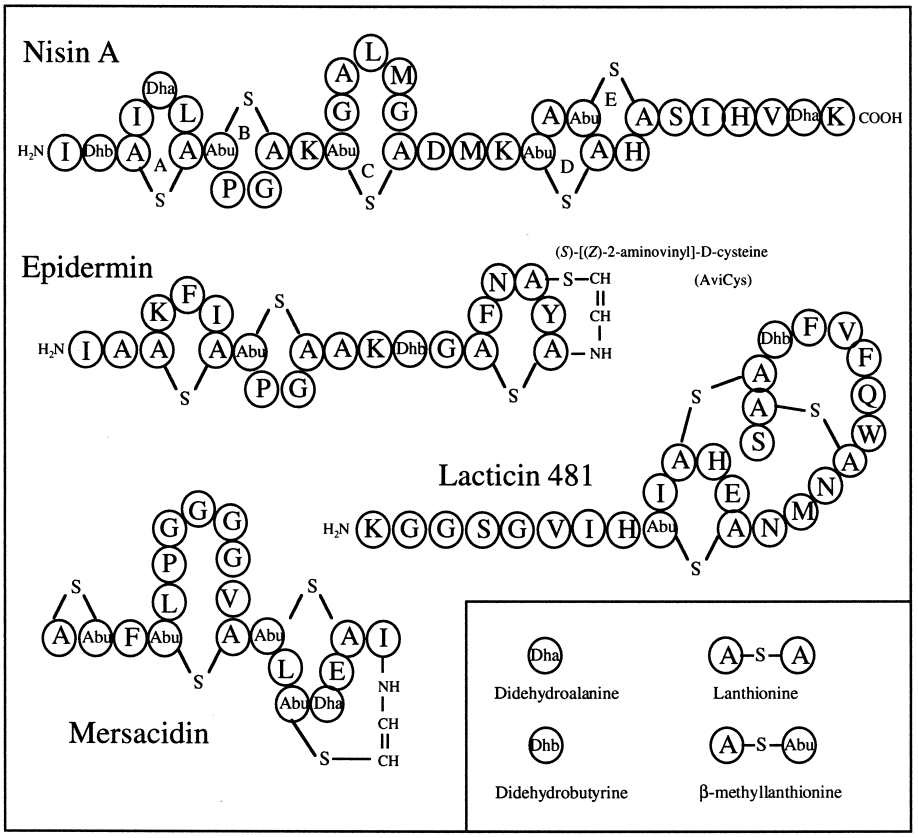

Fig. 2. Selected structures of representative lantibiotics. Nisin A and epidermin are typically elongated, flexible peptides (type-A). Lacticin 481 represents a group with a cross-bridged C-terminus and an unbridged N-terminal domain. Mersacidin is a type-B peptide which are conformationally well defined, globular peptides. Nomenclature of unusual amino acids is based on that which has been previously suggested [59].

was 2–10-fold lower than nisin Z [67], confirming the importance of Dha5 in nisin. Replacement of Dha5 with an alanine did not dramatically affect the pore-forming activity of nisin against vegetative cells, but nisin Ala5 was much less effective as an inhibitor of spore outgrowth [74]. This provides strong evidence that nisin has two distinct biological activities: inhibition of bacterial growth and inhibition of spore outgrowth, produced by two distinct molecular mechanisms. The importance of ring structures in relation to biological activity has also been reported for other lantibiotics [63,75].

2.2. Organisation of lantibiotic gene clusters

The events which lead to the production of a lantibiotic include formation of the prelantibiotic, dehydration and cross-linkage reactions, cleavage of the leader, and secretion. In addition, the cell must be immune to the lantibiotic that it produces [60]. The genetic determinants flanking the structural gene(s) for several linear (type-A) and few type-B lantibiotics have been characterised to date [30,76], and the organisation of a representative number is summarised graphically in Fig. 3. Comparison of the gene clusters indicate the presence of a number of conserved genes proposed to encode similar functions; the products of the genes identified will be discussed in detail in later sections. Following the generic nomenclature used for all lantibiotics, as reported by de Vos et al. [30], these include the precursor peptide (LanA) and enzymes responsible for the specific modification reactions (LanB,C/LanM), accessory proteins including processing proteases responsible for removal of the leader peptide (LanP), ABC-superfamily transport proteins involved in peptide translocation (LanT), regulatory proteins (LanR, K) and dedicated self-protection (immunity) mechanisms (LanI, FEG), in addition to genes with no homologues in the database [31,76,77].

Two classes of genetic organisation have been identified; nisin, epidermin, subtilin and Pep5 are grouped on the basis that they are modified by separate LanB and LanC enzymes, whereas this function is performed by a single LanM enzyme in the subclass containing lacticin 481, lactocin S, cytolysin and mersacidin. Interestingly, the gene cluster of the two-component lacticin 3147 contains two *lanM* genes. In addition, transporters with an associated protease activity have not been found in the gene clusters of the nisin-like lantibiotics.

Lantibiotic genetic determinants may be chromosomally encoded, as in the case of subtilin [78] and SA-FF22 [79], although in most cases, lantibiotic gene clusters are found on large plasmids, e.g. epidermin [62], Pep5 [80], cytolysin [81], and lacticin 3147 [82]. Dufour et al. [83] have recently shown the lacticin 481 gene cluster to be present on a composite transposon, Tn*5271*, on a 70-kb plasmid. The nisin genes are encoded on a number of large (~70 kb) conjugative transposons, Tn*5301* from *L. lactis* NCFB894 [84–86] and Tn*5276* from *L. lactis* NIZO R5 [87,88] which also harbour the genes for sucrose utilisation and which integrate into the recipient chromosome following conjugal transfer [7].

There is evidence that at least parts of the gene clusters of some lantibiotics are organised as operons. Many consist of several transcriptional units [89–97] and a weak terminator structure is often found in the intergenic region between the structural gene(s) and downstream genes. Interestingly, an intragenic triple stem–loop structure has been identified within a crucial gene in the lacticin 3147 biosynthetic operon; this structure acts to control the level of the downstream biosynthetic genes [97]. This transcriptional organisation allows moderate readthrough from the *lanA* promoter, thus ensuring a high level of transcription of the prepeptide mRNA in comparison to the mRNA encoding the biosynthetic enzymes.

2.3. The biosynthetic pathway

2.3.1. Prepeptides and the role of the leader sequence

The *lanA* structural gene(s) found in all lantibiotic gene clusters (*nisA*, *epiA* etc. indicated in Fig. 3) encode ribosomally synthesised precursor peptides referred to as 'prepeptides'. Unlike the mature peptides, these prelantibiotics are biologically inactive and carry an N-terminal extension, or leader peptide, which is attached to the C-terminal propeptide, e.g. *nisA* encodes a 57-amino-acid precursor peptide, 23 of which form the leader sequence which is cleaved from the mature peptide in the last step of nisin biosynthesis [98]. The propeptide domain is that which is modified and corresponds to the mature lantibiotic, but is only activated on proteolytic cleavage of the leader. Weil et al. [99] demonstrated that in Pep5, although the hydroxyamino acids serine and threonine are present in the leader as well as the propeptide, only residues in the propeptide domain undergo modification. The leader peptides of all of the characterised lantibiotics are also devoid of cysteine, in contrast to the cysteine-rich propeptides. The isolation of lantibiotic prepeptides from the cytoplasm of producing strains has proved quite difficult, suggesting that the primary translation product has a short half-life, being dehydrated immediately after synthesis [99]. Lantibiotic leader peptides are typically between 23 and 30 amino acid residues in length and do not resemble the typical *sec*-dependent transport signal sequences (*sec*-signal sequence, [100]) as they lack the hydrophobic membrane-spanning core and the typical processing site. In accordance with de Vos et al. [30], type-A lantibiotics can be classified into two groups, on the basis of size, charge, and sequence of the leader peptides.

2.3.1.1. Type-A(I) leaders. Type-A(I) leaders are generally hydrophilic, possess a high proportion of charged amino acids, some of which are highly conserved, e.g. the FNLDV box, and have a net negative or slightly positive

