Lantibiotics

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Lantibiotics: biosynthesis, mode of action and applications

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1 Introduction

1.1 Discovery and structure of lantibiotics

Just before the discovery of penicillin by Fleming, reports appeared in the literature that described potent antimicrobial substances produced by lactic acid bacteria.[1,2] In those days the nature of the inhibitory compound was not yet elucidated, but in retrospect it seems likely that the inhibitory activity was caused by the production of the special class of peptides that forms the subject of this review. It was found that many Gram-positive bacteria secrete compounds which are specifically active against a wide range of other Gram-positive bacteria.[3–5] This characteristic made these compounds attractive candidates for application in either food preservation, *e.g.* by preventing spoilage or by inhibiting pathogens, or for pharmaceutical use, *e.g.* to prevent or fight infections in humans or animals. These prospects greatly stimulated more detailed research to elucidate the molecular structures of the antimicrobial compounds.

Pioneering work by Gross and Morell in the late sixties and early seventies showed for the first time the unique structural features of the class of bacterial antimicrobial peptides that are called lantibiotics today.[6,7] These small peptides (< 4 kDa) are characterized by their high content of amino acid residues that are uncommon in nature, *i.e.* the thioether bridged amino acid residues lanthionine and 3-methyllanthionine, and the unsaturated amino acid residues dehydroalanine and dehydrobutyrine (Fig. 1). Due to the presence of these modified residues, the elucidation of the primary structure of these peptides by conventional Edman degradation techniques was

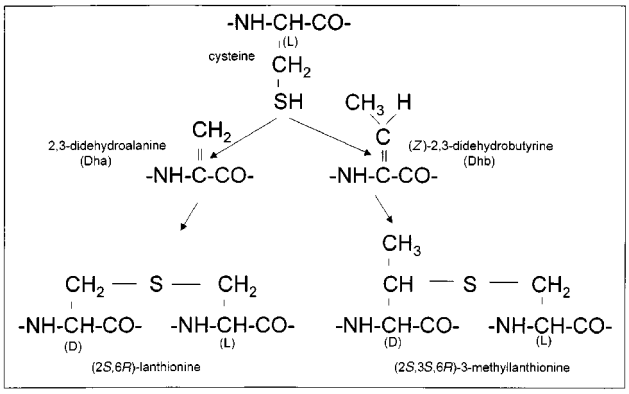

Fig. 1 The structure of some unusual amino acid residues found in lantibiotics. The thiol of Cys-residues attacks the double bond of dehydroalanine or dehydrobutyrine residues.

delayed. Gross and Morrell succeeded in the elucidation of the first primary structures of lantibiotics, *i.e.* that of nisin,[6] produced by *Lactococcus lactis*, and that of subtilin,[7] produced by *Bacillus subtilis*. These 34- and 32-amino acid residue peptides, respectively, showed the presence of three dehydrated residues, *i.e.* dehydroalanine and dehydrobutyrine, and five thioether bridges, forming rings A, B, C, D and E. Fig. 2 shows the primary structures of nisin and subtilin and those of several other well known lantibiotics.[8] All peptides share the common feature of containing lanthionine residues. In addition, a number of other unusual residues is found, which are mostly modified forms of serine, threonine or cysteine residues. The C-terminal Cys-residue of epidermin is oxidized and decarboxylated, resulting in 2-aminovinyl-D-cysteine (AviCys).[8] Lactocin S contains D-Ala residues, which arise from modification of Dha residues. Dha or Dhb residues that are present at position 1 are unstable and undergo additional modifications, as has been found in the lantibiotics epilancin K7, Pep5, epicidin 280 and lactocin S.[8] It should be noted that for some lantibiotics the experimental proof for their structure is not complete and for those lantibiotics the proposed primary structure is indicated.

It was already speculated in the seventies that these peptides could be derived from ribosomally synthesized precursor peptides, which had been posttranslationally modified. In the eighties total chemical synthesis of the lantibiotics subtilin and epidermin was achieved by highly specialized organic chemists from Japan, who were also the first to produce fragments and site-specifically altered species of lantibiotics.[9] Many years later these compounds turned out to be extremely valuable for structure–function relationship studies and even for gene regulation studies.[10] The first 3D-structures of specific lantibiotics in aqueous solution or in DMSO were determined in the late eighties.[11–15] The structure of nisin A was studied in most detail (Fig. 3). The NMR structures showed that there was not a well-defined overall fold of this peptide, but that it is quite flexible in solution. The molecule clearly is amphipathic and the structures of the rings B, D and E are well defined as essentially being beta-turns fixed by a thioether bond. Between rings C and D a flexible hinge region was identified, which could be important for the membrane insertion properties of the molecule.[15]

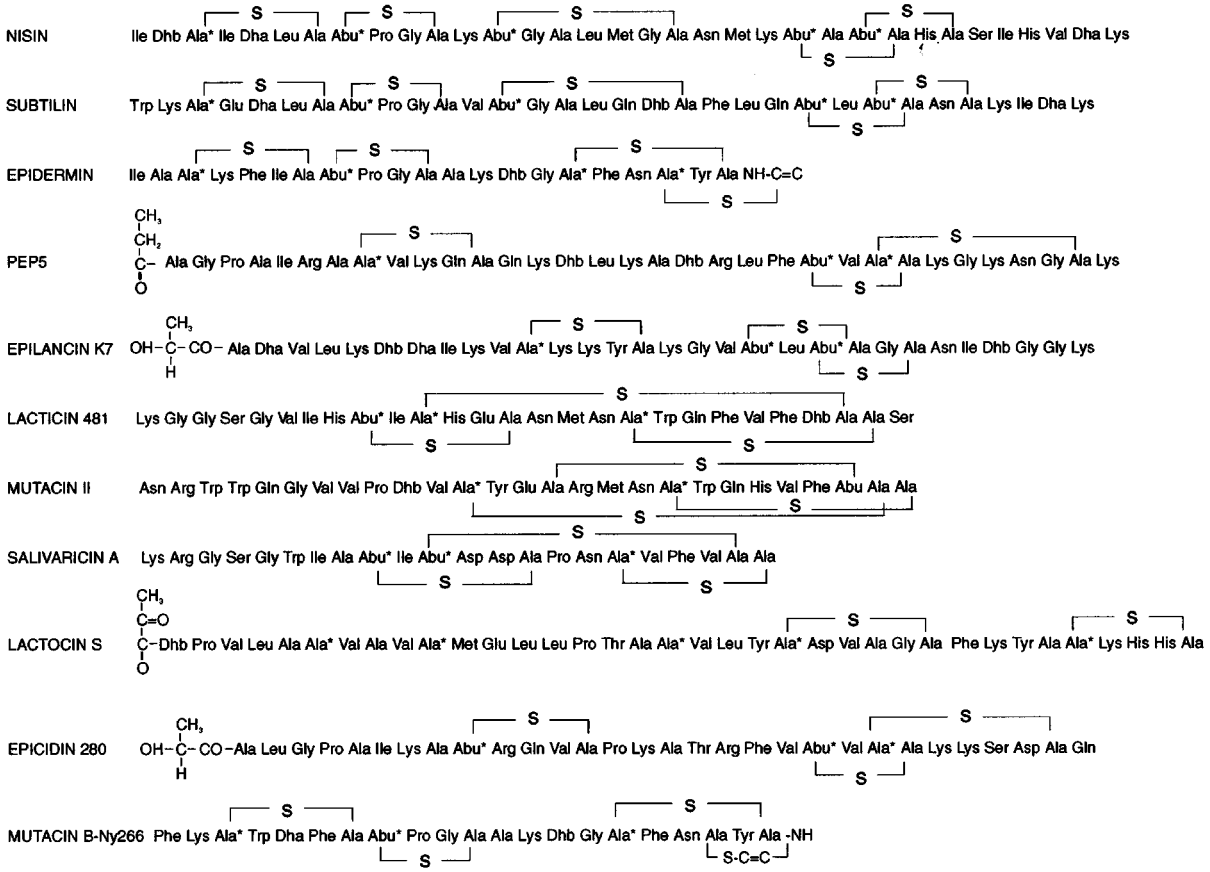

Fig. 2 The primary structure of some type-A lantibiotics. Ala-S-Ala: lanthionine, Abu-S-Ala: β-methyllanthionine, Dha: dehydroalanine, Dhb: dehydrobutyrine. Residues marked with an asterisk are in the D-configuration. For those lantibiotics of which complete structural information is not available, the proposed bridging pattern is indicated.

