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Cellular and Molecular Life Sciences

Review

Biosynthesis, immunity, regulation, mode of action and engineering of the model lantibiotic nisin

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Abstract. This review discusses the state-of-the-art in molecular research on the most prominent and widely applied lantibiotic, *i.e.*, nisin. The developments in understanding its complex biosynthesis and mode of action are highlighted. Moreover, novel applications arising from engineering either nisin itself, or from the construction of totally novel dehydrated and/or lanthionine-containing peptides with desired bioactivities are described. Several challenges still exist in understanding the immunity system and the unique multiple reactions occurring on a single substrate molecule, carried out by the dehydratase NisB and the cyclization enzyme NisC. The recent elucidation of the 3-D structure of NisC forms the exciting beginning of further 3-D-structure determinations of the other biosynthetic enzymes, transporters and immunity proteins. Advances in achieving *in vitro* activities of lanthionine-forming enzymes will greatly enhance our understanding of the molecular characteristics of the biosynthesis process, opening up new avenues for developing unique and novel biocatalytic processes.

Keywords. Nisin, biosynthesis, dehydration, cyclization, thioether-containing peptides, protein engineering, mode of action, therapeutical peptides.

Introduction

Nisin, produced by *Lactococcus lactis*, is one of the oldest known antibiotic compounds. Its antimicrobial capacity against closely related Gram-positive bacteria was described already in 1928 [1]. Sixteen years later this peptide was partially purified and analyzed by Mattick and Hirsch (1944) [2]. However, the isolation of the gene of this lanthionine ring-containing peptide and subsequent characterization of its biosynthetic cluster lasted until the late 80 s early 90 s of the last century [3–9]. The genes required for nisin production, maturation, immunity and regulation are located on a conjugative transposon, *Tn5276*, which also contains the determinants of sucrose metabolism [6]. Nisin biosynthetic genes are transcriptionally organized in four operons, *nisABTCIPRK*, *nisI, nisRK* and *nisFEG* (Fig. 1) [10–12], which are discussed further in the following paragraphs. Nisin is widely used as a food-preservative in a broad range of products, including dairy products, liquid egg, bakery products, vegetables, meat and fish [13]. Its food-grade status, long history of safe use and high efficacy make it one of only a few commercially applied bacteriocins. It effectively kills Gram-positive bacteria including spoilage and pathogenic bacteria, such as *Bacillus cereus, Listeria monocytogenes, Enterococci,*

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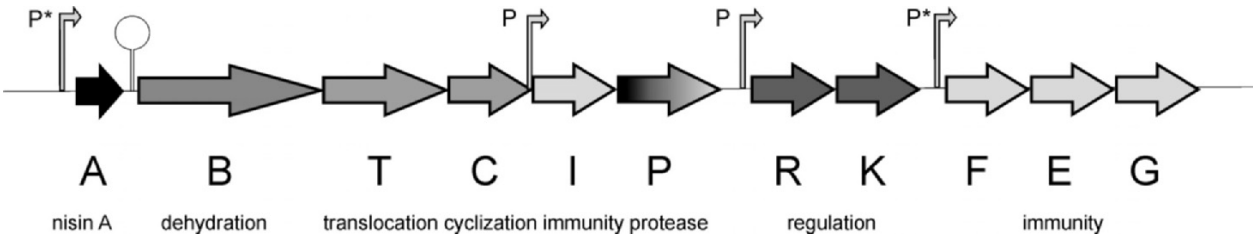

Figure 1. Transcriptional organization of nisin biosynthetic gene cluster. Four separate transcriptional units are present in nisin regulon. The first operon consists of *nisA,* the structural gene of nisin A, which is separated from the modification machinery, *nisBTCP* the embedded gene that encodes the immunity protein *nisI*, and the regulatory genes *nisRK,* by an inverted repeat. *nisI* gene is preceded by an internal promoter, which enables expression of the immunity protein before full nisin production is established. The regulatory two-component system genes, *nisRK*, are preceded by a weak promoter. The fourth operon encodes the immunity proteins *nisFEG.* Promoters marked P* are controlled by a two-component system NisRK, whereas transcription of *nisRK* and *nisI* (P) is constitutive.

Staphylococci and *Streptococci.* More recently, novel pharmaceutical uses of nisin have also been proposed, *e.g.*, using structural elements to develop novel antibiotics [14]. Nisin belongs to the Group A lantibiotics, a class of related elongated post-translationally modified peptides mainly produced by Gram-positive bacteria. Prominent members of this class include subtilin, epidermin, gallidermin, Pep5, lacticin481 and the two-component lantibiotic lacticin 3147. Excellent reviews on the characteristics of these lantibiotics are available [15–20].

In this review we focus on the molecular characteristics of nisin, its biosynthesis, immunity and regulation, its mode of action and engineering novel variants including the production of novel bioactive peptides with increased stability.

Nisin biosynthesis

Nisin A, encoded by *nisA*, is a lanthionine ring-containing peptide that is ribosomally synthesized as a prepeptide of 57 amino acid residues. The unmodified precursor of nisin is processed by a specific maturation machinery that is responsible for dehydration reactions and ring formation (NisBC), transport across the cytoplasmic membrane (NisT) and cleavage of the leader peptide (NisP), which liberates biologically active nisin, consisting of 1 lanthionine, 4 methyllanthionines, 1 dehydrobutyrine, 2 dehydroalanines and 21 unmodified amino acids (Fig. 2) [9]. There have been more than 40 post-translationally modified peptides containing (methyl)lanthionines called lantibiotics reported up to now. These peptides have been classified based on their structure and function into two main groups, A and B. Group A consists of linear, amphipathic and positively charged members and can be further subdivided into subgroups AI and AII. AI members are modified by two separate enzymes, *i.e.*, LanB, responsible for dehydration of serines and threonines, and LanC, which catalyzes (methyl)lanthionine ring formation. Subgroup AII members are modified by LanM enzymes, which perform both modification reactions. Group B lantibiotics are globular peptides and are modified exclusively by LanM enzymes [15, 21]. Nisin is a small post-translationally modified antimicrobial peptide produced by *L. lactis*, which belongs to group A lantibiotics (class I bacteriocins) according to the classification proposed by Kupke [21]. The nisin primary structure was described by Gross and Morell in 1971 [22], and its gene was cloned for the first time in 1988 [3, 4]. The 3-D structure of nisin has been investigated by high-resolution NMR spectroscopy. The structure of nisin shows an elongated conformation with the termini of the peptide pointing towards the middle of the molecule, which is enabled by a flexible hinge region [23, 24].

Unmodified prenisin contains 57 amino acid residues (Fig. 2a), which after translation are targeted to a modification machinery most likely located at the cytoplasmic membrane (Fig. 3) [25]. The N-terminal leader peptide that covers the first 23 amino acid residues appears to be crucial in recognition of unmodified prenisin by the modification and transport proteins [9, 25–27]. The first step in nisin maturation is performed by the NisB dehydratase, which, after interaction with the leader peptide, dehydrates serines and threonines in the nisin pro-peptide (Fig. 2a) [27, 28]. Dehydrated residues may then participate in regioselective cyclization by being coupled to a cysteine with the help of the cyclization enzyme NisC (Fig. 2b) [26, 28]. Modified nisin is subsequently transported *via* the dedicated ABC-type transporter NisT (Fig. 2c) [9, 29, 30]. (Methyl)lanthionine-containing nisin that still contains a leader sequence, remains biologically inactive. Only after the proteolytic cleavage of the N-terminal leader sequence, which is mediated by a protease called NisP (Fig. 2d) [9, 10], nisin becomes active and able to induce NisRK, a two-component system that regulates its biosynthetic and immunity genes.