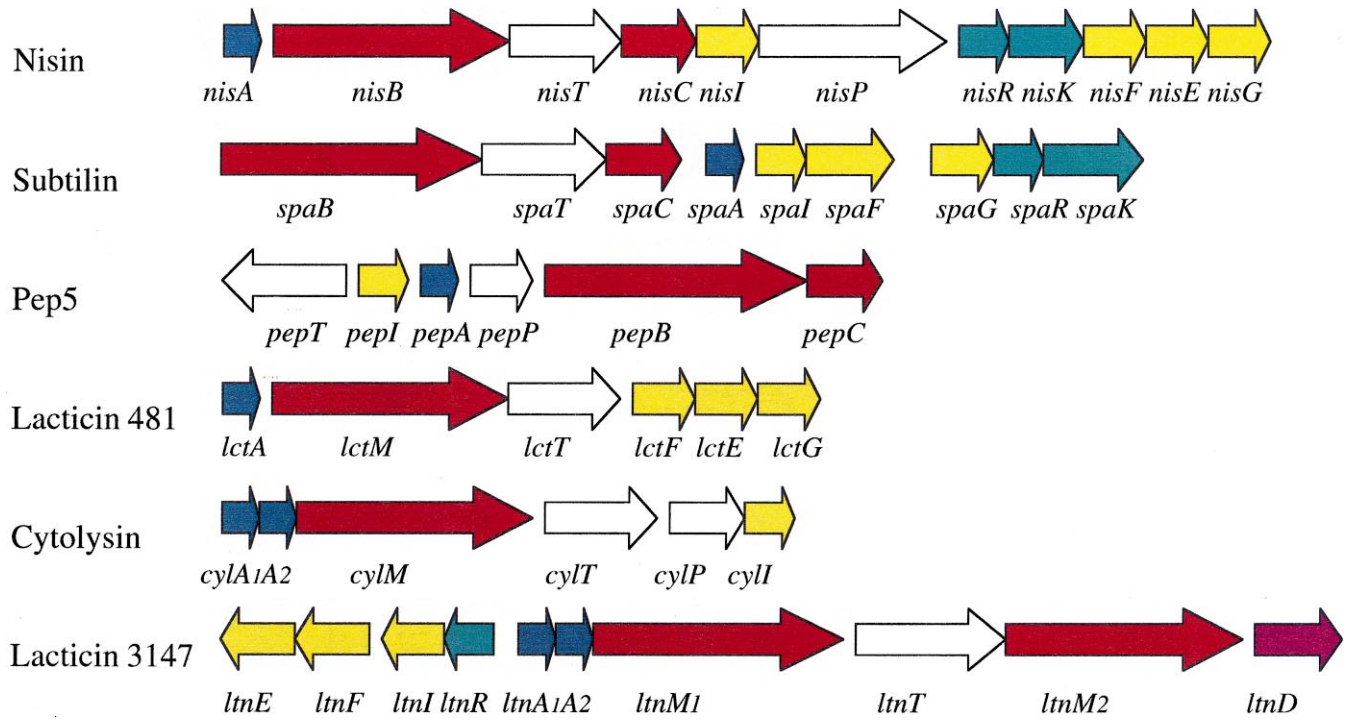

Fig. 3. Organisation of biosynthetic gene clusters of well characterised lantibiotics. Structural genes (not drawn to scale) are highlighted in blue; genes with similar proposed functions are highlighted in the same colour (yellow for immunity, white for transport/processing, green for regulatory red for modification, and blue for unknown function). Gene designations are according to de Vos et al. [30].

charge. This group also possesses a conserved Ser^{6-} and Pro^{2-}. When mutations were created in the −18 to −15 region (i.e., the FNLD consensus of the nisin leader) the secretion or intracellular accumulation of nisin was undetectable, indicating that even conservative changes in this highly conserved region leads to a block in nisin production [101]. In contrast, similar changes in this region of the Pep5 leader did not abolish activity, but production was significantly reduced [102]. In addition, both groups of lantibiotics differ in the residues that precede the propeptide domain; i.e., the cleavage site. Site-directed mutagenesis at Arg^{1-} and Ala^{4-}, but not at the conserved Pro^{2-}, in prenisin strongly affected cleavage of the leader and resulted in the extracellular accumulation of unprocessed, inactive nisin [101]. Consequently, it is probable that these residues are most likely involved in leader protease recognition. Also, the fact that unprocessed nisin was detected in the extracellular environment indicates that cleavage of the leader sequence is not a prerequisite for translocation.

2.3.1.2. Type-A(II) leaders. Typical type-A(II) leader peptides possess highly negative net charges, and have consensus sequences unlike those found in the type-A(I) group, e.g. the conserved ELS/EVS and EL/EM sequences [30]. The leader sequences of the type-A(II) lantibiotics, which are processed concomitantly on export, e.g. cytolysin [12], lacticin 481 [11], lacticin 3147 [82] are more similar to the Class II bacteriocin leader sequences as they contain a 'double-glycine' GG/GA/GS motif immediately preceding the cleavage site. It has been suggested that these lantibiotics and the non-Lan containing bacteriocins may be processed by a peptidase with similar specificity [30,103], and that the LanM type of modification enzymes are present in this group of lantibiotics, rather than LanB/C, because of differences in the leader sequences [76].

A number of potential roles have been suggested for leader sequences in lantibiotic prepeptides, including protection of the producer strain against high concentrations of intracellular bacteriocin since lantibiotic remains inactive with the leader sequence attached [101]. Moreover, the leader peptide may play a necessary role in lantibiotic biosynthesis, in that the consensus sequences within the leader may direct the prepeptide towards the maturation and transport proteins. Also, it has been suggested that the leader sequence may interact with the propeptide domain to ensure a suitable conformation for enzyme–substrate interaction [59], given that the evidence to date indicates that modifications are made at the prepeptide stage.

2.3.2. Enzymes involved in post-translational modification

Most of the genes in the lantibiotic gene clusters described to date have been assigned their potential functions as a result of homology to known genes. The products of the *lanB* and *lanC* genes in the nisin-like lantibiotic systems (*nisB* and *nisC*), and the *lanM* genes in the lacticin 481 (*lctM*), cytolysin (*cylM*), lacticin 3147 (*ltnM1* and *ltnM2*) and lactocin S (*lasM*) systems do not share sequence similarity with any known proteins, and therefore,

their function is unclear. However, disruption of these genes in various lantibiotic systems, e.g. nisin [104,105], epidermin [106], subtilin [107,108], cytolysin [45], has revealed their essential role in biosynthesis, as production of active bacteriocin is abolished in their absence. It has, therefore, been suggested that these enzymes are strong candidates as catalysts of the novel reactions responsible for the dehydration of hydroxyamino acids and thioether ring formation, although the molecular mechanisms involved remain to be elucidated. The enzyme(s) responsible for the formation of D-alanine in lactocin S and lacticin 3147 have yet to be identified.

2.3.2.1. The LanB and LanC enzymes. The *lanB* genes encode large proteins (approximately 1000 residues), which are generally rather hydrophilic in nature, but also have some hydrophobic domains, suggesting a membrane association. This has been demonstrated for NisB, a 115-kDa protein with several amphipathic transmembrane α-helices, which co-sediments with membrane vesicles of *L. lactis* [92], and also for SpaB [108]. It is not known, however, whether these proteins are integral to or simply attached to the membrane surface. In addition, EpiB was shown to have a loose association with the cytoplasmic membrane [109]. An essential role has been proposed for NisB in nisin maturation, as it has been reported that mature nisin is not secreted before NisB can be detected. The synthesis of SpaB is also strictly regulated, but conversely, occurs only when mature subtilin is detected outside the cell [110]. It has been proposed that the dehydration of serine and threonine is catalysed by the LanB enzymes, although experimental evidence is lacking. In strains producing the engineered nisin variants [Trp30]nisin and [Lys27, Lys31]nisin, Ser33 is not dehydrated to Dha33 in approximately 50% of the total peptide produced [111]. When *nisB* was cloned and overexpressed in these strains, only fully modified nisin was isolated, indicating complete conversion of Ser33 to Dha33 in the mature peptide [105]. This illustrates the obvious importance of NisB in the dehydration of Ser33. Despite this fact, however, it was not possible to unequivocally assign a dehydratase function to this enzyme, as overexpression of NisB did not result in the dehydration of Ser29, which remains unmodified in mature nisin. It has been proposed that the nature of the amino acids around the site of dehydration is critical for modification reactions to proceed [112,113].

The *lanC* gene products range in size from 398 (PepC) to 455 (EpiC) amino acids, and several conserved clusters can be identified within the predicted sequences. These proteins share a number of structural motifs, GXA**H**G, W**C**XG, and **CH**G, in which the amino acids histidine and cysteine are conserved. It is thought perhaps that these motifs have some relevance in the catalytic function of these enzymes, but no data have been produced to support this theory. Hydrophobicity plots reveal alternating hydrophobic and hydrophilic regions [92], suggesting a membrane location for these proteins, as demonstrated for NisC [104], although EpiC has not been detected in membrane fractions [114]. The putative role of these enzymes is in thioether formation, following dehydration of the prelantibiotic. Kupke and Gotz [114] reported that while purified EpiC interacted with EpiA in retardation experiments, no modifications occurred. PepC-deficient clones were capable of producing dehydrated peptides, but the majority did not contain thioether residues and none had the correct thioether pattern [115]. This would seem to suggest an essential role for PepC in thioether formation, perhaps in binding to the dehydrated prepeptides and stabilising prepeptide conformation to enable the thiol groups of cysteine to react with the correct dehydroamino acid. These observations, however, are not inconsistent with those reported for EpiC, as purified PepC was not used in this study. Overexpression of NisC did not appear to influence dehydration of nisin prepeptides, and therefore may have a role in thioether formation [105].

It appears that the LanB protein, together with LanC and LanT, the potential peptide translocator, is a component of a membrane-associated multimeric Lan synthetase complex, and the potentially unstable nature of this complex may explain some of the difficulties encountered in elucidating the roles of these enzymes. Using yeast two-hybrid systems, Siegers et al. [104] demonstrated the physical interaction between NisB, NisT, NisC and the NisA prepeptide. While NisC co-precipitated with the nisin prepeptide, NisB protein could not be detected on co-precipitation between NisB and NisA using NisA-specific antibodies. Since these antibodies were directed to the leader of NisA, it was suggested that the leader region of the prepeptide is involved in NisB binding, as discussed previously. On the basis of these and other results, a Lan synthetase complex was proposed, consisting of two molecules of NisT and NisC and a single molecule of NisB, and it is proposed that the nisin prepeptide is matured and translocated at this membrane-bound complex. More recently, the involvement of a similar complex has been proposed in subtilin biosynthesis [116], suggesting that such arrangements could be present in all lantibiotic systems.