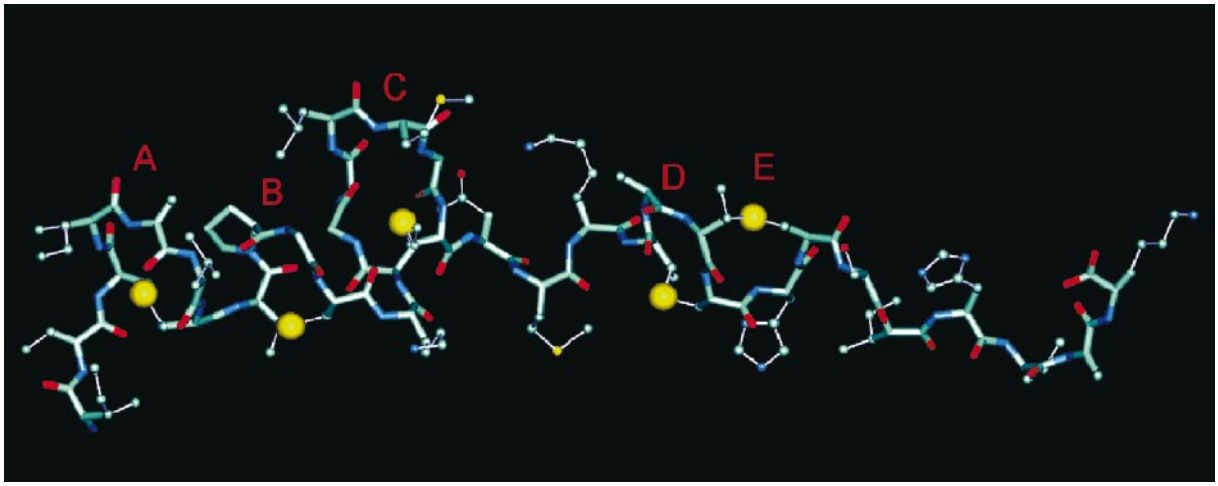

Fig. 3 One of the 3-dimensional structures of nisin A as determined by van de Ven *et al.*[15] The peptide backbone is indicated in liquorice. The side-chains (non-hydrogen atoms) are represented as ball–stick models. The sulfurs of the lanthionine residues are indicated as spheres.

Based on their chemical structures and their antimicrobial activities the lantibiotics are subdivided into type A and type B lantibiotics.[16] Type-A lantibiotics, which are the subject of this overview, are elongated peptides, which have a net positive charge. They exert their activity by permeabilisation of the cytoplasmic membrane of target cells. Type-B lantibiotics are smaller, globular and have a low net positive charge. Their antimicrobial activity is related to the inhibition of specific enzymes. Representatives of this group are mersacidin, duramycin, actagardin and cinnamycin. During the last years the number of newly discovered lantibiotics has increased rapidly. Table 1 lists several recently discovered lantibiotics that were shown to contain lanthionine residues, but of which the structure is still unknown. Cytolysin, produced by *Enterococcus faecalis*[17] and the recently identified bacteriocins lacticin 3147[18] and staphylococcin C55[19] are unique in being two-component lantibiotics. For antimicrobial activity of these lantibiotics, both components are required. In the gene cluster of these lantibiotics two structural genes are present, both encoding a precursor molecule. Both components are post-translationally modified and secreted independently. Salivaricin G32 is considered to be a natural variant of streptococcin A-FF22, in which the residue Lys-2 is absent.[20] The recently discovered lantibiotic sublancin 168, produced by *Bacillus subtilis* is the only lantibiotic species known so far that contains two disulfide bridges next to a thioether bridge.[22]

A great advancement in the study of lantibiotics was the publication of the first gene encoding the precursor of a

Table 1 Type-A lantibiotics that have been isolated and were shown to contain (β-methyl)lanthionine residues. The bridging pattern of the lanthionine residues is not known yet

Lantibiotic	Mass/Da	Producer organism	Ref.
Cytolysin LL	4164 2631	*Enterococcus faecalis*	17
Lacticin 3147	2852 3323	*Lactococcus lactis*	18
Streptococcin C55	3339 2993	*Staphylococcus aureus*	19
Streptococcin A-FF22	2794	*Streptococcus pyogenes*	37
Carnocin	4635	*Carnobacterium piscicola*	33
Variacin	2659	*Micrococcus varians*	35
Salivaricin G-32	2667	*Streptococcus salivarius*	20
Sublancin 168	3877	*Bacillus subtilis*	22

lantibiotic, *i.e.* epidermin produced by *Stapylococcus epidermidis*.[23] The encoded precursor sequence shared high similarity in its C-terminal half with the residues in the known primary structure of epidermin, except for the presence of several Ser, Thr and Cys residues, that were either Dha, Dhb, (3-methyl)lanthionine or *S*-aminovinyl-D-cysteine residues in the active epidermin. This finding unambiguously demonstrated that a mature lantibiotic is ribosomally synthesized as an unmodified precursor protein, which is probably enzymatically converted into the modified precursor. At a certain stage, in several cases shortly after translocation across the bacterial membrane, the leader peptide is removed, yielding the active antimicrobial peptide.

After the finding that lantibiotics are ribosomally synthesized peptides a wealth of novel information was generated in the late eighties and early nineties by sequencing of several gene clusters involved in the biosynthesis of various lantibiotics, among which the gene clusters of nisin A[24] and Z (these lantibiotics differ only in residue 27, being His and Asn, respectively),[25] subtilin,[26] Pep5,[27] gallidermin,[28] streptococcin A-FF22,[29] lacticin 481[30] and epilancin K7.[31] Comparison of the lantibiotic gene clusters generated the overall picture that a lantibiotic is first produced as an unmodified precursor peptide that is intracellularly converted into a modified precursor peptide by the action of one or two dedicated enzymes, commonly denoted as LanB, LanC (two enzymes) or LanM (one enzyme). Subsequently, the modified precursor protein is secreted and processed by dedicated LanT and LanP proteins, or one protein with a dual function. The producing cells protect themselves against the antimicrobial activity through specific immunity proteins (LanI, LanF, LanE, LanG). More recently it was established in some cases that the expression of the genes involved in the biosynthesis is autoregulated by the mature lantibiotic, through the two regulatory proteins LanR and LanK.[10]

The broad application possibilities for lantibiotics have greatly stimulated further research into the details of the complex biosynthesis, mode of regulation, and mechanism of self-protection of the cell (immunity). Moreover, it became clear that it is of crucial importance to understand the mechanism of action of lantibiotics in molecular detail. In this review the latest developments in all these areas will be discussed, with special emphasis on recent advances brought about by the use of protein engineering techniques and membrane biochemistry to elucidate the structure–function relationship of lantibiotics (exemplified by nisin). Last but not least, the broadening area of applications and new possibilities for use that have arisen from fundamental research on lantibiotics will be highlighted in the last section. To keep this review concise we have chosen to highlight mainly research on a limited number of type-A lantibiotics, which are relatively well characterized, *i.e.* nisin, subtilin, epidermin and Pep5. From these we will highlight in most cases nisin as the main example, because this lantibiotic has been characterized in most detail. In particular cases, we have also included additional information on other lantibiotics when these display one or more unique features not encountered in the ones mentioned above. Without any doubt the study of various other lantibiotics, *e.g.* cytolysin LL/LS,[17] mutacin,[32] carnocin UI49,[33] epilancin K7,[29] salivaricin A,[34] variacin,[35] lacticin 481,[36] streptococcin A-FF22,[37] sublancin,[22] epicidin 280,[38] lactocin S[39] and lacticin 3147[18] also generated many new insights in lantibiotic function, biosynthesis and application. However, in view of the limited space available these species will not be discussed here in any detail. For this, the reader is referred to some excellent and elaborate reviews on lantibiotics that have been published previously[16,40,41] and the more recent original research papers that are mentioned above.