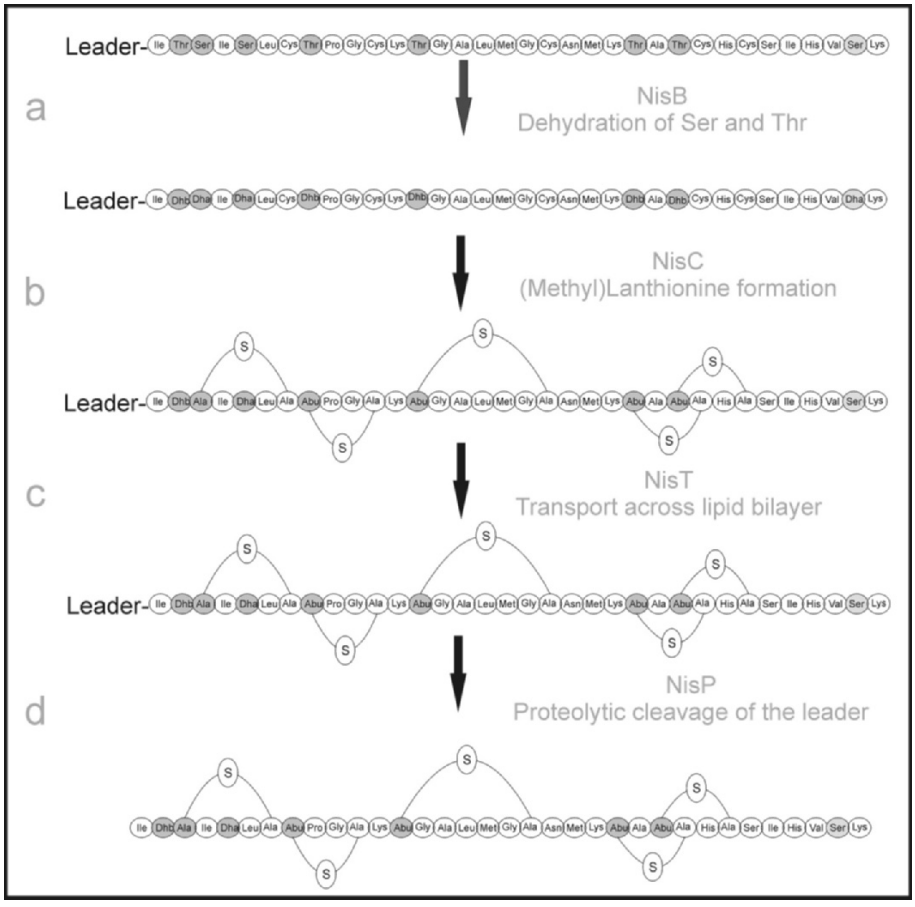

Figure 2. Post-translational processing of nisin. Nisin is ribosomally synthesized as a precursor peptide, which undergoes a series of modifications. (*a*) Specific serines and threonines (shaded gray) within the nisin peptide are dehydrated by a dehydratase, NisB, to dehydroalanines and dehydrobutyrines, respectively. (*b*) The newly formed dehydroamino acids are coupled by a cyclase, NisC, to a specific cysteines, which results in formation of (methyl)lanthionine rings. (*c*) Subsequently, fully modified nisin precursor is transported across cytoplasmic membrane by an ABC transporter, NisT. (*d*) Extracellularly present modified nisin precursor remains inactive until proteolytic cleavage of its leader peptide by NisP.

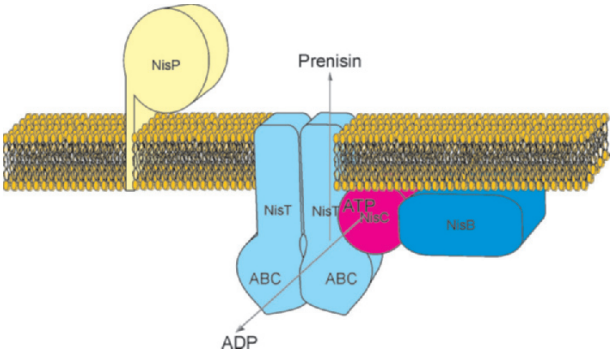

Figure 3. Post-translational processing of nisin occurs most likely at the cytoplasmic membrane. Ribosomally synthesized nisin is first dehydrated by NisB, then cyclized by NisC and transported across the lipid bilayer by NisT. Subsequently, the nisin leader sequence is cleaved off by the protease NisP.

NisB is a dehydratase

NisB is a membrane-associated enzyme that converts serines and threonines to dehydroalanines and dehydrobutyrines, respectively, in the nisin prepeptide. The dehydration reaction is one of the most crucial steps during nisin biosynthesis. Although successful reconstitution of *in vitro* activity of NisB has not yet been reported, there is good evidence indicating involvement of NisB in the dehydration of nisin. Genetic inactivation of the *nisB* gene results in complete loss of nisin production and the NisB protein was shown, using the yeast two-hybrid system, to interact with other members of the nisin modification machinery [25, 31]. Furthermore, elevated levels of NisB by overexpression lead to an increase in the efficiency of nisin dehydration [27, 32]. Mass spectrometric analysis of His-tagged nisin precursor produced by *L. lactis* NZ9700 deficient in NisB, showed production of unmodified prenisin, indicating the role of NisB in the modification reaction [28]. Dehydrated prenisin has been produced by *L. lactis* containing *nisABT* [30]. Finally, NisB has been overproduced together with its substrate, supplied on a separate plasmid, harboring the *sec*-signal sequence. Fully dehydrated prenisin was found in the medium. This suggests transport *via* the Sec machinery, and direct involvement of NisB in the dehydration reaction. Taken together, these data demonstrate that NisB is solely responsible for dehydration of serines and threonines [27].

NisB does not share close sequence similarity with proteins other than members of the LanB family