2.3.2.2. The LanM enzymes. The gene clusters of lacticin 481, lactocin S, mersacidin, SA-FF22 and the two-component lantibiotic cytolysin do not contain *lanB* or *lanC* type genes, but a single *lanM* gene encodes a protein of 900–1000 amino acids which is proposed to be involved in post-translational modification of these lantibiotics. Insertional inactivation of *lctM* (lacticin 481), *cylM* (cytolysin) and *lasM* (lactocin S) genes respectively, led to loss of the bacteriocin production phenotype [45,95,117], indicating an essential role for this enzyme in biosynthesis, although the specific function is as yet unknown. The C-termini of these proteins show striking similarity to the

LanC proteins in the nisin-like lantibiotic systems, but the N-termini do not show homology to the LanB class, excluding the possibility that a gene fusion between *lanB* and *lanC* gave rise to *lanM*. Nevertheless, it is envisaged that the LanM proteins combine the functions of dehydration and thioether formation. Uguen et al. [118] recently used the yeast two-hybrid system to demonstrate direct contact between the prepeptide of lacticin 481 and LctM; this is the first such evidence for LanM proteins and supports their function as modification enzymes.

2.3.2.3. Modification of two-component lantibiotics. An unusual feature of the gene cluster of the two-component lantibiotic, lacticin 3147, is the presence of two *ltnM* genes; the requirement for two LanM proteins is unprecedented [82]. It is very likely that two *lanM* genes are also present in the staphylococcin C55 system, although sequencing of this gene cluster remains to be completed. It could be conceived that one LtnM protein is responsible for dehydration of the hydroxy amino acids, while the other catalysed thioether formation, much like the LanB and LanC enzymes in the type-A(I) group. However, the absence of a second modification enzyme in the lacticin 481, lactocin S and cytolysin systems makes this unlikely. A more probable scenario is that each prepeptide requires a separate modification enzyme for activity; this has been experimentally confirmed recently for lacticin 3147 [119]. On the basis of a number of knockout experiments, it was shown that LtnM1 acts to modify the LtnA1 structural peptide, while LtnA2 is modified by LtnM2 [119]. There is no significant sequence homology between the lacticin 3147 and cytolysin peptides, whereas protein sequence alignments show that LtnA1 and LtnA2 are very closely related to the staphylococcin C55 components, SacαA and SacβA [120]. Furthermore, in the cytolysin system, both peptide components are much more closely related to each other than is the case for lacticin 3147 or staphylococcin C55. This may explain why cytolysin can rely on only one such modification enzyme, while two LanM proteins are necessary in the lacticin 3147 system, and most probably in the staphylococcin C55 system.

2.3.2.4. Oxidative decarboxylation by EpiD. EpiD, a 118-amino-acid enzyme indispensable for epidermin biosynthesis, is the only biosynthetic enzyme involved in lantibiotic formation for which a role and catalytic properties have been determined. This protein is similar to a protein which has been identified in the products of the mersacidin gene cluster [121]; it is not known whether MrsD has a similar function to EpiD. Kupke et al. [122] purified EpiD and demonstrated that it is a flavoprotein which requires flavin mononucleotide as a cofactor, and as such was proposed to catalyse the oxidation–reduction reaction which is an essential step in the formation of AviCys in mature epidermin, i.e. the removal of two reducing equivalents from the C–C group to form a C=C group at the C-terminal cysteine residue. Mass spectroscopic analysis of this novel enzymatic reaction proved that EpiD catalysed the oxidative decarboxylation of EpiA [123], although the decarboxylation of the free cysteine residue may occur spontaneously. Furthermore, this group demonstrated that the recognition site for EpiD is in the propeptide region of EpiA, and therefore, the processing signal for this biosynthetic enzyme is not in the leader region, as has been proposed for the LanB, C, and M proteins. Also, it was shown that EpiD does not react with EpiA with a C-terminal Lan present [124], and therefore, the sequence of events leading to production of mature epidermin is most likely, dehydration and oxidative decarboxylation occurring spontaneously, followed by thioether ring formation.

2.3.3. Activation and translocation

In order for a lantibiotic to exert its antibacterial action, the leader peptide, which renders the prepeptide inactive, must be cleaved and the mature propeptide translocated across the cytoplasmic membrane. Lantibiotics do not use the general secretory pathway [125], as they do not possess the N-terminal *sec*-signal sequence [126]. Instead, a novel system has evolved in the producers of these peptides to translocate the precursor across the membrane. For type-A(I) lantibiotics, proteolytic cleavage of the leader is catalysed by serine proteases [127], termed LanP, prior to or after the peptide is translocated via dedicated transporters of the ABC (ATP-binding cassette)-superfamily, LanT. The type-A(II) lantibiotics, which are characterised by the 'double-glycine' cleavage site, possess LanT transporters which cleave the leader peptide concomitant with export.

2.3.3.1. Proteolytic cleavage by specific leader peptidases. Genes encoding products homologous to peptidases have been identified in many of the lantibiotic gene clusters characterised thus far. The LanP proteins vary in size, from 266 amino acids (LasP) to 682 amino acids (NisP). A number of these proteins possess a preprosequence, implying that the proteins are directed out of the cell, and act extracellularly.

NisP is a 75-kDa protein with striking similarity to the subtilisin-like serine proteases, and contains an N-terminal signal sequence and a C-terminal extension, LPXTGX, that could act as a membrane anchor [98]. This suggests that NisP is a secreted protein which becomes attached to the outside face of the membrane, leading to speculation that cleavage of the leader peptide is the last step in nisin biosynthesis. This was confirmed by gene disruption of *nisP*, which led to production of fully modified nisin with no antibacterial activity, as the leader remained attached [98] The leader peptidase of epidermin, EpiP, also possesses a signal sequence, but lacks the anchor extension observed in the NisP sequence [128], suggesting that this enzyme is exported and active outside the cell. Inactivation

of *epiP* in *Staphylococcus carnosus* revealed that EpiP is not an essential gene product and that this organism apparently contains a protease capable of substituting for the EpiP peptidase [128]. This also appears to be the case in the subtilin biosynthetic pathway, as no specific leader peptidase has been identified among the subtilin gene products. It has been proposed that a variety of secreted proteases produced by *Bacillus subtilis* could be involved in proteolytic activation. In contrast to the results reported for EpiP, PepP is essential for correct cleavage of Pep5 [115]. These experiments showed that PepP is the only protease which is capable of recognising the specific cleavage site of pre-Pep5. Although processing by other host proteases was demonstrated, only truncated peptides were produced which showed significantly reduced biological activity.

2.3.3.2. LanT, the ABC transporter. The proteins responsible for translocating lantibiotics outside the producing cell to where they are biologically active belong to the large family of ABC transporters [129,130]. Based on homology searches, potential transporters of this type have been found in all lantibiotic gene clusters studied to date. These translocators are characterised by four membrane-associated domains; two highly hydrophobic membrane-spanning domains, each consisting of six transmembrane regions, and two ATP-binding domains, with the conserved ATP-binding or Walker motif, GXGLST [131], on the cytoplasmic face of the membrane. Energy for export is provided by ATP hydrolysis, which presumably occurs at the ATP-binding domains. It is generally accepted that the membrane-spanning domains determine the substrate specificity of the transporter, which is in most cases quite relaxed [129]. Each domain may be present on a separate polypeptide; although in almost all lantibiotics characterised to date, with the exception of epidermin, both the membrane-spanning and ATP-binding domains are found on a single polypeptide. These polypeptides dimerise at the cytoplasmic membrane, forming an active translocation complex. Also, it has been shown experimentally that transport is most likely directed by the leader sequence.

The function of a number of LanT proteins has been investigated by gene disruption experiments. Since it was found that inactivation of NisT led to loss of production of and decreased immunity to nisin, no alternative transporter in the host appears to be able to act as a substitute for NisT [132]; however, active nisin could be detected inside producing cells. Similarly, disruption of LasT [95] resulted in loss of lactocin S production, but intracellular accumulation was not verified. In contrast, it seems that host-encoded transporters can substitute for PepT in the Pep5 system [115]; although, the reported reduction of Pep5 production by approximately 10% in the absence of PepT suggests that the host-provided transporters are less efficient than PepT. *epiT′T″*, found in the epidermin gene cluster, would require a frameshift to yield one protein, raising doubts as to whether or not this protein is produced in an active form. Nevertheless, epidermin can be secreted in the absence of this protein [128], again presumably by transporters of the host cell. Unlike the corresponding *epiT*, *gdmT*, from the natural variant gallidermin gene cluster, is not interrupted by a deletion [133]; expression of this gene in an epidermin producer, along with *gdmH*, a hydrophobic protein with no known homologues, resulted in a 7–10-fold increase in epidermin production.