2 Biosynthesis of lantibiotics

2.1 Organization of the gene clusters

The genes involved in biosynthesis of the model lantibiotic nisin are located on a 70 kb conjugative transposon,[42] which also contains the genetic information for sucrose metabolism. The first gene of the nisin gene cluster, *nisA*, encodes the 57 amino acid nisin precursor, consisting of a N-terminal leader sequence followed by the propeptide, from which nisin A is matured. The structural gene is followed by ten other genes *i.e.* *nisB*, *nisT*, *nisC*, *nisI*, *nisP*, *nisR*, *nisK*, *nisF*, *nisE*, *nisG*,[43] encoding regulatory proteins, proteases, transport proteins and immunity proteins (Fig. 4). The proteins that are encoded by

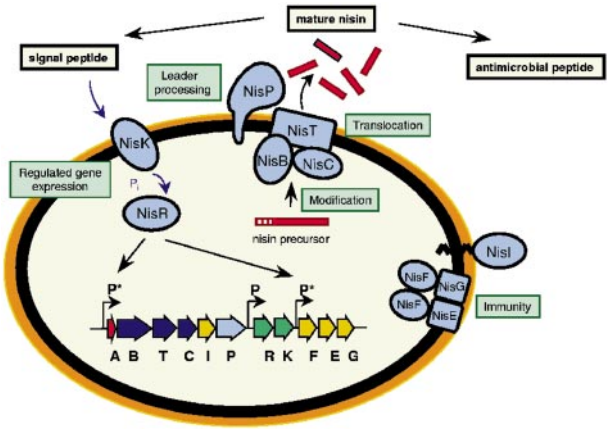

Fig. 4 Model for the biosynthesis of nisin. The nisin precursor is modified by the putative enzymes NisB and NisC and translocated across the membrane by the exporter NisT. The precursor is extracellularly processed by NisP, resulting in the release of mature nisin. NisK senses the presence of nisin in the medium and autophosphorylates. The phosphate-group is transferred to NisR, which activates transcription of the genes *nisABTCIP* and *nisFEG*. NisI, F, E, and G protect the cell from the bacteriocidal activity of nisin. P: promoter region, P*: nisin-regulated promoters.

these genes have been found to be homologous to gene products of the gene clusters of other lantibiotics, such as those of subtilin, epidermin and Pep5. Not all of the mentioned genes have been detected in all gene clusters, so far. Besides, there is no uniform order of the genes in the individual gene clusters. For an overview of the organization of the various gene clusters the reader is referred to Siezen *et al.*[44]

2.2 Modification and transport

The gene clusters for nisin,[45] subtilin,[46] epidermin,[47] Pep5[48] and epicidin 280[38] all contain the genes *lanB* and *lanC*. These genes do not display significant homology to known genes in sequence databases and their function is not completely

understood yet. It has been shown that both genes are essential for the production of lantibiotics, since their disruption results in cessation of lantibiotic biosynthesis.[45,46,49] Most likely, LanB and LanC are involved in post-translational modification of the lantibiotics, involving the formation of dehydrated residues and interaction of these residues with the sulfhydryl group of a nearby Cys-residue to form lanthionine residues. The LanB proteins, generally around 1000 amino acid residues in size, are the putative enzymes that catalyze dehydration of the Ser and Thr residues in the propeptide domain of the prelantibiotic. LanC proteins are all about 400 amino acid residues long. Deletion of the *pepC* gene in *Staphylococcus epidermidis* resulted in production of incorrectly modified Pep5 fragments, that contained only one of three expected lanthionine residues.[47] This suggested that PepC is the thioether-forming protein. Investigations on the proteins SpaB[48] and EpiB[50] revealed that these proteins are membrane-associated, suggesting that lantibiotic biosynthesis occurs at the cytoplasmic membrane. Moreover, by use of the yeast two-hybrid system and co-immunoprecipitation techniques, it was shown that nisin[51] and subtilin[52] precursor peptides are processed by a multimeric protein complex located at the cytoplasmic membrane, consisting of LanB, LanC and LanT proteins.

In the *cyl*, *las*, *lct* and *mut* gene clusters, for biosynthesis of cytolysin, lacticin 481, lacticin S and mutacin II, respectively, LanB is missing. Instead a LanM protein is found,[17,53,54] from which the C-terminal part is homologous to LanC proteins.[44] Possibly, LanM is able to catalyze both the reactions assumed to be catalysed by LanB and LanC. The epidermin-gene cluster is unique in that it contains the gene *epiD*, encoding a 181 amino acid protein that is also involved in post-translational modification of epidermin. To identify its function, EpiD was purified from *Staphylococcus epidermidis* and incubated *in vitro* with epidermin precursor peptide.[55] The results showed that EpiD is a flavoenzyme that catalyzes the oxidative decarboxylation of the C-terminal Cys-residues of epidermin, a modification that is only found in epidermin and its natural variant gallidermin. Another experimental approach in which a variant *epiA* gene encoding His-tag-labelled epidermin was co-expressed with *epiD* established this role of EpiD.[56] In the gene cluster of epicidin 280, which is produced by *Staphylococcus epidermidis*, an additional gene was found, designated *epiO*.[38] Since this gene has similarity to a family of oxidoreductases, it most likely codes for an enzyme that catalyzes the modification of the N-terminal residue of epicidin 280.

All known lantibiotic gene clusters contain a *lanT* gene, which encodes a protein that is involved in transport of the lantibiotic precursor across the cellular membrane to outside the cell. It was first shown for subtilin that disruption of the gene *spaT* resulted in intracellular accumulation of subtilin.[46] A number of transport protein-encoding genes have now been identified in several of the lantibiotic gene clusters, including those for nisin,[45,57] lacticin 481,[53] lactocin S[38] and recently for lacticin 3147.[18] It should be noted that the transport gene in the *cyl* gene cluster is designated *cylB*.[17] The LanT proteins share homology with a large family of transport proteins, characterized by the presence of a cytoplasmic ATP-binding domain and a membrane spanning domain. For all lantibiotic transporters, with the exception of EpiT, both domains are present in one protein, of about 600 amino acid residues in size. The gene encoding EpiT is incomplete and it seems not to be required for transport of epidermin in *Staphylococcus epidermidis*.[37] Possibly, transporter proteins of the host replace its function. Introduction of *gdmT*, the transporter gene of the related lantibiotic gallidermin into an epidermin producing strain strongly increased production yields. The *epi* and *gdm* gene clusters also contain a *lanH* gene, encoding a protein of about 330 amino acids containing several putative transmembrane sections. GdmH is assumed to be involved in secretion of gallidermin, in cooperation with GdmT.[58]

2.3 Processing of precursor peptides

The last step in the biosynthesis of lantibiotics is the removal of the leader peptide from the lantibiotic precursor. In the gene clusters for the lantibiotics nisin,[59] epidermin,[47] Pep5,[48] epilancin K7,[60] lacticin S,[39] cytolysin[17] and epicidin 280[38] a *lanP* gene was found encoding a subtilisin-like protease. The location at which processing of the leader peptide occurs, varies with the lantibiotic. NisP, responsible for cleavage of the nisin precursor is anchored to the cellular membrane at the outside of the host cell.[59] EpiP and CylP are also transported to the outside of the cell, but appear to lack a membrane anchor.[44] In contrast, PepP, ElkP and LasP were reported to act in the cytoplasm of cells, possibly in association with LanB, C and T.[44] Cleavage of lacticin 481 occurs by the protein LctT, which displays a dual function since it is responsible for proteolytic cleavage as well as secretion of the lantibiotic.[61] In contrast to the gene clusters discussed above, the *spa* gene cluster does not contain a gene encoding a peptidase, and it is assumed that processing of subtilin occurs by a general serine protease of the host *Bacillus subtilis*.[44]

The function of the leader peptide is unclear. The fully modified lantibiotic precursor is almost inactive, and it has been suggested that the leader sequence is of importance to keep the lantibiotic in an inactive state, to protect the producer cell from its activity.[16] However, this does not hold for those lantibiotics that are cleaved intracellularly. NMR studies on the nisin precursor have demonstrated that the residues Ile1-Ala19 of the nisin precursor interact differently with membrane-mimicking micelles than the corresponding part of mature nisin.[62] These results indicate that the low *in vivo* activity of the precursor is caused by a less efficient insertion of the peptide into a membrane.[62] The leader peptide might alternatively, or additionally, play a role in modification and excretion of the peptide, for instance by targeting the unmodified precursor to the modification and secretion machinery. This assumption was strengthened by the observation that specific mutations in the leader sequences of nisin[63] and Pep5[64] strongly affected the production level of the lantibiotics. Obviously, a defined leader sequence is of importance for optimal biosynthesis of the lantibiotic.