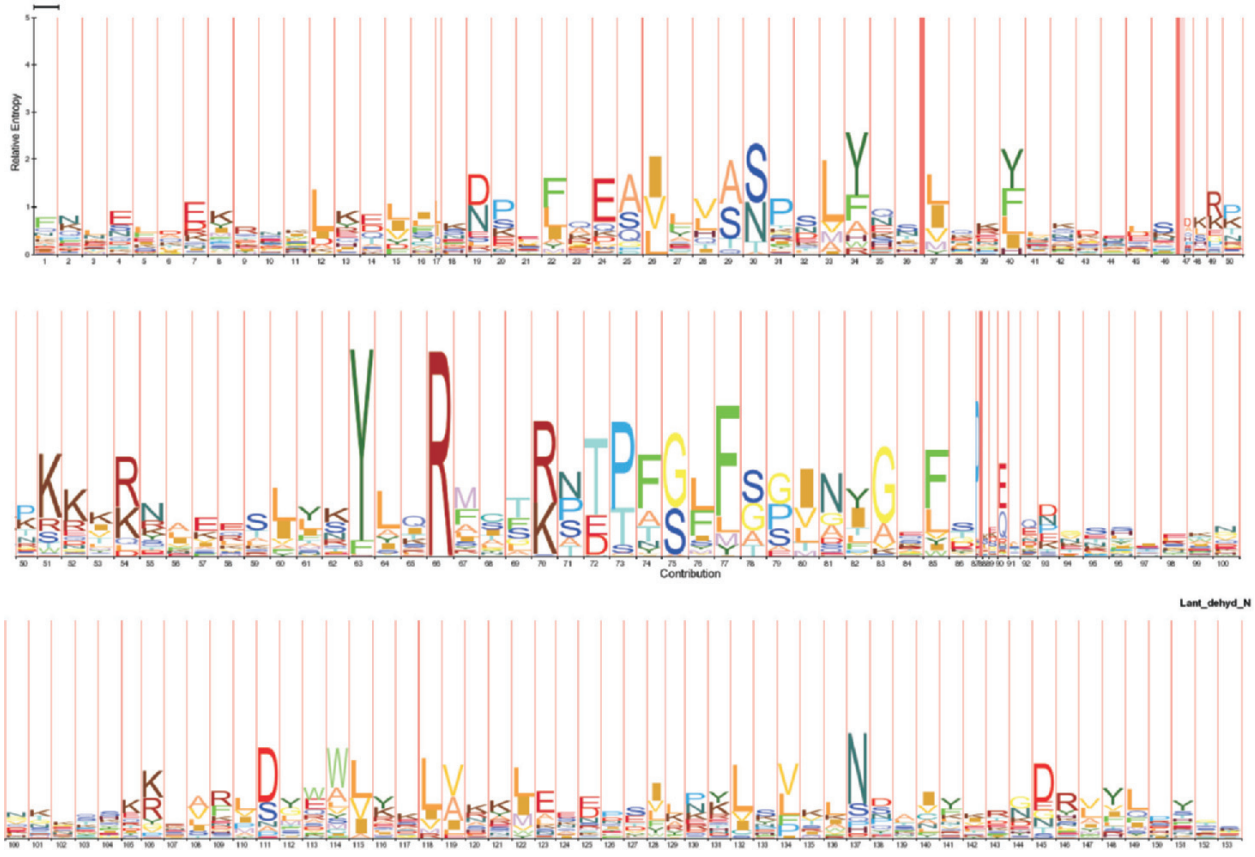

Figure 4. HMM-Logos of the N-terminal part of fifty members of the LanB family. The figure was generated based on Pfam entry Lant_dehyd_N by the software for visualization of proteins families, HMM-Logos [104]. Full alignment of LanB family can be viewed at http://www.sanger.ac.uk/Software/Pfam/ entry: Lant_dehyd_N and Lant_dehyd_C.

present in public protein databases. Even within the LanB family, the sequence identity is rather low and comprises 30 % identical residues. The closest homolog of NisB (43 % identical residues) is the recently described protein NsuB from *Streptococcus uberis* [33]. Although low homology between various members of NisB and lack of homology to other proteins in database makes it difficult to assign putative functionalities to various regions of NisB, alignment of more than 50 LanB enzymes shows some highly conserved residues within the primary sequence of NisB (Fig. 4). These sometimes absolutely invariant amino acid residues might serve as a good starting point for investigation of the leader-interaction site and the catalytic active site of NisB.

Multimeric lanthionine synthetase complex – what is the evidence?

NisB is 117.5-kDa protein that, according to the UniProt database prediction, contains one potential transmembrane segment, ranging from residues 838 to 851. Cellular localization of NisB has been studied and it was suggested that NisB is primarily associated with the cytoplasmic membrane [8]. Antibodies were raised against purified NisB and these were used to probe NisB localization. A cell extract of nisin-producing *L. lactis* was fractionated by centrifugation and NisB was found, by specific antibody detection, in the membrane vesicle fraction, although some signal was also present in the remaining soluble fraction [8]. Studies by Siegers et al. [25] demonstrated that not only NisB localizes at the membrane, NisC, which is responsible for ring formation in the maturation process, is also associated with the cytoplasmic membrane. Interactions between members of the mentioned putative membrane-associated complex were studied by co-immunoprecipitation and a yeast two-hybrid screen [25]. These data together showed interaction between NisA and proteins of the nisin biosynthetic pathway. Physical interactions of NisC with NisA, NisB and NisT were demonstrated. NisC also showed interaction with itself, suggesting at least a dimeric arrangement of this protein. NisB interaction with NisC and also a somewhat weaker interaction with NisA were also reported. Based on these interaction studies, the authors concluded that matu-

ration of nisin and likely other related lantibiotics is performed at a membrane-associated multimeric lanthionine synthetase complex, which consists of one molecule of NisB, dimeric NisC and dimeric NisT [25].

Similarly, SpaB, which is a NisB homolog responsible for maturation of subtilin, a closely related lantibiotic, was also shown to localize at the cytoplasmic membrane [34]. Moreover, the proteins SpaB, SpaC and SpaT, responsible for subtilin processing, were also demonstrated to form membrane-associated complexes [35]. Interactions of SpaB with itself and with SpaC were also observed when both proteins were overexpressed in *E. coli* [36]. Finally, a multimeric protein complex was also shown to exist for members of type AII lantibiotics, *e.g.*, nukacin ISK-1. Here, the yeast two-hybrid system and surface plasmon resonance were applied to study interactions between NukA, NukM and NukT [37].

Even though a body of evidence was reported in support of the existence of a multimeric lanthionine synthetase protein complex consisting of LanB, LanC and LanT enzymes, direct isolation of such a complex with the blue-native gel electrophoresis method and co-purification methods was reported to be unsuccessful [25, 32]. Recently, modification processes of nisin such as dehydration, cyclization and secretion were dissected *in vivo*. It was demonstrated that NisB alone is capable of performing dehydration reactions entirely independently of the proposed lanthionine synthetase complex [27]. Also, the dedicated nisin ABC-transporter NisT can transport unmodified peptides without presence of either NisB or NisC [30]. Activity of NisC was demonstrated *in vitro* in the absence of NisT and NisB [26]. Moreover, recent data shows an exclusive cytoplasmic localization of SpaC and SpaB [38]. Taken together, these data suggest that the proposed multimeric lanthionine synthetase complex is not a prerequisite for functioning of any of the modification and transport enzymes, and is likely highly unstable and transient in nature.

NisB has relaxed substrate specificity

For a long time it was believed that the lantibiotic transport and modification machinery is highly specific for a dedicated substrate. However, recent reports clearly demonstrated that nisin-modifying enzymes possess rather broad substrate specificity. NisT, a nisin-dedicated ABC transporter, was demonstrated to be a broad spectrum (poly)peptide transporter, which is able to transport a variety of non-lantibiotic peptides if fused to the nisin leader sequence [30]. Overexpression of only the *nisBTC* genes in *L. lactis* was shown to be sufficient for dehydration, cyclization and export peptides totally unrelated to nisin provided that they were fused to the nisin leader sequence [39]. These findings opened the possibility for the biotechnological application of the nisin modifying enzymes as novel tools to introduce dehydrated amino acids and (methyl)lanthionine rings into a variety of non-lantibiotic peptides. The presence of these unusual amino acids can substantially modify biological activity of biotechnologically valuable peptides and protect them against proteolysis [39].

The function of NisB is to dehydrate serines and threonines in the nisin precursor. Co-expression of genetic fusions of the nisin leader to a variety of non-lantibiotic peptides, with *nisBTC* genes results in production and transport of modified peptides [27, 30, 39–41]. This clearly demonstrates that the activity of NisB is not restricted to the nisin prepeptide and that this enzyme can dehydrate serines and threonines occurring in various amino acid sequence templates. These studies raised intriguing questions. What is the substrate specificity of NisB? Is there a sequence motif that encompasses the modification signal? Do the flanking amino acids of Ser and Thr influence the dehydration reaction or does NisB dehydrate any serines and threonines that follow the leader sequence? Some of these questions were addressed in studies by Rink et al. [41]. The authors computationally analyzed 37 primary lantibiotic structures to search for modification rules. Although no specific sequences encoding a modification signal could be identified, it was shown that the immediate surroundings of dehydratable serines and threonines contain hydrophobic residues rather than hydrophilic ones. This was experimentally supported by the construction of designed hexapeptides fused to the nisin leader peptide, which were predicted to be either dehydrated or not. Mass spectrometric analyses of the exported leader peptide fusions concerning the dehydration pattern supported the *in silico* predictions [41]. This study demonstrates that the nature of residues close to dehydratable serines and threonines may influence the dehydration. However, even if the modifiable residue is surrounded by hydrophobic residues it still does not necessarily becomes dehydrated. In nisin, Ser29 is invariably not dehydrated even though it is surrounded by a hydrophobic isoleucine and by cysteine. In contrast, Ser33, which is surrounded by a hydrophobic valine and a hydrophilic lysine, is partly dehydrated. Interestingly, six serines and threonines, which are encoded in the leader sequence, are never dehydrated. This most likely relates to the binding function of this stretch of amino acids that may preclude its interaction with the catalytic site of NisB, thereby protecting these serines and threonines against dehydration.