As mentioned earlier, a second group of LanT transport proteins are found in the products of the gene clusters of the lantibiotics with the 'double-glycine' cleavage site. These ABC transporters have a dual function, in that they remove the leader while translocating the substrate. Members of this family include LctT from lacticin 481 [117], CylT (also known as CylB) from cytolysin [45], LtnT from lacticin 3147 [82], ScnT from SA-FF22 [79] and MrsT from mersacidin [121]. Lactocin S is the obvious exception, as this is processed and translocated by separate LasP and LasT proteins [95]. In order to achieve this dual function, this new family of transporters contains a proteolytic N-terminal domain belonging to the family of cysteine proteases (containing the sequence motifs QX_4D/$ECX_2AX_3MX_4Y$/FGX_4I/L and HY/FY/VVX_{10}I/LXDP), in addition to the integral membrane and ATP-binding domains [103]. This domain is located, along with the ATP-binding domain, inside the membrane. Similar proteins are present in many of the gene clusters of non-lantibiotic bacteriocins, e.g. lactococcin G [103], pediocin PA-1 [134] and plantaricin G (unpublished results cited by [103]). It is interesting to note that these bacteriocins also possess leader sequences with a 'double-glycine' cleavage site.

The *spaT* gene found in the subtilin gene cluster, produces a transporter which does not have this recognised proteolytic domain, and it was generally accepted that this lantibiotic was processed by host-encoded proteases. However, it was later observed that inactivation of *spaT* did not disrupt translocation. Interestingly, it is instead proteolytic cleavage of the leader segment which is disrupted [135]. Sequence homologies clearly identify SpaT as an ABC transporter, and not a protease. In addition, the subtilin leader does not possess the diglycine and other motifs that are typical of these chimeric transporters. In order for SpaT to have proteolytic activity in addition to transport activity, it would have to constitute a new class of dual-function translocators.

2.3.3.3. Activation and export of cytolysin. Cytolysin is a two-component lantibiotic, produced by *E. faecalis*, with both bactericidal and haemolytic biological activities. An interesting feature of the cytolysin biosynthetic pathway is the presence of both a LanP-type serine protease, termed CylP (formerly CylA) [136], and a chimeric transporter

with an associated proteolytic activity, termed CylT (previously CylB) [12]. CylT was the first ABC exporter identified in Gram-positive bacteria [12]. Detailed mutagenesis studies demonstrated that while the C-terminal resident ATP-binding activity of CylT is not essential for CylT-mediated secretion of one of the components, CylA1, it is a prerequisite for secretion of the other, CylA2 [45]. CylT also mediates removal of the cytolysin-subunit leader peptide during secretion [103]. In addition, CylP acts extracellularly to activate the cytolysin precursors, CylA1 and CylA2, through further N-terminal proteolytic cleavage. Hence, a two-step process has been proposed for the activation of this bacteriocin [137,138]; both cytolysin precursors are externalised by CylT in a form possessing an N-terminal truncation, and both cytolysin subunits are further trimmed at the N-terminus by CylP in the process of activation.

2.4. Regulation of lantibiotic biosynthesis

The synthesis of a number of lantibiotics has been shown to be growth-phase-dependent [110,139] and appears to be under the control of a large family of two-component signal transduction systems [140,141]. In their simplest form, these intracellular signalling systems have two protein components: a membrane-bound sensor, a histidine protein kinase (HPK), which monitors an environmental signal; and a cytoplasmic response regulator (RR) that mediates an adaptive response which is usually a change in gene expression [141]. In response to the external signal, the HPK autophosphorylates a conserved His residue in the C-terminal cytoplasmic domain of the protein. The phosphoryl group is then transferred to a conserved Asp of the corresponding intracellular RR which is generally a transcriptional activator. Genes encoding both HPKs (LanK) and RRs (LanR) have been identified in the gene clusters of nisin [110], subtilin [139], SA-FF22 [79] and mersacidin [121]. In the case of nisin and subtilin, inactivation studies confirmed that these genes were essential for production. It is proposed that the most likely target for the RR is the promoter of *lanA*, the lantibiotic structural gene. Even though the nature of the signalling molecule is unknown in the subtilin biosynthesis pathway, it has been demonstrated that fully modified nisin autoregulates its own biosynthesis [142], by acting as a peptide pheromone for quorum sensing involving NisK and NisR [143]. A protein with some similarities to RRs, EpiQ, was shown to regulate the production of epidermin [144]; however, EpiQ does not appear to act as a RR. These, and other aspects of lantibiotic regulation, are discussed below.

2.4.1. Autoregulation of nisin production

As mentioned previously, the proteins encoded by *nisR* and *nisK* have been shown to be involved in the regulation of nisin biosynthesis [98,110]. It has recently been demonstrated that fully modified nisin, mutant nisin species and nisin analogues can act as inducers of transcription of the *nisA* structural gene as well as the downstream genes, via signal transduction, by acting as an extracellular signal for the sensor histidine kinase, NisK [142]. Previously, it had been reported that a 260-bp transcript was produced from the *nisA* gene, and that creation of a deletion within this gene completely abolished *nisA* transcription [93]. Δ*nisA* transcription could be restored on supplying exogenous nisin in the culture medium [142]. Interestingly, unmodified prenisin, in addition to some other related lantibiotics, was unable to restore transcription, suggesting that the modifications found in nisin are critical for induction. It was proposed that residues 1–11, which comprise the first two rings of mature nisin, may interact directly with the membrane-located NisK, thus signalling NisR to activate transcription at the *nisA* promoter. Gel mobility shift assays have shown that overproduced His-tagged NisR binds to direct repeats in the *nisA* promoter region, possibly as a dimer and from there, triggers gene expression [145].

2.4.2. Regulation of lacticin 3147 immunity

Analysis of the lacticin 3147 operons identified a candidate regulator, LtnR; this 79-residue-protein has a high degree of homology to the PBSX (Xre) family of transcriptional repressors [146]. It has recently been demonstrated that LtnR is responsible for repression of its own transcription and that of the downstream immunity genes, *ltnIFE* [97]. Regulation of expression from the promoter controlling the immunity genes (P_{imm}) is achieved through binding of LtnR to the intergenic region between *ltnR* and the first lacticin 3147 structural gene, *ltnA1*. This region encompasses the divergent lacticin 3147 promoters and an inverted repeat has been identified within this region which may serve as a specific operator site. The binding of LtnR to this region results in a 90% decrease in the levels of expression of the lacticin 3147 immunity genes. It was also established that the biosynthesis of lacticin 3147 is not regulated by LtnR [97]. However, while expression from the promoter preceding the biosynthetic genes (P_{bac}) appears to be constitutive, an intragenic rho-independent terminator identified within the modification gene, *ltnM1*, most likely controls the level of transcription of the downstream biosynthetic genes. This transcriptional organisation ensures the correct stoichiometry is maintained between the abundant prepeptide mRNA and the mRNA for the biosynthetic genes. To our knowledge, this is the first report of negative regulation of gene expression through a repressor in a lantibiotic gene cluster. A regulator which appears to be repressor in nature has also been identified in the cytolysin gene cluster [147], but has not been further characterised.

2.4.3. Other regulatory proteins

Production of epidermin is regulated by EpiQ, which

possesses some similarities to RRs at the C-terminus, but lacks the highly conserved phosphoryl acceptor Asp residue [90]. In addition, no corresponding histidine kinase has been identified; however, it has been proposed that EpiQ may direct epidermin biosynthesis following phosphorylation by an intrinsic histidine kinase [106]. Despite the lack of a cognate HPK, the function of EpiQ as a transcriptional activator has been demonstrated. Peschel et al. [144] reported that EpiQ activates the *epiA* promoter by binding to an inverted repeat (ANAATTAC-N_6GTAATTNT) immediately upstream of the −35 region of this promoter. Similar repeats have been identified in other promoters in the epidermin gene cluster. In addition, the transcription of *epiFEG*, suggested to be involved in immunity, and *epiHT*, putatively involved in transport, is increased in the presence of EpiQ [133,148].

Altena et al. [121] have recently reported the identification of three regulatory genes, *mrsR1*, *mrsR2* and *mrsK2*, in the mersacidin gene cluster. *mrsR2* and *mrsK2* are components of a two-component regulatory system which appears to be necessary for the transcription of the mersacidin immunity operon, *mrsFGE*. *mrsR1* encodes a protein with similarity to RRs but it is unknown whether this protein plays a role in regulation of production and/or immunity of this lantibiotic.

Open reading frame (ORF) 239 in the lactocin S gene cluster has a significant level of identity (22%) to WrbA, which is involved in regulation of *trp* operon expression in *Escherichia coli* [95]. Also, in the mutacin II gene cluster, produced by *Streptococcus mutans*, a gene product with 25% identity to a positive transcriptional regulator, Rgg, has been identified [149]. At present, it is not known if these proteins play a role in regulating biosynthesis of their respective lantibiotics. To date, regulatory proteins have not been identified in the gene clusters of Pep5 or lacticin 481.

2.5. Producer immunity to lantibiotics

Bacteriocin production is invariably linked to the expression of specific immunity proteins required to protect the producing strain against the inhibitory action of its own product [150,151]. To date, the mechanisms by which these proteins confer immunity remain relatively unknown. Two distinct systems of lantibiotic immunity have been identified to date. Protection can be mediated by the first of these, the so-called 'immunity' proteins, LanI [89,93,152–156], while the second constitute specialised ABC-transport proteins, LanFEG [79,152,155,148], which can be encoded on two or three separate ORFs.