2.4 Immunity of producer organisms

One of the most intriguing questions in lantibiotic production concerns the mechanism of self-protection. Obviously, without efficient ways to protect themselves from the pore-forming activity of the peptides, the producing cells would be suicidal. In all gene clusters studied so far small (50–70 AA) or medium-sized (160–250 AA) hydrophobic proteins (LanI) are encoded that are attached to the outside of the membrane and are involved in the immunity process. So far, LanI proteins have been found in nisin A[45,65] and Z,[66] subtilin,[67] Pep5[68] and epicidin 280[38] producing cells. Possibly, these molecules play a role in the recognition of the active lantibiotic present at the outside of the cell, either directly from solution or upon adsorption to the membrane. Overexpression of LanI proteins in cells that do not possess the lantibiotic biosynthesis machinery only yields very low protection levels (1–4%) against the corresponding lantibiotic.[45,65,69] An in-frame disruption of *nisI* in *Lactococcus lactis* yielded a strain that could still produce nisin, albeit to levels five times lower than wild-type,[70] which suggests that the immunity level could be the first limiting factor in reaching high production levels. These results already indicate that additional factors are involved in the acquirement of full self-protection.

In several lantibiotic gene clusters three other proteins are encoded, named LanF, LanE and LanG. The LanFEG proteins belong to the group of ABC transporters, where LanF contains the intracellular ATP-binding domain and LanEG the mem-

brane-spanning subunits. The genes *lanFEG* have been found in the gene clusters for nisin,[71] subtilin,[67] lacticin 481,[72] epidermin[73] and lacticin 3147.[18] It is tempting to speculate that these proteins have a function in the removal of the corresponding lantibiotic at a certain stage of its membrane interaction. Expression of the LanFEG proteins, without LanI, yields a significant level of immunity although it remains below the wild-type level.[74] The synergistic function of all immunity proteins could thus reside in the first recognition and binding or 'immobilization' of the lantibiotic in the membrane by NisI, followed by active removal, in which the LanFEG proteins are involved. It cannot be excluded, however, that also other proteins encoded in the lantibiotic gene clusters display a synergistic effect in the immunity mechanism, for instance by being involved in a multi-protein complex that spans the membrane. Candidates to be involved in such a complex are LanBTC proteins, which have already been shown to form a membrane associated complex.[51,52]

Interestingly, in some cases, *e.g.* nisin and subtilin production, the expression of immunity genes is also regulated by the concentration of the lantibiotic in the medium (see also section 2.5), which means that by sensing low (subinhibitory) amounts of the antimicrobial peptide in the medium, cells can rapidly increase their immunity level, concomitant with or even faster than the biosynthesis rate.[75] The challenge still exists to unravel the complete mechanism of immunity at the molecular level, the understanding of which could in the end result in engineering increased immunity and possibly increased production levels of lantibiotics.

2.5 Regulation of biosynthesis

In 1995 it was reported that apart from displaying a strong antimicrobial activity, the lantibiotic nisin also plays an important role in the regulation of its own biosynthesis.[10] In fact nisin can be regarded as a peptide pheromone which is sensed by the histidine kinase NisK, which resides at the outer side of the membrane, probably by direct protein–peptide interaction. By analogy with other known two-component regulatory systems[76–78] NisK will autophosphorylate at a specific histidine residue when it senses a certain nisin concentration in the medium and subsequently transfer the phosphate moiety to the response regulator NisR. The response regulator is assumed to get phosphorylated at a specific Asp residue, which is supposed to trigger its binding to two regulated promoters in the nisin gene cluster, *i.e.* the *nisA* and the *nisF* promoter, thereby activating transcription of the structural gene *nisA* and the downstream genes *nisBTCIP* by limited readthrough from *nisA*, and the genes *nisFEG* located at the end of the gene cluster.[10,75] The regulatory genes *nisRK* themselves are transcribed from their own promoter which is assumed to be not dependent on nisin induction.[75] Although not all molecular details have been unraveled yet, it is clear that this autoregulatory process resembles the quorum-sensing phenomenon found in several Gram-negative bacteria, for which the bioluminescence phenotype of *Photobacterium fischeri* is the paradigm.[78,79]

The transcription from the *nisA* and *nisF* promoter in the nisin gene cluster is directly related to the concentration of nisin in the medium. This property is extremely useful for the development of controlled gene expression systems, since a linear dose–response for the expression of target genes is highly desirable in industrially relevant production organisms. Especially when toxic gene products should be produced, the nisin-controlled expression (NICE) system is ideally suited, since the *nisA* promoter is tightly shut off in the uninduced state.[80–82] Moreover, very high expression levels of up to 60% of total intracellular protein can be reached in *Lactococcus lactis*. The NICE system has also been succesfully implemented in some heterologous hosts like the lactic acid bacteria *Leuconostoc lactis* and *Lactobacillus helveticus*[83] and the Gram-positive bacteria *Streptococcus pyogenes*, *Bacillus subtilis* and *Enterococcus faecalis*.[84]

Since in many other lantibiotic and non-lantibiotic gene clusters the counterparts of the two-component regulatory proteins NisR and NisK are found, it is reasonable to assume that also in these cases an autoregulatory process takes place. In fact for the production of several non-lantibiotic antimicrobial peptides, *e.g.* carnocin, plantaricin and sakacin the peptide pheromone concept has been shown to be valid.[78,85–87] In the case of subtilin production the autoregulatory process has been shown to be very similar to the nisin case, because the *spaB*, *spaI* and *SpaS* promoters could be activated by subtilin in the medium.[88] Knock-outs of *spaRK* and *nisRK* have been shown to be detrimental for lantibiotic production[10,88,89] An exception to the general rule is found in the case of epidermin, since in that biosynthetic gene cluster only the response regulator encoding EpiQ was found to be present.[90] Possibly, a high expression level of only the response regulator results in effective expression of the target genes, probably because this protein will be phosphorylated in an aspecific way. Also by overexpression of *nisR* in a strain lacking *nisK*, efficient transcription from the *nisA* promoter was observed.[59]

Both inducer and sensor engineering have been used to study the molecular interaction between these molecules. First, it was shown that variants and fragments of nisin were able to act as inducer with variable efficiencies and that their induction capacity was unrelated to their antimicrobial activity, demonstrating that the mechanisms for induction and pore formation are different[10,91,92] In a recent study a plasmid containing the reporter gene *gusA* under control of the *nisA* promoter was introduced in a strain containing the *nisR* gene integrated on the chromosome. Introduction of a *nisK* expressing plasmid in this strain resulted in a fully functional nisin induction system. Surprisingly, also introduction of *spaK*, combined with subtilin as inducer, led to a functional signal transduction, showing that cross-talk between SpaK and NisR occurs, albeit with decreased efficiency. In an extension of this study hybrids of NisK and SpaK were constructed. It was demonstrated that the subdomain needed for inducer interaction is located in the N-terminal domain of the sensor protein and includes at least both transmembrane domains and the external loop.[88] A better understanding of these interactions will open the way to the rational design of more effective sensors and perhaps even to sensors with a loosened specificity for the inducer molecule. Undoubtedly, in the coming years the autoregulatory processes found to occur in many lantibiotic biosynthesis processes will yield valuable spin-off for a variety of industrial applications, *e.g.* increased production levels, development of novel biosensors and improved controlled gene expression systems.

3 Protein engineering of lantibiotics

3.1 Expression systems for modified lantibiotic structural genes

Knowledge of the organisation of the gene clusters and the biosynthesis pathways of lantibiotics enabled the development of various expression systems for lantibiotic structural genes. This has opened up possibilities to produce engineered lantibiotic molecules with altered biological properties. Different strategies have been described for the production of mutant species. In the first report a plasmid-encoded mutant copy of the nisin structural gene is introduced in a nisin-producing strain, which results in simultaneous production of wild-type nisin and the mutant species.[93] In this way, the biosynthesis machinery was assured to be functional. However, this approach had the disadvantage that the mutant species could not always be separated from the wild-type easily. Therefore, a common approach was developed for nisin,[94] Pep5[95] epidermin and gallidermin[96] which involves the introduction of a disruption in

the structural gene of the lantibiotic gene cluster, located either on the chromosome of the host-strain, or in case of Pep5, on a plasmid. The deficient gene is complemented with a plasmid-encoded variant of the structural gene. The plasmid-encoded gene is easily manipulated by molecular techniques and can be expressed under control of a strong promotor, independent of the biosynthetic genes. Alternatively, gene replacement expression systems were developed for nisin A,[10,97] subtilin[98] gallidermin[99] and recently for mutacin II,[100] in which the chromosomally located structural gene was replaced by a mutant copy of this gene. The latter strategy has the advantage that the balance between the expression of the structural gene and the biosynthesis genes is maintained, because the variant gene is expressed at its natural locus.