In vitro reconstitution of the lantibiotic synthetase activity of LctM

Although LctM produced by lactococci does not show close amino acid sequence similarity to members of the LanB family, one of its activities is similar to that of NisB. LctM performs both dehydration and cyclization of lacticin 481, a lantibiotic that belongs to group A(II). Purified LctM was demonstrated to dehydrate serines and threonines and establish thioether rings in His-tagged purified LctA. *In vitro* activity of this bifunctional enzyme was dependent on the presence of ATP and Mg^{2+} ions. Moreover, lacticin 481 was capable of processing a wide variety of point mutants of LctA, demonstrating a low substrate specificity [42]. Subsequently also activity of HalM1 and HalM2 was reconstituted *in vitro* [42b].

NisC is a cyclase

NisC shares only limited homology, roughly 30%, with members of the LanC family and with the C terminus of proteins that belong to the LanM family that, besides the cyclization reaction, also perform the dehydration reaction. There are, however, several highly conserved residues including two cysteines (Cys284 and Cys330) and two histidines (His212 and His331) suggesting a Zn^{2+}-binding site. NisC has been shown to be membrane associated and to interact physically with other members of the nisin transport and modification machinery as well as with nisin itself [25]. Chromosomal deletion of *nisC* results in a lack of nisin secretion [31]. Moreover, analysis of His-tagged purified nisin precursor from the cytoplasm shows dehydrated prenisin species lacking lanthionine ring formation [28]. This indicates the role of NisC in catalyzing the reaction of dehydrated amino acids with the free cysteines. In addition, Kuipers et al. [30], who compared plasmid-based overexpression of *nisABT* with *nisABTC* genes, concluded that NisC catalyzes (methyl)lanthionine formation.

NisC has been overexpressed and purified from *E. coli.* NisC was in the monomeric state in solution, whereas previous reports from yeast two-hybrid studies suggested a strong interaction between the NisC proteins [25]. This difference could be related to the experimental set up, *e.g.*, a lack of other members of the putative synthetase complex [43]. Interestingly, spectrophotometric assays suggest that NisC contains stoichiometric amounts of zinc, and a model for the mechanism of NisC-catalyzed cyclization involving zinc ions has been proposed [43].

The high-resolution crystal structure and the *in vitro* reconstitution of the cyclization reaction have recently been presented by Van der Donk and co-workers [26]. Heterologously produced NisC was shown to be capable of catalyzing cyclization within the dehydrated nisin prepeptide *in vitro,* as evidenced by mass spectrometry. Moreover, the modifications introduced were demonstrated to fully recover nisin's antimicrobial activity following tryptic cleavage of the leader sequence. When NisC or trypsin was omitted from the *in vitro* reaction, no antimicrobial activity could be observed [26]. Cleavage of the leader sequence of prenisin by trypsin prior to the *in vitro* cyclization resulted in a lack of the cyclized product, which indicates the importance of the leader sequence in the binding and recognition by NisC [26]. It was also demonstrated that NisC does not require ATP for activity [26].

NisC was crystallized and its X-ray structure examined at 2.5 Å resolution. The overall structure shows a monomer with two domains, *i.e.*, an α-toroid domain that consists of two layers of seven α-helices forming a barrel-like bowl that contains a centrally positioned Zn ion, and an extended domain (SH2-like domain), which consists of three antiparallel β-strands surrounded by two α-helices (Fig. 5) [26]. Two conserved cysteines (Cys284 and Cys330) together with His331 bind the zinc ion in the shallow bowl formed by the surface of the α-thyroid and SH2-like domain and are complemented by His212 and Arg280. These residues appear to be responsible for the activation of cysteine and its addition to the β-carbon of a dehydrated residue [26]. The leader sequence of nisin was shown to be indispensable for recognition and targeting to the modifying enzymes, and the structure of NisC indicates a possible binding site for the leader sequence. This putative binding groove is localized next to the catalytic site and is lined with a number of hydrophobic and negatively charged residues forming a channel in which the positively charged leader sequence is supposed to be trapped [26].

Recently, crystallographic findings that indicate that several conserved residues in NisC form a catalytic site, were corroborated by biochemical analysis of a close homolog of NisC, namely SpaC [38]. Residues, which, based on the available crystal structure of NisC and conservation among the LanC family, were shown to be important for coordination of the zinc ion and the proposed general acid/base catalysis that eventually leads to the lanthionine ring formation, were separately replaced by alanines [38]. Mutagenesis of these residues showed that they are essential in subtilin biosynthesis [38]. Recently essential catalytic residues of NisC were identified [38b].

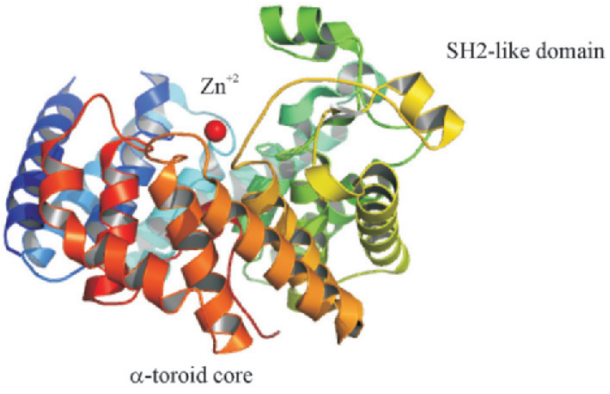

Figure 5. Crystal structure of NisC. A ribbon representation of two domains of NisC is shown. An α-toroid domain creates the main body of a NisC, forming a shallow bowl on top, next to extended domain, in which a catalytic Zn^{+2} ion is localized [26]. This figure has been prepared using the PyMol program from the 2G02 pdb file.

NisC and SpaC were shown by co-immunoprecipitation assays and yeast-two hybrid screens to interact with their substrates. Helfrich et al. [38] visualized these interactions by employing an *in vitro* co-purification assay. A Ni-NTA immobilized double-tagged subtilin precursor peptide was incubated with various mutants of SpaC and subsequently specific interactions were visualized by Western blotting. Five C-terminal residues and Trp320 were shown to be crucial for SpaC and subtilin-precursor interactions, and thus crucial for activity of SpaC. Conversely, mutations of residues that were postulated to play role in catalysis did not abolish substrate-enzyme interactions, which indicates that these catalytically inactive SpaC mutants were properly folded [38].

Precursor nisin is transported across the membrane by NisT

NisT is an ABC half-transporter that consists of putative α-helices that transverse the cytoplasmic membrane five times and a hydrophilic nucleotide-binding domain that binds ATP and is needed to energize the transport. A typical ABC transporter includes four modules, *i.e.*, two transmembrane segments and two nucleotide-binding domains. Thus, NisT, which is a half-transporter and contains only two out of four modules commonly found in ABC transporters, most likely requires another half-transporter to form an active unit. This putative partner is likely another molecule of NisT that is required to form a homodimer.