2.5.1. The immunity proteins, LanI

The first LanI protein described for a lantibiotic was PepI, which is encoded by the Pep5 operon [89]. This 69-amino-acid protein has a hydrophobic N-terminal domain and a strongly hydrophilic C-terminal part, suggesting that PepI is membrane-associated [153]. This protein is most likely attached to the outer surface of the membrane where it can antagonise the pore-forming activity of Pep5. In this respect, the Pep5 immunity peptide and the proposed molecular mechanism of immunity are more closely related to the immunity systems of the non-lantibiotic peptide bacteriocins [7,150] than that of other lantibiotics. However, no suggestion of a direct interaction between Pep5 and PepI could be observed using circular dichroism (unpublished results cited in [30]). Recent evidence has established that the apparent coupling of immunity to Pep5 production, initially reported by Reis et al. [153], is achieved through the stabilisation of *pepI*-containing transcripts by an inverted repeat, which in the wild-type is located downstream of *pepA* [156]. PepI displays a high degree of similarity (74.2%) to EciI, the epicidin 280 immunity protein [154], and also confers cross-immunity to epicidin 280, suggesting a similar self-protection mechanism for both lantibiotics. This is the only reported case of cross-immunity between lantibiotic producers.

Immunity proteins of 165 (SpaI) and 245 (NisI) amino acids are found in the gene products of the subtilin [152] and nisin [93] gene clusters. There is no sequence similarity between these proteins and PepI, except for the rather hydrophobic N-terminus found in all three proteins, which may serve as a membrane anchor. The presence of typical lipoprotein signal sequences [126] in both SpaI and NisI suggest that these proteins become peripheral membrane proteins, attached to the membrane by a lipid-modified N-terminal cysteine [93,152,157]. It is interesting to note that apart from the signal sequence and characteristic consensus cleavage site, there is no homology between SpaI and NisI, despite the fact that subtilin and nisin share approximately 60% sequence similarity; this indicates the highly specific interactions between these immunity proteins and their respective lantibiotic. Expression of *nisI* in both *L. lactis* and *E. coli* provided the cells with a significant level of protection against exogenous nisin in liquid cultures [93] and in plate assays [110]. However, full immunity was only observed when mature nisin, or the *nisA* structural gene was present [93]. This has subsequently been shown to be a result of nisin autoregulation, as mature nisin is required to activate transcription of the nisin gene cluster [142]. Again, the levels of immunity conferred on these strains by NisI were only a fraction of the wild-type levels, providing evidence that other gene products play a role in producer self-protection in both the subtilin and nisin systems.

Recently, a 116-amino-acid protein termed LtnI has been shown to confer immunity to lacticin 3147 [146]. When the *ltnI* gene was cloned downstream of the strong P32 promoter in pMG36e and transformed into the lacticin-sensitive *L. lactis* MG1363, levels of immunity comparable to that of the wild-type producer strain were observed. The presence of three putative transmembrane domains suggests that LtnI is most likely localised at the

cytoplasmic membrane where it may serve to hinder the insertion of the bacteriocin molecules in the membrane. Also, a leucine-zipper motif has been identified at the C-terminus of LtnI. Leucine zippers are usually associated with DNA-binding proteins where they promote dimerisation [158]; it is conceivable that LtnI forms homodimers in order to create an active complex, but the mode of action of this protein remains to be determined. Sequence analysis of the region downstream of the cytolysin activator *cylP* revealed a gene encoding a 327-amino-acid protein, CylI, which was shown to be essential for immunity to cytolysin [147]. A protein of similar size and hydrophobicity to PepI is found in the lactocin S gene cluster [95], but as yet, a role in immunity has not been demonstrated for this protein.

2.5.2. Involvement of LanFEG in immunity

In addition to LanT, genes encoding a second ABC transporter have been identified in a number of lantibiotic systems; however, in this case the individual domains are encoded by separate genes. *lanF* encodes the intracellular ATP-binding domain, whereas LanE and LanG represent the membrane-spanning subunits. ABC-transporters of this kind are found in the gene clusters of nisin [155], epidermin [148], lacticin 481 [159], and mersacidin [121]. In the subtilin system, the ABC transporter involved in immunity is encoded by two genes, *spaF* and *spaG* [152]. In this instance, SpaF contains both an ATP-binding domain at the N-terminus, and a membrane-spanning domain at the C-terminus [152], similar to the HisP family of translocators [160]. In the lacticin 3147 gene cluster, genes encoding an ATP-binding domain (*ltnF*) and a membrane-spanning domain (*ltnE*) of an ABC transporter have been identified [82,146]; the role of these gene products in lacticin 3147 immunity remains to be investigated.

Insertional inactivation of the *nisFEG* genes resulted in a decrease in both nisin production and immunity [155]; similar observations were reported for interruption of *spaFG* [150]. In both cases, the mutant strains survived the relatively high amounts of bacteriocin they produced, confirming the role of other factors, most probably the LanI proteins, in conferring immunity to these lantibiotics. The *epiFEG* genes conferred increased tolerance to epidermin on *S. carnosus*, and all three genes were required for expression of the immunity phenotype [148]. However, EpiFEG seemed insufficient to provide complete self-protection, suggesting the existence of other immunity factors. As yet, no other putative immunity proteins have been identified in the epidermin system. Peschel and Gotz [148] proposed that the LanFEG proteins could mediate immunity by either active extrusion of the respective peptide, which would keep the lantibiotic concentration in the membrane below a critical level, or by uptake and intracellular degradation. Recently, it has been demonstrated using peptide release assays based on HPLC analysis, that the former appears to be the case. Otto et al. [161] reported that on incubation of cells with gallidermin, the extracellular gallidermin concentration was 4-fold higher for an *epiFEG*-expressing strain than for a control strain, strongly suggesting that the EpiFEG transporter works by expulsion of its substrate into the surrounding medium. These experiments provide the first evidence supporting export of the respective peptide from the membrane as the molecular mechanism of immunity in strains possessing LanFEG transporters.

3. Mechanism of action of bacteriocins

In general, the action of bacteriocins produced by Gram-positive bacteria is directed primarily against other Gram-positive species. The range of organisms inhibited by each bacteriocin varies greatly; while nisin is active against a broad variety of bacteria including strains of *Lactococcus*, *Streptococcus*, *Staphylococcus*, *Listeria* and *Mycobacterium*, as well as the vegetative cells and outgrowing spores of *Bacillus* and *Clostridium* species [2,68,162], the Class II bacteriocin, lactococcin A, specifically kills lactococci [162,163]. Under normal circumstances, bacteriocins produced by Gram-positive bacteria do not have a bactericidal effect on Gram-negative species. However, in some cases, activity against Gram-negatives can be observed on disruption of the outer membrane, as reported for nisin [165]. It has been established that the primary target for many of these small, cationic peptides is the cytoplasmic membrane of sensitive cells [164,166–170], where they act to dissipate the proton motive force (PMF) through the formation of discrete pores in the cytoplasmic membrane, and thus deprive cells of an essential energy source [171]. The PMF, which is composed of a chemical component (the pH gradient; ΔpH) and an electrical component (the membrane potential; $\Delta\psi$), drives ATP synthesis and the accumulation of ions and other metabolites through PMF-driven transport systems in the membrane [172]. Collapse of the PMF, induced by bacteriocin action, leads to cell death through cessation of energy-requiring reactions. Such a mode of action has been demonstrated for the type-A lantibiotics and the Class II bacteriocins. The type-A lantibiotics act in a voltage-dependent manner without the requirement for a specific protein receptor [173–177]; however, recent work has shown that the activity of nisin is dependent on the concentration of lipid II (undecaprenyl-pyrophosphoryl-Mur*N*Ac-(pentapeptide)-Glc*N*Ac) in the membrane of sensitive cells [178,179]. The Class II bacteriocins are thought to interact with membrane receptor proteins prior to insertion into the cytoplasmic membrane in a voltage-independent fashion [164,167]. In contrast, the type-B lantibiotics do not form membrane pores; instead, these peptides act by interfering with essential enzyme activities [180].

3.1. Pore-forming lantibiotics

Studies with intact bacterial cells, membrane vesicles and artificial liposomes have demonstrated that the type-A lantibiotics including nisin, Pep5, subtilin, lacticin 3147 and streptococcin FF22 act to disrupt the PMF, inhibit transport of amino acids and cause the release of intracellular low-molecular-mass compounds, such as amino acids, ions and ATP, by forming short-lived, non-selective, transmembrane pores [177,181–184]. It appears that in vivo, no binding to a putative target cell protein receptor is necessary for the action of the above mentioned lantibiotics, since liposomes can be used to study pore formation. Formation of such pores is energy-dependent, and much of the current knowledge on the mechanism of pore formation has come from work with nisin in artificial membrane systems.