The approaches in which only a mutant copy of the structural gene is expressed by the host strain ensure production of solely the engineered lantibiotic molecule by the bacteria, which facilitates purification of the mutant peptide. Problems may arise when the produced mutant lantibiotic has lost its capacity to act as signaling molecule, due to the introduced mutation.[10] For many mutant nisin species it has been reported that their signaling capacity was reduced, probably due to a disturbed interaction with the sensor histidine kinase NisK.[10] As a consequence, the transcription of the biosynthetic and immunity genes of the gene cluster is not fully activated, resulting in a low production level of the engineered lantibiotic. Addition of low amounts of wild-type nisin to the growth medium of the expression strain stimulated the production of these mutants.[10] For production of some mutant species it appeared to be essential to add external nisin, because production of these species could otherwise not be detected.[92]

3.2 Site-directed mutants of lantibiotics

In the past decade, a large variety of engineered lantibiotic molecules has been produced and characterised. The physical, biochemical and biological properties of the more than 40 lantibiotic mutants that were known up till 1994, as well as the expression systems that were used to obtain these variants, are discussed in detail in an overview by Kuipers *et al.*[101] Despite the well-developed expression systems for the different lantibiotics, it appears that the number of succesful mutations one can apply in these peptides is limited. Many designed mutants could not be produced, probably due to disturbed interaction of the peptides with biosynthetic enzymes or the secretion machinery. In particular, a strongly reduced production level was obtained for lantibiotics in which residues were modified that are involved in thioether formation.[101] It was concluded at that time that a correct formation of all thioether residues is of importance for the completion of biosynthesis and secretion of lantibiotics. Nevertheless, several lantibiotic mutants have been isolated in which thioether residues were substituted or deleted. Characterisation of these mutants provided insight into the importance of the unusual lanthionine residues of lantibiotics. Two mutants of Pep5 were purified in which cysteine residues were replaced by alanines, resulting in peptides that were devoid of either ring B or C. These mutant peptides were produced as a mixture of incorrectly modified peptides as well as degradation products.[102] Furthermore, a Pep5 mutant was generated that contained a novel thioether ring, due to interaction of an introduced Cys-residue with the naturally occurring Dhb-16. The production level as well as the biological activity of all these ring-mutants was strongly reduced, indicating that the presence of the lanthionine rings in lantibiotics is of importance for antimicrobial activity as well as recognition by the proteins involved in biosynthesis and secretion. Furthermore, it was shown that deletion of a lanthionine ring resulted in increased susceptibility of Pep5 for proteolytic degradation.[102] Recently, mutants of mutacin II were isolated in which each of the three Cys-residues was replaced by an Ala, each time resulting in deletion of one of the three thioether rings.[100] The production level of these mutacin II variants, as well as their capacity to exert antimicrobial activity against *Streptococcus sobrinus* was very low. Introduction of a Cys-residue on position 13 of nisin Z resulted in the formation of a nisin analog that contains a disulfide bond in ring C, instead of the usual thioether bond. NMR analysis of this mutant revealed that the structure of this mutant was similar to the wild-type peptide, with the exception of ring C. The peptide exhibited less than 1% of the wild-type activity, indicating that the thioether ring C plays a profound role in antimicrobial activity.[103]

Recently, cysteine-scanning mutagenesis was applied to the antimicrobial peptide nisin, for the purpose of the production of nisin analogs that are suitable for chemical modification with thiol-specific probes. For 8 Cys-mutants of nisin a lack of production was observed. Nevertheless, two Cys-mutants of nisin Z, *i.e.* S5C and M17C nisin Z, could be succesfully isolated, by maintaining reducing conditions during the production and purification procedure. In view of the possibilities to chemically modify Cys-residues, these results significantly expand the perspectives of protein engineering in the lantibiotic field.[103]

Of great interest in the engineering of lantibiotics is the possibility to design new peptides that have improved activity against specific undesirable microorganisms, and therefore could find commercial applications. Up till now, only a few mutants, such as T2S nisin Z, M17Q/G18T nisin Z and L6V gallidermin, displayed higher antimicrobial activity towards some target strains.[101] A difficulty of the rational design of species that have increased bacteriocidal activity towards specific strains is the fact that the mechanism of action of lantibiotics is not yet fully understood. For this reason, research to obtain better insight in the mode of action of lantibiotics has gained much interest. Several mutants of nisin were designed especially for the use as a research tool in mechanistic studies (Fig. 5). The introduction of specific markers, such as fluorescent labels, radioactive labels, tags, or charged residues in nisin, has made it possible to study the interaction of the peptide with the cytoplasmic membrane of target bacteria. How these modifications contributed to a better understanding of the mode of action of lantibiotics will be discussed in the next section.

4 Mode of action of lantibiotics

4.1 Bacteriocidal activity towards Gram-positive bacteria

Type A lantibiotics exert bacteriocidal activity towards a broad range of Gram-positive bacterial strains, including streptococci, bacilli, listeriae, clostridia and staphylococci. The antibacterial effect is strong: the addition of nM concentrations of a lantibiotic is sufficient to kill bacterial cells. In contrast, other well-known antibacterial compounds, such as melittin, magainin or cecropin, are active in μM concentrations. Fungal cells, yeast cells or human cells are very insensitive to lantibiotics, even when treated with mM concentrations of the peptides.[104] In general, Gram-negative bacteria are also insensitive to lantibiotics. It has been shown that their outer membrane functions as a barrier for the lantibiotics. When the outer membrane of the Gram-negative strains *Escherichia coli*[104] or *Salmonella* species[105] was weakened by treatment with EDTA or osmotic shock, the susceptibility of the cells towards nisin or Pep5 strongly increased.

The primary target for the activity of the lantibiotics appears to be the bacterial cytoplasmic membrane. The peptides interfere with the membrane function of sensitive cells by increasing the permeability of the bilayer for small molecules and disrupting the membrane potential, resulting in cell death. Within several minutes, the lantibiotics induce the release of

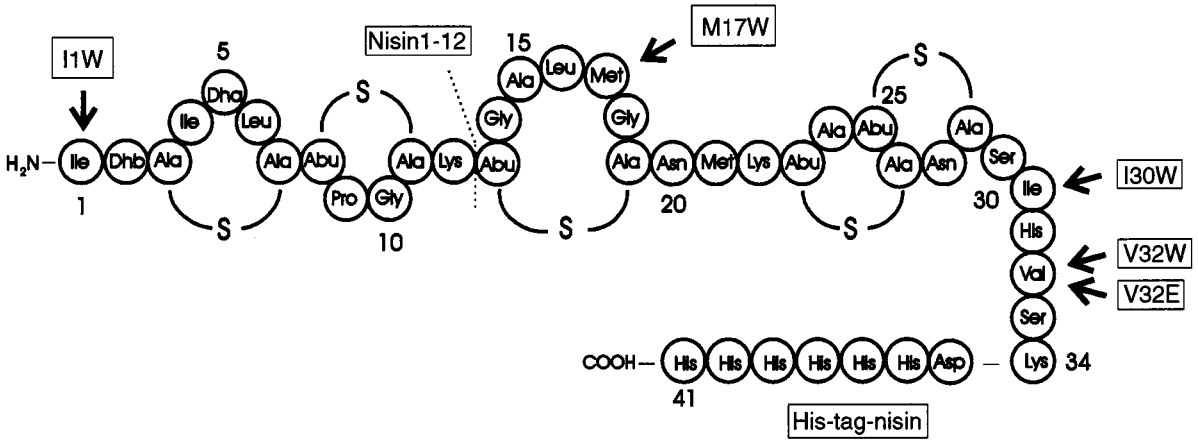

Fig. 5 Primary structure of nisin Z showing the position of some site-directed mutations that have been introduced. Sites of proteolytic cleavage by trypsin are indicated by dotted lines.

ions, small molecules and ATP from sensitive bacterial cells.[106] The efflux of high-molecular-weight compounds (> 500 Da) was not observed,[107] indicating that lantibiotics do not completely disrupt the barrier function of the membrane, as detergents do. Also, other mechanisms could be involved in their biological activity, since it has been reported that lantibiotics also inhibit outgrowth of bacterial spores,[108] activate autolytic enzymes[109] and might inhibit cell wall biosynthesis.[110] However, these mechanisms seem to be secondary effects, since these processes are relatively slow and require relatively high concentrations of lantibiotics.