NisT was shown by means of yeast two-hybrid screens to interact with NisC and was implicated to be a part of a putative membrane modification and transport complex [25]. Deletion/disruption of *nisT* abolishes secretion of nisin, and as a result of the inability of the cell to secrete it, nisin accumulates in the cytoplasm [29, 31]. This phenotype can be restored by providing the *nisT* gene on a plasmid [29]. Although the knockout analysis and overexpression studies [30] clearly show the necessity of NisT for transport of prenisin, direct evidence that transport is mediated by NisT has not been provided either *in vivo* or *in vitro*. It has been assumed that ABC transporters that are responsible for secretion of lantibiotics are rather specific and dedicated to transport of a specific polypeptide. However, it has been demonstrated that NisT possesses a broad substrate specificity [30]. It cannot only transport fully modified nisin but also partially modified or completely unmodified peptides. Moreover, various non-lantibiotic peptides were successfully transported by NisT provided that they were fused to the leader sequence of nisin [30].

Processing of precursor nisin by NisP

Disruption of the *nisP* gene results in a lack of leader-peptide cleavage. Fully maturated prenisin can be found in the extracellular medium as has been confirmed by N-terminal sequencing [10]. The presence of the leader sequence attached to the fully modified nisin keeps the peptide in an inactive form. Activity of the secreted prenisin could be restored by incubation with cells producing NisP. This suggests involvement of NisP in nisin leader peptide processing [44].

NisP belongs to the subtilisin family of serine proteases (Pfam entry: Peptidase_S8). It contains an N-terminally located Sec-signal sequence (residues 1–22) that is likely responsible for targeting and transport of NisP out of the cell *via* the Sec pathway. The N-terminal residues up to 195 constitute a propeptide sequence that is cleaved during maturation. Expression of NisP in *E. coli* resulted in a protein with a molecular mass of 54 kDa, which is substantially lower than expected (74 kDa) [44]. It has been suggested that the differences in predicted and obtained mass relates to the N-terminal prosequence cleavage [10, 44]. Cells expressing NisP can cleave extracellularly furnished fully modified prenisin, which indicates that NisP activity is not obligatorily linked to export *via* NisT [30]. NisP contains a C-terminally located LPXTG sequence, which suggests that it is anchored to the cell surface [10, 45]. The nisin prepeptide with the leader sequence attached does not show significant antimicrobial activity [10, 46]. Treatment of the purified nisin prepeptide with heterologously expressed NisP generates an active nisin molecule,

which suggests involvement of NisP in the proteolytic processing of nisin [44]. This activity was found to be restricted to the cell surface of *L. lactis* or *E. coli* expressing NisP, and was not observed with membrane-free cell extracts of *L. lactis* expressing NisP [44]. This further supports surface localization of NisP as predicted based on amino acid sequence analysis [44].

NisP is assumed to be a protease with rather narrow substrate specificity; however, this has not been extensively studied experimentally. It has been demonstrated that, even though subtilin and nisin leader sequences share substantial sequence homology, they are not interchangeable. NisZ fused to the subtilin leader is not processed by NisP, which has been attributed to the differences between the subtilin and nisin leaders at the cleavage positions at –1, Gln and Arg, respectively [46]. The importance of residues in the immediate surroundings of the cleavage site has also been shown by Kuipers et al. [47]. Mutations of Arg–1 to Gln or Ala–4 to Asp resulted in production of fully modified nisin, which contains the leader sequence still attached, highlighting the importance of these residues for NisP specificity [47]. In contrast, mutation of the conserved Pro–2 and many other residues within the leader sequence did not influence any of the biosynthetic steps of nisin including proteolytic cleavage by NisP. Alteration of the conserved residues from –18 to –16 resulted in lack of nisin production, which indicates a disturbance of early biosynthetic processes [47]. Not much is known about the requirements at the propeptide side of prenisin for NisP cleavage. Neither unmodified prenisin nor dehydrated prenisin could be cleaved by NisP, indicating that one or more thioether rings are required for NisP activity [30]. Future research has to establish which thioether rings are required for NisP activity.

Immunity of nisin-producing strains

Strains of *L. lactis* that produce nisin have developed so-called immunity against the bactericidal activity of nisin. Immunity is conferred by two different systems: lipoprotein NisI and ABC transporter NisFEG [48]. The relative contributions of the two systems were estimated by a knockout approach of either one of the systems [31, 48], showing that each system alone only accounts for 5–20% of the full immunity level provided by the two entities together, indicating strong synergistic action.

NisI was first described in an early work on the characterization of the nisin gene cluster [9]. It is a 245 amino acid lipoprotein with a consensus lipoprotein signal sequence, which is post-translationally removed. Subsequently, the protein is anchored to the extracellular side of the cell membrane *via* lipid modification of the N-terminal cysteine residue [49]. Circular dichroism (CD) studies and biomolecular interaction analysis have provided evidence for physical interaction of nisin and NisI [50, 51]. Having no homology to other LanI proteins, purified NisI was demonstrated to bind specifically to nisin but not to subtilin, which is closely related to nisin, using native SDS-PAGE, and to form an insoluble and unstable complex [52]. These data suggest a function of NisI as a nisin intercepting molecule. In the same studies, NisI was shown to exist in two forms: a lipid-free form secreted into the growth medium and a membrane-associated lipoprotein, a situation not uncommon for lipoproteins [52, 53]. Both of the forms were shown to bind nisin [52] and, interestingly, lipid-free NisI enhanced immunity of *L. lactis* more efficiently in the strain expressing *nisEFG* as compared to the strain lacking these genes. [51]. These findings suggest that lipid-free NisI either supported NisEFG in exporting nisin from the cytoplasmic membrane or NisFEG assisted the effect of lipid-free NisI by providing high local concentrations of nisin close to the cytoplasmic membrane that lipid-free NisI could intercept and by diffusion move away to the environment. To further investigate the interaction of lipid-free NisI-nisin complexes surface plasmon resonance studies were performed, which indicated an equilibrium dissociation constant (K_D) in the micromolar range (0.6–2.0 μM), pointing at a weak interaction between NisI and nisin [51]. Surprisingly, Koponen et al. [53] reported that activity of nisin can be enhanced by an external addition of lipid-free NisI, which is in contrast to the immunity function of NisI. This effect was, however, only observed in a specific condition, namely, on a solid surface. To localize a specific site for NisI–nisin interaction, Takala et al. [54] made a series of constructs with C-terminal truncations of NisI and transformed them into a nisin-sensitive *L. lactis* strain. The levels of immunity were measured according to the growth inhibition by the different concentrations of nisin in the medium. The shortest deletion of five amino acids resulted in approximately 22% immunity of native NisI, and deletion of 21 C-terminal amino acids resulted in approximately 14% native NisI immunity level. The longer deletion provided the same level of immunity as the 21-amino acid C-terminal deletion. Interestingly, to determine if the 21 C-terminal amino acids of NisI could protect cells against nisin, the authors replaced the first 21 C-terminal amino acids of SpaI with the 21 amino acids of NisI. The obtained hybrid significantly increased the immunity level for nisin in *L. lactis*,

which indicates direct involvement of these particular residues in specific binding of nisin [54].

NisFEG immunity proteins were first described by Siegers and Entian [48]. NisFEG proteins form an ABC transporter complex, where NisE and NisF are homologous to the ABC transporters of the HisP family, NisF is a cytoplasmic ATP binding protein and NisG together with NisE are integral membrane proteins [48]. Since many ABC transporters consist of four domains, two of which are hydrophobic and two are ATPases, it is hypothesized that a $NisF_2EG$ complex is formed [55].

To investigate the role of NisFEG, Stein et al. [52] integrated different combinations of nisin immunity genes into the chromosome of the nisin-sensitive *Bacillus subtilis* strain and expressed them under the control of an inducible promoter. The nisin tolerance level suggested the additive action of the lipoprotein NisI and the transporter NisFEG. The quantitative peptide release assay showed that the quantity of cell-associated nisin was significantly reduced after the expression of NisFEG or NisIFEG, pointing at nisin expelling properties of NisFEG [52].