3.1.1. Biological activity of nisin

As mentioned previously, the lantibiotic nisin is bactericidal against a wide range of Gram-positive bacteria, in addition to preventing the outgrowth of spores [10]. It was demonstrated that in vitro, nisin inhibited bacterial cell wall biosynthesis [185]. Subsequently, it has been shown that nisin kills bacterial cells by interfering with basic energy transduction occurring at the cytoplasmic membrane [166,173,181]. It was found that pores formed in the membrane by the nisin molecules allowed the diffusion of small compounds, since no transport system for ATP has been reported [173,186]. The increase in membrane permeability results in the collapse of the PMF; in the case of nisin, both the ΔpH and the $\Delta\psi$ are completely dissipated leading to a rapid cessation of all biosynthetic processes [173,175,187,188]. In contrast, the inhibition of cell wall biosynthesis is a comparatively slow process. Thus, pore formation is considered the primary mode of action of nisin.

3.1.1.1. Effect of membrane composition. The interaction of nisin with membrane components of sensitive cells is considered a vital step in its mode of action. It has been reported that even in the absence of an energised membrane, nisin can associate tightly with lipid bilayers through electrostatic interactions with the phospholipid head groups [189]. Model membrane systems have been used extensively to study the lipid dependency of the nisin–membrane interaction [176,189–192]. The degree of association of nisin with the membrane is largely dependent on the type of lipids present, and most importantly, the charge carried by those lipids. Several groups have demonstrated that due to the cationic nature of nisin, its activity in vitro is most efficient when a high percentage of anionic, or negatively charged, membrane lipids are present [176,193,194]. More recent studies have substantiated this evidence, confirming the ability of nisin to insert into lipid monolayers in an anionic lipid-dependent way [195]. Therefore, the composition of membranes is likely to be an important determinant in the sensitivity of different bacterial species to nisin. Breukink et al. [194] reported that this initial interaction with anionic phospholipids is mediated by the C-terminal domain of the peptide, since this region contains the bulk of the positive charge carried by the nisin molecule. Replacing valine at position 32 of nisin Z with a negatively charged glutamate residue abolished the negatively charged dependency of this interaction, thus decreasing the antimicrobial activity of this mutant nisin Z species compared to wild-type nisin Z [194]. Thus, the C-terminal domain of nisin constitutes an important anionic phospholipid binding site. Nisin also displays anion carrier activity in carboxyfluorescein-filled liposomes composed of cationic phospholipids [189]. Essentially, the positively charged nisin molecules bind the negatively charged carboxyfluorescein molecules at the inside of the membrane, and cross the membrane as a nisin–anion complex. On the outer membrane surface, the anion is released and nisin returns to bind another molecule. This activity is strongly inhibited in liposomes composed of anionic phospholipids, presumably because the positively charged nisin is involved in interactions with the negatively charged phospholipids [176,189]. Little or no anion carrier activity would be expected in vivo, since bacterial cell membranes are rich in anionic phospholipids.

3.1.1.2. Energy requirements for pore formation. The membrane interactions described above are followed by insertion of nisin into the membrane in an energy-dependent manner. The energisation state of a sensitive cell is critical for the pore-forming activity of nisin; energy is required for both formation and opening of pores. The electrical transmembrane potential ($\Delta\psi$), as generated by metabolising bacterial cells, is considered the major driving force for activity. Sahl et al. [173] reported that a $\Delta\psi$ is required for nisin action and similarly, studies with carboxyfluorescein-loaded liposomes confirmed that a $\Delta\psi$ is necessary for nisin's membrane-disruptive abilities [176]. Black-lipid membrane experiments revealed that nisin can form pores only when a *trans*-negative (inside negative) electrical potential is applied, the same orientation as occurs at the bacterial cytoplasmic membrane [173,190]. From these experiments, it was also possible to determine the threshold potential required for pore formation, and to estimate the size of the pores formed. Nisin appears to form quite stable pores with a diameter of approximately 1 nm, with a threshold potential of approximately −80 mV [173,190]. At low $\Delta\psi$ within the range of −10 to −40 mV, nisin fails to induce any permeability in artificial membranes. Nevertheless, one study has demonstrated $\Delta\psi$ dissipation by nisin in *E. coli* liposomes in the absence of a threshold potential [192]. Driessen et al. [189] proposed that the presence of a $\Delta\psi$ may change the orientation of

nisin in the membrane prior to pore formation. More recent evidence suggests that nisin is not exclusively a voltage-dependent bacteriocin. Moll et al. [196] demonstrated the ability of nisin to dissipate the ΔpH of *L. lactis* cells, even in the absence of a $\Delta\psi$. Therefore, while it remains the case that efflux and depolarisation by nisin is more efficient with energised cells, both the $\Delta\psi$ and the ΔpH can serve to promote this activity.

3.1.1.3. Secondary mode of action. Study of nisin-treated cells over a longer time course revealed that this cationic peptide is capable of inducing autolysis of some *Staphylococcus* species [197]. Nisin was shown to competitively release lytic enzymes from their inhibitors, the polyanionic cell wall constituents such as teichoic-, lipoteichoic-, and teichuronic acids. Under normal conditions, these enzymes, which include *N*-acetylmuramoyl-L-alanine-amidase and β-*N*-acetylglucosaminidase, strictly regulate cell wall division and turnover [198]. The non-specific activation by nisin results in the degradation of areas of the cell wall, particularly in the area of the septum [199–201]. The combination of increased osmotic pressure, which results from pore formation, and a weakened cell wall encourages subsequent cell lysis. Similar observations have been reported for Pep5 [197,199–201].

3.1.1.4. Models for pore formation. A number of tentative models have been proposed for the mechanism of pore formation by lantibiotics, but as yet very little is known about the in vivo situation [202,203]. Pore formation by the 'barrel-stave' mechanism, used by a number of cytolytic pore-forming toxins [204], has been predicted (Fig. 4). This model involves the initial accumulation of the peptide at the membrane surface through ionic interactions with the phospholipid head groups. The presence of these peptides induces significant thinning of the membrane in these areas, due to localised displacement of the phospholipids. On application of a $\Delta\psi$, the molecules adopt a transmembrane orientation. As lantibiotics are small peptides that can span the membrane only once, it is assumed that several molecules associate with the membrane to form a pore. Whether this aggregation of molecules occurs prior to insertion, or in the membrane after insertion is unknown. It has been demonstrated that at high pH, nisin monomers aggregate outside the membrane, significantly reducing biological activity [176]. Thus, insertion followed by aggregation is the favoured model. As yet, it is unknown how many monomers are required to form a pore, but it is believed that it is a dynamic process with peptides joining and leaving the pore complex. The peptides are thought to align around a central channel, with the hydrophobic faces towards the lipid bilayer and the hydrophilic faces towards the pore centre.

A wedge model has also been proposed for pore formation by nisin. Here, the positively charged C-terminus, together with the bound lipids, enter into the membrane forming a wedge-like pore composed of multiple nisin

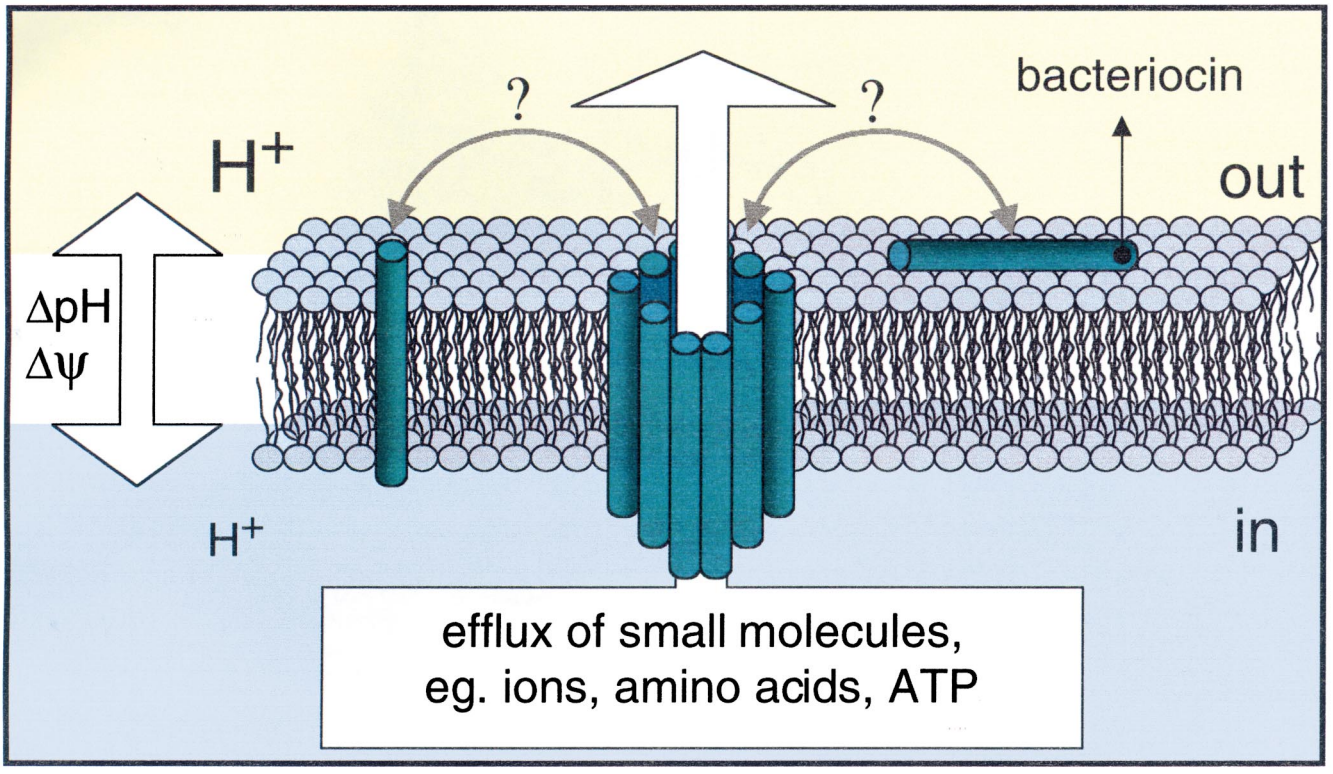

Fig. 4. General model of the 'barrel-stave' mechanism of pore formation by peptides. Adapted from [203].

molecules [189,196,202]. Moll et al. [196] suggested that the hinge region of the nisin molecule may act as a twist that allows nisin to bend the lipid surface. More recently, this group have reported that nisin induces rapid movement of a fluorescent phospholipid from the inner leaflet to the outer leaflet of unilamellar phospholipid vesicles [205]. This supports the theory of nisin-induced disturbance of the phospholipid organisation in the membrane, as proposed by the wedge-like model.