Since the lantibiotics act on the bacterial cytoplasmic membrane, they first have to pass the bacterial cell wall. Many non-lantibiotic bacteriocins, such as lactococcin[111] appear to interact with membrane-associated receptor proteins, prior to their membrane perturbing activity. In contrast, a specific proteinaceous receptor for lantibiotic species on the outer surface of bacteria has not been found so far. Also, the fact that lantibiotics are able to permeabilize lipid vesicles implies that a specific receptor is not essential for activity. In general, it is assumed that lantibiotics reach the membrane by diffusion through the peptidoglycan layer of Gram-positive bacteria. Recently, it was reported that lipid-bound peptidoglycan precursors play a role in efficient pore-formation by lantibiotics.[112] The presence of this membrane-bound precursor in liposomes substantially increased the susceptibility of the liposomes to nisin and epidermin, but not to Pep5 or epilancin K7. It was postulated that lipid II, which is present at the outer surface of Gram-positive bacteria, serves as a docking molecule for nisin and epidermin, facilitating specific binding to the bacterial membrane. Additionally, the interaction with lipid II could promote the pore-forming process, by facilitating the insertion of the peptides into a conducting state. The concept that nisin and epidermin specifically interact with the membrane-bound lipid II, prior to membrane-disruption is very novel. The molecular interaction and its role in the mechanism of pore formation has to be investigated in more detail. Furthermore, it remains to be investigated whether the use of an integral membrane compound as a docking molecule for specific binding to target bacteria is a general phenomenon for type-A lantibiotics.

To understand the interaction of lantibiotics with the cytoplasmic membrane at the molecular level, the interaction of the peptides with various membrane-mimicking systems, such as lipid monolayers, liposomes, black lipid membranes and SDS-micelles was studied. From these studies a general picture of the membrane interaction of lantibiotics has emerged, in which the following steps can be distinguished; 1) binding of the peptide to the membrane, 2) insertion of the peptide into the membrane, 3) membrane permeabilisation. The mechanism of these steps will be discussed for the prototype lantibiotic nisin.

4.2 Binding to membranes

The first step in the mechanism of action of nisin is considered to be the binding of the peptide to the cytoplasmic membrane of target bacteria. Electrostatic interactions between the cationic lantibiotic and negatively charged phospholipids appear to be involved in this initial peptide–membrane interaction.[113–115] Binding studies of nisin using fluorescently labeled phospholipids established that nisin tightly interacts with membranes containing the negatively charged lipid phosphatidylglycerol, and has little affinity for zwitterionic lipids.[107] The binding affinity was maximal on lipid vesicles containing > 60% negatively charged phospholipids.[114] Along with these observations, it should be noted that the cytoplasmic membrane of Gram-positive bacteria generally contains a high content of negatively charged lipids, such as phosphatidylglycerol and cardiolipin, while Gram-negative bacteria, yeast cells and human cells generally contain primarily zwitterionic lipids, and less negatively charged lipids in their membrane. This has led to the suggestion that negatively charged phospholipids, most likely phosphatidylglycerol, might form binding sites for nisin.[114] On the other hand, the susceptibility of bacterial strains, or strains within one species, varies greatly, more than one would expect on the basis of a pore formation model for which the only prerequisite is the presence of anionic lipids. It has been suggested that differences in the accessibility of the lipid II molecule in bacterial strains could explain this phenomenon.[112]

The C-terminal region of nisin was shown to play a dominant role in the membrane-binding step of nisin.[114] This part of the molecule contains 4 out of the 6 positively charged residues of nisin A (Lys-22, His-27, His-31, Lys-34), which are likely to be involved in ionic interactions with membrane phospholipids. The introduction of a negatively charged glutamic acid residue in the C-terminus of nisin Z severely reduced the affinity of the peptide for negatively charged phospholipids, indicating that the negative charge interferes with binding to these lipids. Concomitantly, the antimicrobial activity of this nisin analog towards various indicator strains was strongly reduced.[92] Removal of the C-terminal part of nisin, as in the peptide fragments nisin1-12, resulted in almost complete loss of activity.[10,115] This fragment of nisin could be isolated after prolonged incubation of nisin with high concentrations of trypsin. Interestingly, nisin1-12 acts as an antagonist of the antimicrobial activity of the wild-type peptide.[116] It remains to be clarified whether this truncated nisin molecule can compete for binding at the cytoplasmic membrane, or competes in

another process of the nisin–membrane interaction, for instance aggegation of nisin molecules. Alternatively, the fragment might also inhibit the *in vivo* activity of nisin by competing for binding to the putative docking molecule lipid II.

4.3 Insertion into membranes

Binding of nisin molecules to the surface of the cytoplasmic membrane is assumed to be followed by insertion of the peptides into the membrane. This insertion step appears to be an essential step in the membrane permeabilizing effect of the peptide and involves the penetration of (part of) the peptides into the outer leaflet of the bilayer. To study the membrane-insertion of nisin and nisin analogs, the lipid monolayer model system has been used, which provides information on the penetration of membrane-active peptides into the lipid layer, while binding to the lipids is not reflected.[117] Nisin efficiently inserted into monolayers containing phosphatidylglycerol or cardiolipin, whereas the interaction with zwitterionic lipids was significantly lower. For mutant nisin species, particularly those that contain N-terminal modifications, a good correlation was found between the interaction with anionic monolayers and the antimicrobial activity against *Micrococcus flavus*.[117] The effect of C-terminal modifications of nisin as in V32E nisin Z[114] and nisin Z-Asp-His_6[118] on membrane insertion was less pronounced. From these results it was concluded that the monolayer data predominantly reflect the membrane insertion of the N-terminal region, and that the C-terminal part of nisin contributes to a lesser extent to the process of membrane insertion.

4.4 The orientation of nisin in membranes

Studies of the nisin–membrane interaction at the molecular level have shown that the molecules have an orientation that is parallel to the surface of the membrane. NMR spectroscopic studies on nisin complexed to membrane-mimicking micelles showed that the peptides are located at the surface of these micelles, such that the hydrophobic sites are immersed below the surface, while the more polar or charged residues have an outward orientation.[119,120] More recently, tryptophan fluorescence spectroscopy was applied to determine the topology of nisin at the surface of a lipid bilayer.[121] Since nisin does not naturally contain Trp residues, tryptophan-containing variants of nisin were developed by protein engineering techniques.[92,101] Fluoresence studies on three Trp-mutants of nisin Z, containing a unique Trp residue either at position 1, 17 or 32, indicated a parallel orientation of the molecule with respect to the membrane surface, similar to what was found for the micellar systems.[121] By using spin-labelled lipids, the insertion depth of the different tryptophan residues in the lipid bilayer could be determined, and revealed that all three tryptophans were embedded in the hydrophobic part of the bilayer. The results were in good agreement with that of a fluorescence quenching study with the mutant Ile30Trp nisin A.[122] The calculated insertion depths of the different tryptophan residues and the data of the NMR structure of nisin at the surface of micelles were used to model the orientation of nisin at the membrane surface. The results indicate that the molecule is inserted in the bilayer in an overall parallel orientation with respect to the bilayer surface, with the N-terminal part inserted slightly deeper than the C-terminal part.[121]

The insertion of peptides at the *cis*-site of the membrane–water interface is most likely not directly responsible for the permeabilising effect of nisin, as has been proposed for the mechanism of action of melittin and several other membrane-active peptides.[123] In contrast, it has been shown that the C-terminal part of nisin translocates across the membrane, a process that appears to be related to the membrane-permeabilizing activity of the peptide.[118] This was demonstrated by using an engineered nisin Z molecule, containing the C-terminal extension Asp-His_6 (His-tag-nisin Z). Addition of this peptide to large unilamellar vesicles, which had trypsin encapsulated in the lumen, resulted in proteolytic cleavage of the C-terminal extension from part of the peptides. From these results it was concluded that the C-terminal part of these peptides was at least temporarily exposed to the vesicle lumen. The translocation process was proposed to be an essential step in the pore-forming mechanism of nisin. It was observed that complexing the C-terminal histidine residues with nickel ions or increasing the positive charge of the histidines by lowering the pH, resulted in blocking of the translocation process, and concomitantly the permeabilizing capacity of the peptide was strongly decreased. The interpretation of these results was that the formation of bulky Ni–histidine complexes or the presence of strong electrostatic interactions leads to anchoring of the C-terminus at the membrane surface, which prohibits the translocation of this region across the membrane.[118] The results further suggest that for the membrane permeabilizing activity of nisin, at least in vesicles consisting solely of phospholipids, the molecules have at some point in time a transbilayer orientation. In view of the results of the tryptophan fluorescence experiments it is considered unlikely that the nisin molecules exist in a stable transmembrane orientation, as has been proposed for the pore-forming peptide alamethicin.[123]