Immunity and production of nisin in *L. lactis* is regulated by the NisRK two-component system [56]. NisK phosphorylates itself in the presence of nisin and transfers a phosphoryl group to an aspartate of NisR, which triggers binding of the response regulator to *nisA* and *nisF* operators [56, 57]. This initiates transcription of *nisABTCIPRK* operon as well as *nisFEG* [10, 57, 58]. Until recently it was believed that transcription of *nisI* is controlled only by the *nisA* operator in *nisABTCIPRK* operon. However, it was recently shown by a Northern blot analysis that *nisI* mRNA was present in the absence of *nisA* transcription. This observation suggests the presence of an internal promoter within the operon [12].

Between the two immunity systems NisI seems to play a more dominant role since disruption in the *nisI* gene resulted in a higher level of sensitivity to nisin as compared to the disruption in *nisFEG* genes [48].

Upon deletion of the *nisI* gene the immunity level is approximately 20 % of that of the wild type, which indicates that the NisFEG transporter system provides 20 % of the immunity. On the other hand, overexpression of *nisI* in a strain lacking any *nis*-encoded genes gave only 1–4 % of the wild-type immunity level [9]. Thus, the severe effect of the interruption of the *nisI* gene can be only explained by the cooperative effect between the NisFEG and NisI systems. Inhibition of *nisEG* or *nisG* translation by antisense RNA resulted in a strongly decreased immunity level further supporting this statement [59]. Expression of *nisI* together with the *nisABTC* genes resulted in a higher immunity level of 8–20 % [31] due to the autoinduction effect of the produced nisin by quorum sensing. Furthermore, Takala et al. [60] showed that plasmid-based overexpression of the *nisI* gene can lead to elevated immunity levels up to 25 %.

In conclusion, the complete picture of nisin immunity has not been revealed yet, and currently two opinions exist: Stein et al. [52] suggest that the two *L. lactis* immunity systems are independent of each other and their activities are additive; on the other hand, knockout studies and several other reports point at cooperative effects between NisI and NisEFG [31, 51, 54]. Collectively, to achieve full nisin immunity two factors are required: nisin production and involvement of both NisI and NisFEG protective mechanisms [31, 52].

Autoregulation of nisin biosynthesis by the two-component regulatory system NisRK

NisK is a histidine sensor kinase that is localized in the cytoplasmic membrane and serves as a receptor of fully maturated nisin [57]. Extracellularly present and modified nisin binds to NisK and initiates a signal transduction cascade, which starts with autophosphorylation of histidine of NisK [44, 56, 57]. Subsequently, phosphate is transferred to NisR, which is a transcriptional activator that binds to promoter regions of *nisABTCPRK* and *nisFEG* inducing transcription of genes that are required for nisin biosynthesis and immunity [10, 57, 58]. The promoter of the *nisRK* operon was shown to be independent of nisin regulation and the genes *nisRK* to be constitutively expressed [58]. Recently, it has been reported that prenisin, which is produced by a translocator deficient strain and accumulates intracellularly, can also induce NisK and initiate a signal transduction pathway. It was suggested by the authors that prenisin is cleaved by an unidentified intracellular protease(s) and that subsequently active nisin from inside of the cell *via* the membrane can activate extracellularly located NisK [61].

The first genetic evidence demonstrating that the *nisR* gene could be a response regulator involved in nisin biosynthesis regulation came from reports published by van der Meer et al. [44]. The authors demonstrated that disruption of *nisR* leads to a lack of nisin production [44]. One year later the missing sensor kinase, NisK was identified [56]. Autoinduction of nisin biosynthetic genes by the fully modified nisin molecule was demonstrated by a short (4 bp) deletion in the structural gene of nisin, which led to the lack of transcription of *nisA* [57]. This transcription can be restored by external nisin addition [57]. Disruption of

nisK, which is involved in signal transduction, led to lack of inducibility of *nisA* transcription [57]. A *nisA* promoter fusion with reporter gene *gusA* was used to determine the capacity of various antimicrobial peptides and nisin mutants to induce a signal transduction cascade mediated by NisRK [57]. It has been noted that NisK has a rather stringent substrate specificity and a variety of other lantibiotics such as subtilin, pep5, lacticin481 and lactococcin A as well as unmodified synthetic prenisin did not induce transcription of the reporter gene. Next, a variety of point mutants of nisin and a shorter version of the nisin molecule indicated the importance of the N-terminal residues and of post-translational modification for the functional interaction between inducing nisin and NisK [57].

Tight regulation of genes that are under the *nisA* or *nisF* promoters *via* externally acting inducers led to the development of the nisin-controlled expression (NICE) system [58, 62–64]. This system was successfully used for high overexpression of a variety of proteins including notoriously difficult-to-overproduce membrane proteins [65–68].

Nisin structure and natural variants

Five natural nisin variants have been described thus far: nisin A [22], nisin Z [69], nisin Q [70] and two variants of nisin U [33] (Fig. 6). Nisin A, Z and Q are produced by some strains of *L. lactis*. Nisin U and U2 are produced by *S. uberis*. The amino acid positions in prenisin are indicated using the leader peptide cleavage site as reference. Prenisin, composed of 23 amino acids of leader peptide and 34 amino acids of propeptide, has from N–C-terminal positions: –23 to +34 (no residue is labeled '0'). Nisin Z differs from nisin A by having a glutamine instead of a histidine in position 27. Nisin Q differs from nisin A in the positions: –8 (K→T), –2 (P→T), 15 (A→V), 21 (M→L), 27 (H→N) and 30 (I→V). The leader peptide of nisin U and U2 is one amino acid longer than the leader of nisin A, Z and Q. An additional Glu seems inserted between positions –8 and –9 of nisin A, Z and Q. After alignment of the leader peptides 9 (nisin U) or 10 (nisin U2) other positions of the leader peptide differ from the leader peptide of nisin A. Nisin U and U2 propeptides are three amino acids shorter than nisin A, Z and Q and differ from nisin A in positions 15 (A→I), 18 (G→Dhb), 20 (N→P), 21 (M→L), 27 (H→G), 29 (S→H) 30 (I→F) and 31 (H→G). Nisin U2 additionally differs from nisin A in position 1 (I→V) (Fig. 6). Little is known about the structural features and requirements of the leader peptides. The percentage of alpha helicity of the nisin A leader peptide in solution containing 90–0% trifluoroethanol (TFE) varied from ~30–5% [71]. Five constituents have been reported to be present in a commercial nisin A sample: [2-hydroxy-Ala5]nisin, [Ile4-amide, pyruvyl-Leu6]des-Dha5-nisin, [Met(-O)21]nisin, [Ser33]nisin and nisin–(1–32)-peptide amide [72]. After freeze drying in an acid solution [2-hydroxy-Ala5]nisin-(1–32)-peptide amide was also formed. The 2-hydroxyalanine-containing variants and the desdehydroalanine variant were strongly reduced in antimicrobial activity. The activity of [Ser33]nisin was identical to nisin, [Met(O)21] nisin had slightly reduced activity and nisin-(1–32)-peptide amide seemed slightly more active against *S. thermophilus*, but slightly less active against *C. tyrobutyricum*. Two other degradation products of nisin have been found: (nisin$^{1-32}$) and *des*-ΔAla5-nisin$^{1-32}$ [73]. Both peptides have an overall flexible structure in solution [74]. Recently, a synthetic nisin analog, in which dehydroresidues were replaced by alanines and methyllanthionines by lanthionines, has been published in patent literature [75].