Some contradictory evidence exists which makes the proposal of a pore formation model difficult. Breukink et al. [206] studied the topology of nisin in model membranes using site-directed tryptophan fluorescence spectroscopy. This work revealed an overall parallel orientation for nisin, suggesting that nisin causes membrane leakage by destabilising the bilayer while remaining parallel to the membrane, as has been proposed for other membrane-active peptides [207]. However, more recently, van Kraaij et al. [208] demonstrated that the C-terminus of nisin translocates the membrane, a process which would agree with the 'barrel-stave' model outlined above. It was suggested that the most likely event after transient pore formation by nisin would be translocation of the whole molecule to the inside of the membrane, as has been described for magainin [209].

3.1.1.5. The role of lipid II in nisin activity. While the model outlined above was based on results obtained with model membrane systems, a number of the observations made using intact bacterial cells remained unexplained, e.g. the killing efficiency of nisin in nanomolar concentrations in vivo, compared to its activity at micromolar concentrations in membrane systems [210]. As previously mentioned, it is believed that nisin also mediates inhibition of cell wall biosynthesis, by forming a complex with the bactoprenol-bound peptidoglycan precursor, Lipid II [185]. Brotz et al. [211] reported that ramoplanin-treated cells of *Micrococcus luteus*, i.e. cells in which accessibility to Lipid II is reduced, were resistant to the inhibitory effect of nisin, suggesting that nisin may use Lipid II as a 'docking molecule' for binding to specific membranes. In a recent comparative study with the defense peptide magainin 2, Breukink et al. [178] demonstrated that increasing the concentration of Lipid II in isolated model membranes, in the range of 0.001–0.1 mol percent, increases the sensitivity of the membrane to nisin. The results of this study also showed that not only does Lipid II function in the activity of nisin, but that it appears to be the sole target of nisin. The effect of Lipid II was specific for nisin, suggesting a specific, high-affinity interaction of Lipid II with one or more of the structural elements of nisin, resulting in pore formation. A study examining the interaction of Lipid II and a number of mutant nisin species to identify structural elements of the nisin molecule involved found that mutations affecting the conformation of rings A through C ([S3T]nisin) led to reduced binding of Lipid II and and increased the concentration needed for pore formation [212]. In contrast, peptides mutated in the flexible hinge region ([N20/M21]nisin) were unable to form pores, but surprisingly had only a slightly reduced activity in vivo. This in vivo activity was a result of the unaltered ability of this mutant nisin species to bind Lipid II, inhibiting its incorporation into the peptidoglycan chain [212]. Thus, nisin demonstrates a Lipid-II-mediated duality, combining two killing mechanisms in one molecule and therefore, the model of pore formation has to be revised. Certainly, many questions relating to lantibiotic-induced pore formation remain unanswered, and considerably more study is needed to understand these mechanisms at the molecular level.

3.1.2. Other pore-forming lantibiotics

Other lantibiotics that have been characterised as voltage-dependent pore-formers have mechanisms of action similar to that described above for nisin. Nevertheless, some significant differences have been reported. As previously discussed, the orientation of the $\Delta\psi$ applied across the membrane is critical for pore formation. Pep5, like nisin, forms pores only with the application of a *trans*-negative $\Delta\psi$ [182]; in contrast, subtilin, epidermin and streptococcin FF22 (SA-FF22) can act irrespective of the orientation of the potential [174,183,184]. Furthermore, subtilin and epidermin have been reported to form larger pores than nisin, e.g. subtilin can form pores of up to 2 nm with lifetimes of up to 10 s. In contrast, SA-FF22 forms pores of approximately 0.5–0.6 nm in diameter and with lifetimes of only milliseconds [184]. Consequently, SA-FF22-treated cells demonstrate efflux of ions only, while it is likely that amino acids and ATP remain inside the cells. It is proposed, therefore, that cell death due to the action of SA-FF22 results from disruption of the PMF, rather than immediate loss of metabolites [184].

Carnocin U149, a lantibiotic produced by *Carnobacterium pisicola*, also acts at the cytoplasmic membrane in a similar fashion to nisin [50,51]. It was observed that nisin-producing lactococci were highly sensitive to the inhibitory action of carnocin U149. Subsequent examination of the sensitivities of *L. lactis* MG1614 transformants harbouring plasmids with varying amounts of the nisin biosynthetic gene cluster revealed that NisP, the proteolytic activator of nisin is the only protein in the nisin biosynthesis pathway capable of enhancing carnocin U149 activity. Hence, NisP may act as a membrane receptor for carnocin U149 [51]. This proposed receptor-mediated action, however, seems to be specific for the bacteriocin carnocin U149 and nisin-producing *L. lactis* strains, as other LAB, though inhibited by carnocin U149, are up to 10 times more resistant to its action than nisin-producing lactococci [51].

In addition to their ability to kill vegetative cells by pore formation, nisin and subtilin also inhibit the outgrowth of bacterial endospores; however, the exact mode of inhibition remains to be elucidated. It has been reported that in

the case of subtilin, the molecular mechanism responsible for preventing spore outgrowth is not the same used to inhibit vegetative cells. This activity can be clearly attributed to the Dha residue at position 5. It is assumed that the double bond provides a reactive group for an interaction with a spore-associated factor that is essential for outgrowth [213].

3.2. Type-B lantibiotics

3.2.1. The cinnamycin subtype

This subtype of the type-B lantibiotics includes cinnamycin, ancovenin and the duramycins which demonstrate antimicrobial activity against relatively few bacterial strains, in particular *B. subtilis* [214–216]. Treatment of sensitive cells with members of this bacteriocin group results in increased membrane permeability, reduced ATP-dependent calcium uptake, and ATP-dependent protein transport [217–219]. However, the mode of action of this group is not strictly limited to bacterial cells as lysis of red blood cells has also been observed on treatment with cinnamycin. This effect could be significantly reduced by first incubating the lantibiotic in the presence of the phospholipid phosphatidylethanolamine, suggesting that cinnamycin has an ability to interact specifically with this phospholipid [220]. Similar observations were made with duramycin [221,222]. In addition to these membrane effects, this group of lantibiotics have also been shown to inhibit the enzyme phospholipase A2, an enzyme involved in the synthesis of prostaglandins and leukotrienes in the human immune system [223]. Since phospahtidylethanolamine is the primary substrate for this enzyme, binding of the lantibiotic renders the phospholipid unavailable for conversion by phospholipase A2.

3.2.2. Mode of action of mersacidin

Mersacidin, a type-B lantibiotic produced by a species of *Bacillus*, is active against several Gram-positive bacteria including methicillin-resistant *S. aureus* [224]. The mode of action of mersacidin differs not only from the type-A lantibiotics but also from those of the type-B group mentioned above. Treatment with mersacidin results in the cessation of growth and a slow induction of lysis [225]. Macromolecular biosynthetic processes are uninhibited, except for the synthesis of peptidoglycan, indicated by a block on glucose and D-alanine incorporation. In addition, the thickness of the cell wall of treated cells was markedly reduced. Thus, inhibition of cell wall biosynthesis is the primary mode of action of mersacidin [225].

Comparison of the cytoplasmic pool of peptidoglycan precursors in mersacidin-treated cells revealed that the level of UDP-Mur*N*Ac-pentapeptide, the ultimate soluble peptidoglycan precursor, was unaffected by the action of the lantibiotic [180], suggesting that mersacidin inhibits a later membrane-associated step of the biosynthesis pathway. In the case of *Bacillus megaterium*, this step was identified as the transglycosylation step, or first polymerisation reaction, due to an accumulation of lipid II in the membrane (Fig. 5; [180]). Subsequent experiments have shown that the molecular basis for this inhibition is the interaction of mersacidin with lipid II, and not with the transglycosylase enzyme [226]. The existing glycopeptide antibiotics, such as vancomycin, inhibit transglycosylation by binding to lipid II at the peptide side-chain of this molecule [227,228]. It is known that mersacidin does not bind to this side-chain [226], or to any a site targeted by any antibiotic currently in use. Indeed, the exact binding site for this bacteriocin remains to be identified. Thus, mersacidin and lantibiotics with similar modes of action, e.g. actagardine, may prove useful in the treatment of emerging multi-drug-resistant pathogens, such as methicillin-resistant *S. aureus* and vancomycin-resistant enterococci.