4.5 Permeabilisation of membranes

The membrane-permeabilising activity of lantibiotics has been extensively studied on intact bacterial cells, protoplasts, liposomes, proteoliposomes and black lipid membranes. The results of the *in vivo* and *in vitro* experiments are all consistent with the conclusion that lantibiotics induce efflux of low molecular weight compounds and a rapid dissipation of the proton motive force.[113–116,24,125] For an overview of these studies the reader is referred to Moll *et al*.[126] The addition of nisin to liposomes results in a rapid release of the entrapped solutes. The nisin-induced leakage is initially fast and levels off in time. A comparison of the nisin-induced release of potassium and carboxyfluorescein revealed that the rate and extent of carboxyfluorescein leakage is higher, suggesting that the pores are to some extent anion-selective.[114] This suggestion was supported by the results of planar membrane studies, showing that the replacement of residue Lys-12 by an uncharged Leu in nisin A resulted in a strongly increased membrane conductance, which indicated that due to the removal of the charged Lys-12 increased passage of ions through the nisin pore was allowed.[115]

The presence of anionic lipids is a prerequisite for the pore-forming activity of nisin, as was demonstrated in model membrane systems, such as liposomes,[113,114] lipid monolayers[114,119] and planar lipid membranes[115,124] as well as in bacterial membrane systems.[114] In contrast, the permeabilizing activity of nisin on liposomes composed of zwitterionic lipids is very low. It has been suggested that nisin acts as an anion-carrier on these vesicles, such that it translocates across the membrane, binds anionic compounds at the inside, and subsequently translocates back to the outer surface, where the anion is released.[107] However, negatively charged lipids were shown to inhibit this process, and therefore the anion-carrier activity is expected to be low *in vivo*.[107]

Several studies have demonstrated that the presence of a transmembrane electrical potential ($\Delta\psi$) or pH gradient (ΔpH) is not essential for nisin activity, since in the absence of a $\Delta\psi$, nisin effectively induced permeabilisation of liposomes and bacterial membranes.[113,114,116,120,123,124] However, upon the imposition of a $\Delta\psi$ (negative inside), the membrane-disruptive activity of nisin in model membrane systems and in bacterial membranes was increased.[114,127,128] Measurements on sensitive *Lactococcus lactis* cells and proteoliposomes, using fluorescent

pH-indicators, revealed that the nisin activity also increases with the magnitude of the ΔpH (inside alkaline).[116] The role of both these components of the proton motive force in the molecular mechanism of action of nisin is still unclear. No effect of the $\Delta\psi$ was observed on binding affinity of nisin for the membrane,[114] or on the orientation of nisin at the membrane surface.[123] Possibly, the $\Delta\psi$ and ΔpH act on the membrane-associated nisin molecules by promoting a switch from a parallel orientation into a membrane-spanning orientation. An inside-negative electrochemical potential might accelerate the translocation of the positively charged C-terminal part of the peptide across the membrane.

It is likely that the pore-forming activity of lantibiotics involves the aggregation of several peptides. Since lantibiotics have a length of approximately 5 nm, they can span the membrane only once and therefore it can be excluded that a membrane channel is formed by a single molecule. Indications for a possible aggregation process were obtained in binding studies of nisin to liposomes.[114] and planar lipid bilayer studies.[125] However, aggregates of nisin have not been detected so far. An explanation for this could be that only a minor fraction of the peptides is involved in aggregation, or that the aggregates are short-lived, and therefore difficult to trap. Single channel recordings in planar membrane experiments indicated that lantibiotics form transient channels with a lifetime in the order of milliseconds.[129] The diameter of the channels was calculated to be 0.2–1 nm. Different conductance levels were observed with nisin, indicating that pore complexes with varying numbers of participitating molecules are formed, resulting in variable pore diameters. Similar results were obtained with the lantibiotics Pep5,[130] subtilin[131] and streptococcin A-FF22.[132]

4.6 Model for the mode of action of lantibiotics

Several models have been proposed to explain the membrane-activity of nisin. Most of the models are based on the 'barrel-stave' model, initially proposed for nisin by Sahl *et al*.[133,134] In current models it is assumed that nisin, in particular the C-terminal region of the molecule, binds to anionic phospholipids by means of electrostatic interactions (Fig. 6, step 1). *In vivo*, the binding of nisin and epidermin to bacterial membranes might also involve the interaction with the peptidoglycan precursor lipid II. This more specific interaction was suggested to be mediated by the N-terminal part of nisin, as nisin and epidermin share a homologous N-terminal ring pattern. The membrane-associated peptides, or at least part of them, insert into the membrane, thereby taking an orientation parallel to the membrane surface.[121–123] (step 2). The hydrophobic residues of nisin interact with the fatty acyl chains of the lipids, while the polar residues are located at the membrane–water interface. According to the barrel-stave model, the peptides subsequently switch into a membrane-spanning orientation (step 3), and form a cluster around a central water-filled pore (step 4a). It is likely that this process is preceeded by aggregation of peptides, although this process remains speculative. In an alternative so-called 'wedge-model' it was proposed that the bound anionic lipids co-insert with the peptide, resulting in bending of the lipid surface, giving rise to wedge-like pores[114] (step 4b). Both models assume that nisin exposes its charged residues to the lumen of the pore, which is expected to result in anion selectivity of the nisin pore. It has been proposed that the C-terminal region of the peptides switches across the membrane to form a pore.[120] Possibly, the electrochemical membrane gradient facilitates this process, by pulling the charged C-terminus across the membrane. The transmembrane orientation of the peptides is presumably only transient. Upon disassembly of the pores, the peptides are assumed to flip back to an orientation parallel to the membrane surface. Since the concentration of nisin molecules in the outer leaflet of the membrane is high, peptides could flip to the inner leaflet of the membrane, similar to the mechanism of action proposed for the α-helical peptides magainin, mastoporan and melittin.[135]

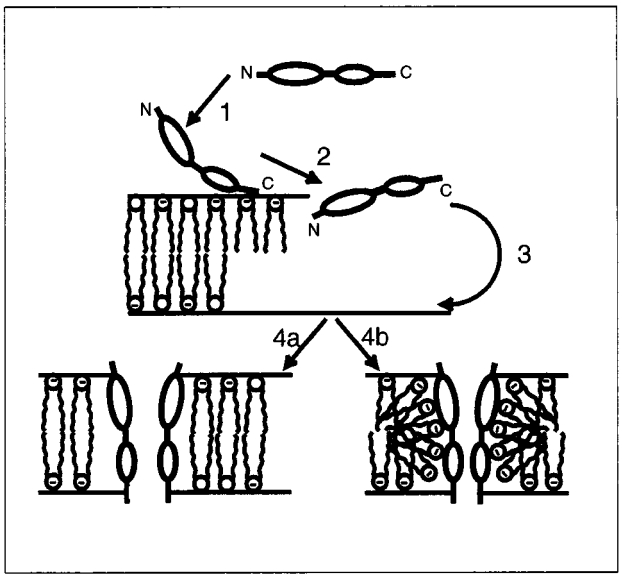

Fig. 6 Model for the mechanism of action of nisin. At first nisin binds to the surface of the membrane (1), followed by insertion of the molecule into the membrane (2). The peptides switch to a membrane spanning orientation, involving translocation of the C-terminus of the peptides across the membrane (3). A transmembrane aqueous pore is formed, most likely consisting of several peptides (4). Membrane phospholipids might co-insert, resulting in bending of the lipid surface (4b).

5 Applications and prospects

Ever since the discovery of the first member of the lantibiotics, nisin A, the application of these antimicrobial peptides has received considerable attention. Presently both type A and type B lantibiotics are used in agricultural, veterinary and medical practice and, more recently, also in personal care products (Table 2). Important factors that underlie the application of lantibiotics include a desired antimicrobial efficiency, such as indicated by minimal inhibitory concentrations and spectrum as discussed above, appropriate stability, and cost-effective production level. Moreover, several lantibiotics are produced by bacteria that have a long history of safe use in foods which allow these to be applied in food products. Below an overview is given of the different lantibiotics that have reached the stage of application, have potential to become applied, or have specific prospects that are relevant to discuss with respect to their application in pharma or food (Table 2).