The 3-D structure of nisin A in aqueous solution has been first derived from NMR measurements [23]. The main part of the molecule consists of the lanthionine ring A, and the methyllanthionine rings B, C (residues 3–19) linked by a "hinge" region to two intertwined double methyllanthionine rings (D, E), residues 23–28. These N-terminal and C-terminal parts are both quite flexible. It is remarkable that rings A, B and C share the feature that the hydrophobic part is situated opposite to the thioether bonds. The hydrophobic face is composed of the residues Ile4, Leu6, Pro9, Leu16 and Met17 and the hydrophilic Lys12 is located at the opposite face. In addition, the region from residue 21 to 28 is amphipathic: Lys22 and His27 are on one side and Met21 and His21 on the opposite side. The amphiphilicity of the amino acids Ser29–Lys34 is not directly clear. Nevertheless, in a mixed water-TFE solvent, the α-helical character of the residues 23–28 seems to extend to the C terminus. In the latter case, the helix is amphipathic with His27, His 31 and Lys34 on one side. There are four positively charged side chains, Lys22, His27, His31 and Lys34, in the C-terminal half of the molecule at a pH lower than 6. Taken together, the molecule is amphipathic in two ways: first, most of the residues in the N-terminal part are hydrophobic and only a single charged residue is present, Lys12, whereas the charged and hydrophilic amino acids are mainly located in the C-terminal half of the molecule. Secondly, both the N-sided domain, containing rings A, B and C as well as the C-sided domain, containing rings D and E, have a hydrophobic and a hydrophilic side. Interestingly the peptide bonds

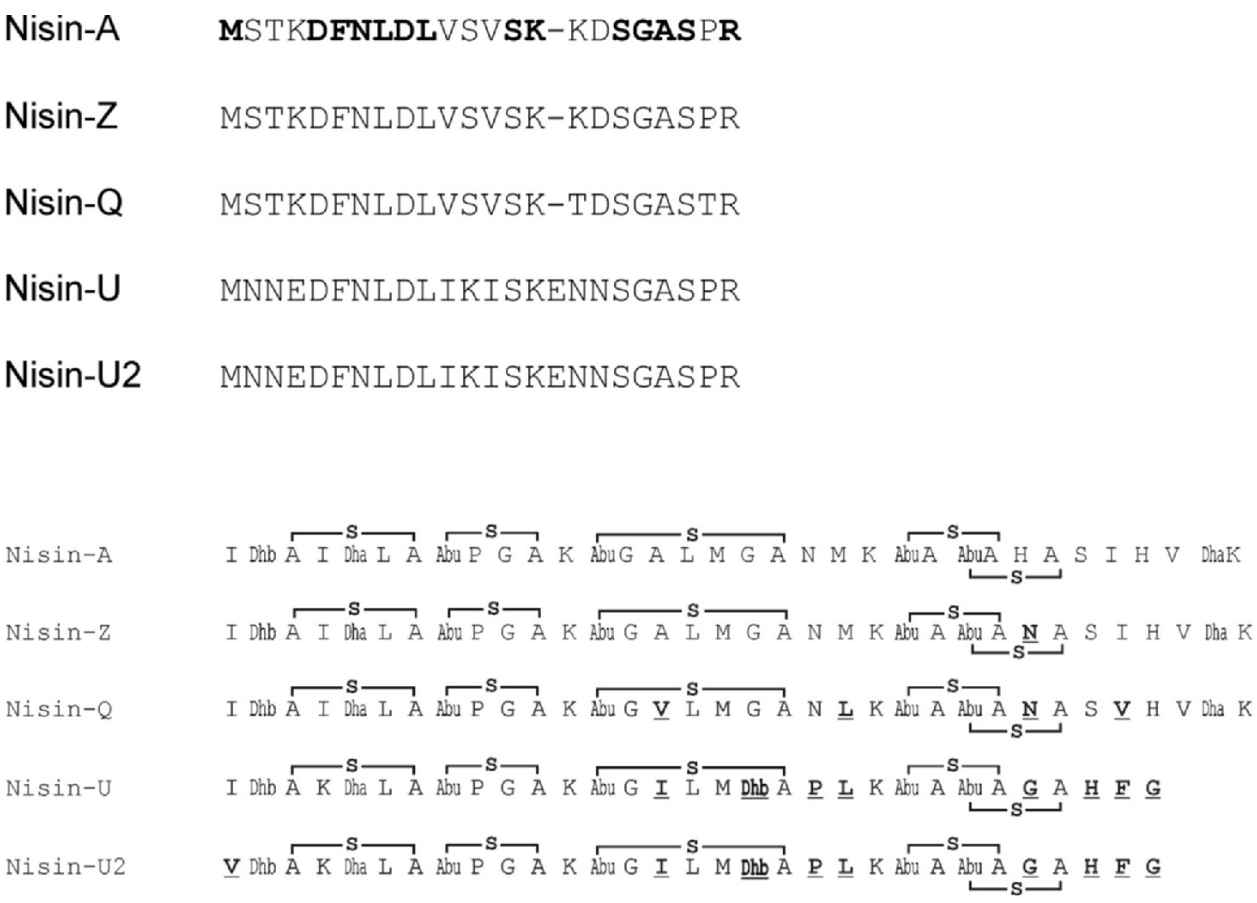

Figure 6. Leader sequences and structures of nisin A, nisin Z and proposed structures of nisin variants.

between residues 23–24, 25–26 and 27–28, which form the intertwined D and E rings, are parallel.

Nisin binds to micelles of zwitterionic dodecylphosphocholine micelles (DPC) and anionic sodium dodecylsulfate (SDS) and the structure of nisin has been determined in the following membrane-mimicking models: (1) DPC micelles, (2) SDS micelles and (3) a mixture of TFE and water [76]. The largest differences in chemical shift between nisin in solution and nisin complexed to DPC micelles concern the amide protons of Dha5, Leu6 and Abu8 and H^{β} protons of Dha5. Various facts may cause these observations, such as a varied interaction with the micelles, an altered intrinsic susceptibility to changes in chemical surrounding and/or altered hydrogen bridging. In the structure of nisin in aqueous solution a γ-turn is observed around Dha5. In contrast, for nisin complexed to both micelles no typical β- or γ-turns are present. In this respect it is of relevance to note studies with a Dha5Dhb mutant [77] and with [α-OH-Ala5]nisin. Both the bactericidal activity as well as the extent of structural changes following interaction with micelles decrease in the following order: nisin A > Dha5Dhb mutant > [α-OH-Ala5]nisin [78]. This indicates that the micelle-bound conformation of ring A might correctly mimic the membrane-bound nisin. Nisin complexed to DPC as well as nisin in TFE/water (3/1) is in the monomeric form; nisin complexed to SDS micelles is mostly in the monomeric form (>90 %). Following an increasing addition of TFE or DPC micelles, the conformation of nisin changes gradually, until 70 % TFE or a DPC/nisin ratio of 30. No titration is possible with SDS micelles. The NMR data were complemented with CD measurements. The CD spectra of nisin in the three model systems, TFE/water 3/1, SDS micelles, DPC micelles, differ significantly from the spectrum of nisin in aqueous solution, which suggests a structural change [76].

The interaction of nisin with the DPC and SDS micelles and in particular the surface location and orientation have been further investigated. The hydrophobic amino acids are slightly immersed into the micelles and oriented towards the center, whereas the more polar or charged amino acids have an outward orientation. The entire nisin molecule itself does not seem to be embedded into the micelles. Nevertheless, the temperature coefficients of the amide protons indicate that both dehydroalanines (residues 5 and 33)

as well as the residues Ile30–Lys34, which surround the second dehydroalanine, are shielded to some degree from the solvent. Furthermore, some experiments suggest that the interactions of the residues Ser29–Lys34 with the micelles are the strongest [78]. Since nisin$^{1-32}$ has full activity, the membrane interaction of the last two residues cannot be essential for activity. Taken together the structural analyses indicate a structural change in ring A upon the membrane interaction of nisin.