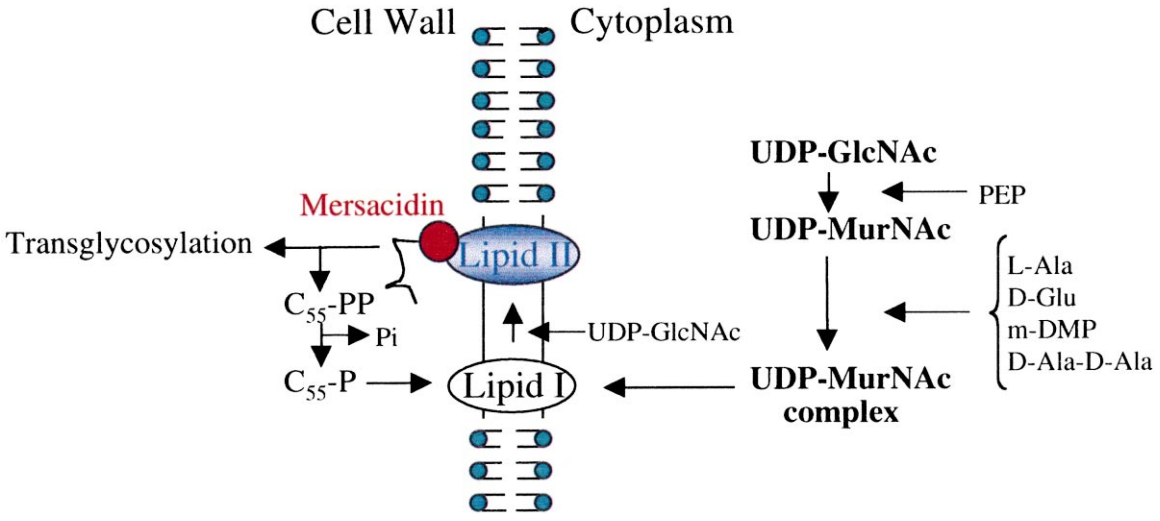

Fig. 5. Schematic representation of the major events occurring during cell wall biosynthesis in bacteria. The likely site of inhibition of cell wall biosynthesis by mersacidin is indicated. Adapted from [203].

3.3. Two-component bacteriocins

A number of Class II bacteriocins which require the complementary action of two peptides for activity have been characterised to date. These bacteriocins are referred to as the Class IIb group, and the mode of action of some of these bacteriocins has been described. Two-component bacteriocins can be further classified into two types; the type-E class (Enhancing), where one of the peptides functions only to enhance the activity of the other; and the type-S class (Synergy), when the activity is believed to require the complementary action of both peptides [229].

3.3.1. Lactococcin G

Lactococcin G, produced by *L. lactis* LMG2081, was the first two-component bacteriocin to be identified and characterised [18]. The inhibition spectrum of this bacteriocin includes a number of LAB and *Clostridium* sp. Moll et al. [170] determined that equivalent amounts of both the α and β peptides were required to yield biologically active lactococcin G. This classifies lactococcin G as a type-S two-component bacteriocin [229], i.e. when present separately, neither peptide is active. It was observed that lactococcin G inhibited the uptake of amino acids, dissipated the $\Delta\psi$ component of the PMF, and reduced the intracellular level of ATP. In addition, the pH gradient was unaffected by lactococcin G action, implying that membranes become permeable to ions other than protons. Moll et al. [170] suggested that this was due to the formation of highly specific K^+ pores in sensitive cell membranes by the α and β peptides, as immediate efflux of preaccumulated rubidium was observed on addition of lactococcin G. Ultimate cell death is a result of a futile cycle of ATP-driven K^+ uptake and thus, increased ATP hydrolysis [170]. More recently, it has been reported that lactococcin G also permeablises cells to Na^+ ions [230] which results in an osmotic imbalance in treated cells and prevents Na^+-coupled transport. It is likely that lactococcin G requires a cell wall component for activity, as attempts to demonstrate an effect on membrane vesicles and liposomes have been unsuccessful [170,230]. However, it cannot be excluded that lactococcin G acts on a transport system which functions in intact cells but not in cell membrane vesicles and which is absent from liposomes.

3.3.2. Lacticin F

Lacticin F activity occurs through the action of two peptides LafA and LafX [7,231]. This two-component bacteriocin, produced by *Lactobacillus johnsonii* VPI11088, has a narrow inhibitory spectrum, exhibiting bactericidal activity against a number of *Lactobacillus* species and *E. faecalis*. Such a narrow inhibition spectrum has led to speculation that the action of lacticin F is receptor-mediated [168]. In contrast to lactococcin G, one of the peptides, LafA, has some biological activity against *Lactobacillus helveticus*. Thus, lacticin F can be described as a type-E two-component bacteriocin [229]. Lacticin F induces membrane permeability, leading to K^+ and phosphate efflux and PMF dissipation in *E. faecalis* cells [168], demonstrating that the cytoplasmic membrane is the primary target for the bacteriocin. Although membrane pores do not allow an efflux of ATP, intracellular ATP levels are slowly reduced in a futile effort to regenerate the PMF. Similar results were also reported using *Lactobacillus delbrueckii* as the target organism [168]. As with other Class II bacteriocins, lacticin F action appears to be PMF-independent [175]. Furthermore, the bactericidal activity is pH-dependent and optimal under acidic conditions.

3.3.3. Acidocin J1132

This narrow-spectrum, two-component bacteriocin, produced by *Lactobacillus acidophilus* JCM 1132, also acts by forming pores in the cytoplasmic membrane [232]. Total dissipation of the PMF was observed from cells of *L. acidophilus* JCM 2010 treated with this bacteriocin. In addition, acidocin J1132 induced the efflux of glutamate, suggesting that pores large enough to allow amino acid release are formed. Like lacticin F, acidocin J1132 acts in a voltage-independent manner, as efflux of glutamate could also be observed in cells which had been pre-treated with valinomycin and nigericin [232].

3.3.4. Thermophilin 13

In contrast to other two-component bacteriocins, thermophilin 13, produced by *Streptococcus thermophilus*, has a broad host-range activity [229]. In addition, this bacteriocin was reported to form pores in cytochrome *c* oxidase-containing liposomes, an activity only previously observed for lantibiotics. This indicates that thermophilin 13 does not require a receptor, either proteinaceous or lipid, for activity. However, unlike the lantibiotics, thermophilin 13 acts in a voltage-independent manner. Marciset et al. [229] classified thermophilin 13 as a type-E bacteriocin, as one of the peptides in isolation, ThmA, possesses bactericidal activity against *S. thermophilus*, *C. botulinum*, *L. monocytogenes*, and *B. cereus*. However, the activity of ThmA is enhanced 40-fold when equivalent amounts of ThmB are present.

3.3.5. Lacticin 3147

The mode of action of the two-component lantibiotic, lacticin 3147, has also been determined. McAuliffe et al. [177] demonstrated that this lantibiotic exhibits bactericidal activity against a broad range of Gram-positive species, which is enhanced when target cells are energised. The pores formed by lacticin 3147 were shown to be selective for ions and not larger compounds such as ATP. The resultant loss of ions results in immediate dissipation of the $\Delta\psi$ and hydrolysis of internal ATP, leading to the eventual collapse of the ΔpH and, ultimately to cell death.

4. Future prospects

Fuelled mainly by the success of nisin, the enormous research efforts of recent years have led to a wealth of information on both modified and unmodified bacteriocins. However, despite this, understanding the molecular mechanisms of these peptides is far from complete. This is especially true for the lantibiotics, where many questions regarding the function of dehydrated amino acids, the enzymic reactions responsible for their formation, and mechanism of action remain unanswered. The understanding of such molecular mechanisms will be particularly important in the area of biotechnology. Already, the potential of genetic engineering for rational drug design has been demonstrated for subtilin [75], resulting in enhanced stability and activity. With the continuing development of expression systems, the production of mutant peptides will accelerate our knowledge of the biological activities of lantibiotics. Moreover, it may one day be possible to employ the unique modification systems found in lantibiotic producers in vitro to design novel, modified peptides displaying unique properties.

Although the commercial exploitation of bacteriocins to date is mainly restricted to the food applications of nisin, potential novel applications for lantibiotics and unmodified bacteriocins continue to be developed [233]. In particular, problems such as low production levels, and instability of bacteriocins in certain foods/environments need to be addressed. In addition, the cytolytic abilities of these peptides must be assessed, especially in the wake of the identification and characterisation of the haemolysin/bacteriocin, cytolysin, produced by *E. faecalis*. Possibly one of the main obstacles to the use of other bacteriocins in food is a regulatory one. To this end, use of bacteriocin-producing cultures in food might be of considerable advantage over using purified bacteriocin preparations, which would be considered food additives. In these respects, bacteriocins, both modified and unmodified, deserve further, in-depth scientific attention.

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