Table 2 Overview of present and potential future applications of several type-A and type-B lantibiotics

Lantibiotics	Present or future applications			
	Food	Medical	Veterinary	Personal care
Nisin A / Z	×	×	×	×
Epidermin/Gallidermin		×		×
Lacticin 481 / Variacin	×			
Lacticin 3147	×		×	
Mersacidin		×		
Ancovenin		×		
Cinnamycin		×		
Duramycin		×		

5.1 Pharmaceutical applications

While food applications are limited to type A lantibiotics that are produced by food-grade bacteria, both type A and type B

lantibiotics are used in pharmaceutical applications (Table 2). In fact the first applications of nisin were ventured in clinical and veterinary therapies.[136] However, the intravenous use of nisin has not been further developed since nisin shows a low stability at physiological pH. However, several protein-engineered derivatives of nisin Z have been generated in recent years that show improved stability and these or others may extend the medical application of nisin.[93,137] Various alternative pharmaceutical applications of nisin have been considered. One that builds on the high activity at low pH relates to the use of nisin to antagonize *Helicobacter pylori* which is the causative agent of gastric ulcers.[138] Since this is a high value market, it is feasible that the development of specific nisin species that have been improved by protein engineering could be rewarding. The same holds for the use of nisin to inhibit growth of multidrug-resistant pathogens. In a recent survey, the capacity of nisin to prevent growth of resistant *Staphylococcus aureus* or *Streptococcus pneumoniae* was found to be promising, although resistance development was observed.[139] Another application is the use of nisin as a sanitizer against mastitis pathogens in cows that also include *Streptococcus* and *Staphylococcus* spp.[140] Finally, the use of nisin in personal-care products such as mouth wash or deodorants has also been suggested.

Pharmaceutical applications of other lantibiotics are limited and include mersacidin and the related peptides gallidermin and epidermin. Mersacidin is produced by a *Bacillus subtilis* strain and inhibits the cell wall synthesis of methicillin-resistant staphylococci with a similar efficiency to vancomycin.[141] However, its mode of action differs from vancomycin which allows combination with this important last option antibiotic.[142] The lantibiotics epidermin and gallidermin that are produced by the related *Staphylcoccus epidermidis* and *gallinarum*, respectively, and differ in a single residue, have been tested in medical and personal care products. These lantibiotics show activity against *Propionibacterium acnei*, the causative agent of juvenile acne.[16] In addition, they have antagonistic activity against staphylococci and streptococci isolated from skin and hence have been implied in deodorants. Recently, several mutants of gallidermin have been described that have improved antimicrobial activity and these may have potential since the efficiency of this lantibiotic is not so high as that of nisin.[143]

The type B lantibiotics exclusively have pharmaceutical potential and the lantibiotics duramycin (including duramycin B and C) and cinnamycin were found to inhibit phospholipase A2. This enzyme plays a major role in the release of arachidonic acid from phospholipids that may be oxidized resulting in the formation of prostaglandins and leukotrienes, both potent promoters of inflammations and allergies. As a consequence, the lantibiotics duramycin and cinnamycin have potential as antiinflammatory drugs. Their mode of action has been shown to involve inhibition of phospholipase A2 indirectly by sequestering the substrate phosphatidylethanolamine, analogous to the protein lipocortin.[144] The other type B lantibiotic with medical application is ancovenin that has reported to be an inhibitor of the angiotensin converting enzyme and hence can be used to treat high blood pressure.[16]

5.2 Food applications

Many applications concern the use of nisin, not only for historical reasons, since this lantibiotic couples a high antimicrobial efficiency to a wide host-range including vegetative cells and spores of many Gram-positive bacteria that are either pathogenic or otherwise undesired. Since they only differ in the solubility at neutral pH values, the applications of the two natural variants of nisin, nisin A and nisin Z, are discussed here simultaneously, although superiority of nisin Z in some applications is to be expected.[145] The fact that the solubility of nisin improves at low pH has been exploited in the use of nisin in acidified food products. These applications were facilitated by the fact that the production host of nisin belongs to the species *Lactococcus lactis*, strains of which are used as starter cultures for industrial cheese fermentations. Notably, the use of nisin-producing strains to control the outgrowth of spores of the cheese-spoilage bacterium *Clostridium tyrobutryricum* appeared to be a promising application and has been followed by many others, including control of *Clostridium botulinum* and other pathogens in processed or canned foods.[146] The observation that the property to produce nisin could be transferred to specific industrial *L. lactis* strains by conjugation, since the *nis* gene cluster is located on so-called conjugative transposons, further enhanced the applications of nisin-producing strains.[147,148] Another development that has contributed to the expanded use of nisin is the finding that its antimicrobial activity and spectrum can be enhanced by the addition of several compounds. A prominent synergistic activity is observed when divalent cations are eliminated by the use of chelating agents such as EDTA or citrate, thus destabilizing the outer membrane of Gram-negative bacteria that hence become sensitive to nisin.[105] Remarkably, not only Gram-negative bacteria, which include many pathogens, may thus become sensitized to nisin, but also yeast cells may become sensitive to nisin when devoid of their cell wall, although high doses are required.[149] In addition, several other compounds, such as magainin II amide or sucrose fatty esters, have been found to enhance the antimicrobial activity of nisin against relevant pathogens.[150,151] Finally, synergistic activities of nisin and other antimicrobial peptides, including the lantibiotic lacticin 481, has been reported.[152] Presently, nisin is used world-wide as a food preservative and is by far the most widely applied lantibiotic. It is used in more than 50 different countries in products ranging from processed cheese to salad dressings and bread to beer, wine, and other alcoholic drinks.[138] Some of the applications of nisin, however, are jeopardized since the use of antimicrobials in foods is presently under political pressure. Nonetheless, it should be noted that there has not been any indication of cross-resistance to clinically relevant antibiotics nor any sign of nisin-resistant pathogens.

Various other lantibiotics couple production by food-grade hosts to high antimicrobial activity to relevant pathogens or food-spoilage organisms. These include lacticin 481 produced by *Lactococcus lactis*[153] and the highly homologous lantibiotic variacin, produced by the meat starter *Micrococcus varians*.[35] The recently discovered antimicrobial peptide lacticin 3147 has a high activity against *Listeria* spp. Although evidence for the presence of lanthionine residues in lacticin 3147 has not been provided yet, the genetic information on its structural gene and flanking sequences which are located on a fully sequenced plasmid, strongly argues for the presence of the post-translational modifications found in lantibiotics.[18] Since this plasmid can be conjugationally transferred to other *L. lactis* strains, similar strategies as with nisin-producing strains are feasible and the use of lacticin 3147 transconjugants as alternative salami starters has been documented.[154]

Presently, no lantibiotics that have been improved by protein engineering are applied in foods but the future use of nisin mutants with improved antimicrobial efficiency remains a realistic option for specific applications.[155]

6 Concluding remarks

Investigations into a wide range of lantibiotics and their producing organisms have demonstrated an enormous progress in the lantibiotic field. In the last five years, great advances have been made in studies on production, biological activity and applications of lantibiotics. Still, fundamental questions remain to be answered in the field. The mechanism by which lantibiotics are post-translationally modified and the role of the proteins LanB, C, M, D and O in these processes is still unclear. Further insight into this subject could extend the possibilities for

mutagenesis of lantibiotics, since currently biosynthetic problems are encountered with the production of many mutant peptides. Also, unraveling of the mechanism by which the immunity proteins LanI, F, E and G protect the producing cell from the bacteriocidal activity is a great scientific challenge, since this mechanism is very poorly understood. The use of site-directed mutants to study structure–function relationships and the mode of action of lantibiotics has proven to be valuable. Great progress was made in elucidation of the molecular mechanism of action of lantibiotics. Future studies will continue to be directed towards understanding of the interaction of the peptides with target membranes and unraveling of the pore complex. Another great challenge arises from the recent findings that some lantibiotics specifically interact with a docking molecule, present at the outside of target cells. This fascinating topic is currently under further investigation. Possibly, these studies will improve insight into the factors that determine sensitivity of bacteria to lantibiotics. As indicated in the last section of this review broader applications of lantibiotics are to be expected in the future. Regarding this, more insight into the development of resistance by undesired organisms will be required. Also, a better stability and effectiveness of lantibiotics in biological systems should be realized, which could for instance be accomplished by protein engineering of lantibiotics and by searching for new synergistic combinations with other compounds.

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