Studies on fully modified prenisin by NMR have also been performed [79]. In aqueous solution the leader part of the fully modified prenisin has a predominantly random coil structure, like the leader peptide itself. Also the nisin part of the fully modified prenisin adopts a structure similar to that of nisin itself. Hence the leader peptide and the nisin part of fully modified prenisin do not seem to influence each other. The structure of fully modified prenisin was compared with nisin when each was interacting with DPC micelles. This indicated a different interaction with micelles of the N-terminal part of the nisin part of prenisin. In view of the present insights in the modes of action of nisin, the contribution of the presence of the leader peptide more likely results from interfering with nisin's docking (see following section).

Mode of action

By binding to the peptidoglycan precursor lipid II, nisin exerts two killing mechanisms: it permeabilizes the membrane and inhibits the cell wall synthesis [80–82]. Sahl and co-workers [80] and Breukink et al. [81] discovered that nisin utilizes lipid II as a docking molecule. By Breukink and co-workers, it was found that the presence of lipid II in liposomes dramatically lowers the concentration of nisin required for liposome permeabilization. Therefore, the interaction with lipid II explains why nanomolar concentrations of nisin are sufficient to permeabilize cell membranes, whereas μM concentrations are needed for the permeabilization of artificial membranes. The binding to lipid II induces a transmembrane orientation of nisin [83]. Lipid II and nisin form a hybrid pore, composed of eight nisin molecules and four lipid II molecules [84]. Inhibition of cell wall synthesis results from nisin-mediated displacement of lipid II from the septa [85]. *In vitro* nisin and mutacin 1140 each cause segregation of NBD-labeled lipid II incorporated in giant unilamellar vesicles. In the case of nisin this is preceded by pore formation, in the case of mutacin 1140, which is a shorter lantibiotic, no pore formation was observed. *In vivo* fluorescein-labeled nisin A as well as N20PM21P nisin A, which is unable to form pores, were found to displace lipid II from the septa in *L. lactis* 1104, *B. subtilis* and *B. megaterium* cells. Control experiments showed that the fluorescently labeled nisin only bound to lipid II and that fluorescently labeled vancomycin clearly revealed pools of lipid II at the septum [85]. These results demonstrated that nisin, and mutacin 1140, segregated lipid II into abnormal domains both *in vitro* as well as *in vivo*, thus constituting a new bactericidal mechanism [85].

In another study the role of lipid II in membrane binding was studied using dioleoylphosphatidylglycerol (DOPG) and dioleoylphosphatidylcholine (DOPC) membranes [86]. Lipid II strongly increased nisin binding affinity to DOPC membranes but not, or in less pronounced manner, to DOPG membranes. The absolute amount of bound nisin was, however, not affected by lipid II. In the presence of 0.1 mol% lipid II nanomolar concentrations of nisin were sufficient to form pores, whereas in the absence of lipid II micromolar concentrations of nisin were required. Unspecific destruction of pure DOPG membranes occurring by micromolar concentrations of nisin was prevented by the presence of lipid II [86].

The interaction of nisin Z with the docking molecule of nisin, lipid II, in SDS micelles has been studied using solution NMR [87]. Lipid II is a bacterial cell wall component, and composed of a membrane-anchoring undecaprenyl chain, linked to diphosphate, which is coupled to *N*-acetylmuramic acid. The latter is coupled to *N*-acetyl glucosamine. The carboxylic group of *N*-acetylmuramic acid is substituted with a stem pentapeptide. For instance, in *S. aureus*, this stem pentapeptide is L-Ala-D-Glu-L-Lys-D-Ala-D-Ala. The last constituent of lipid II is a pentaglycine interpeptide that can connect the ε-amino group of the L-Lys of the stem peptide to D-Ala of a neighboring stem pentapeptide of another lipid II molecule. The peptidoglycan network results from the interconnecting lipid II molecules and the growing chain of alternating *N*-acetylmuramic acid and *N*-acetyl glucosamine. It comprises a large part of the cell wall of many Gram-positive bacteria protecting them from osmotic pressure and determining their shape.

The first two thioether rings in nisin, *i.e.*, ring A and ring B, form a cage-like structure that binds the pyrophosphate of lipid II [87]. The lanthionine part at position 3 is in the D configuration, which allows hydrogen bond formation between the backbone NH and the pyrophosphate. This structure also provides an explanation why S3T nisin lost 12-fold activity since the additional methyl group would point in the space surrounded by the cage structure of the rings.

Upon binding of nisin to lipid II large chemical shift perturbations were found for the first two rings, while

the C-terminal part appeared unaffected. The C-terminal part of nisin appeared to remain flexible and solvent-exposed. Apart from the positions 3 and 7–11 all observed differences in this position between homologs of nisin reside at the circumference of the pyrophosphate cage. This indicates that mutations would be possible in positions 4–6. The dehydroalanine at position 5 would have a hydrogen bond with the pyrophosphate moiety [87].

Protein engineering of nisin

Shortly after cloning of the nisin biosynthetic cluster, the nisin structural gene was subjected to mutagenesis to understand its mode of action, substrate specificity of its biosynthetic machinery and, last but not least, to obtain new nisin variants with altered specificities and activities.

Initially available expression systems allowed investigation of only the final mutated product. That made it difficult to exactly pinpoint the influence of the mutagenic changes on the molecular mode of action of a peptide. However, continuous improvements of nisin expression systems methodology, allowed controlling separate biosynthetic steps, such as introduction of dehydrated residues, rings, transport and proteolysis.

Even though a variety of nisin mutants were produced (Table 1), also by random mutagenesis [88], not many of them showed a significant increase in antimicrobial activity. This may relate to the fact that nature has already optimized the nisin peptide for activity and further changes will not add to it. Alternatively, putative nisin mutants with improved antimicrobial activity could overcome producer immunity, which in turn could shut down the production. Recently, an overexpression system was developed [41] that can overcome this obstacle. With that system, nisin is produced in an inactive form with the leader sequence still attached to the peptide, and such a prenisin can be processed *in vitro* later on for downstream application. Indeed the latter expression system combined with a rings A and B involving random mutagenesis approach established a breakthrough method for generating improved nisin variants and most recently yielded a large number of interesting mutants [88b]. Even before this recently developed method, already an impressive collection of nisin mutants had been generated (Table 1).

An impressive collection of nisin mutants has been generated so far (see Table 1). The mutants presented have been investigated to different degrees and it is difficult in some cases to establish the exact role of the described mutations. So far, any alteration of the residues that take part in formation of (methyl)lanthionine such as S3T, T13C mutations [89] has a strong negative influence on nisin antimicrobial activity. Thus, nisin ring structures seem a prerequisite for its antimicrobial activity. However, changes to dehydratable residues that do not form rings (S5 and S33) are by far less severe for nisin antimicrobial activity [77, 89–91]. Interestingly, the alteration of some residues can differentially influence the various nisin activities. An S5A mutation influences the activity of nisin against vegetative cells only slightly, whereas the same mutant has a strongly reduced ability for inhibition of spore outgrowth [92]. Introduction of an additional positive charge in the hinge region N20K and M21K slightly decreases the activity against Gram-positive species, but improves the activity against several Gram-negative bacteria [93]. In nisin A all cysteine residues are engaged in formation of thioether bridges with dehydrated residues and *in vivo* engineering of additional cysteines residues in the nisin prepeptide resulted in a dramatic reduction in antimicrobial activity [94].

The N-terminal part of nisin, which was demonstrated to be responsible for the formation of a "pyrophosphate cage", seems less amenable to mutagenic changes than the C-terminal part. The gradual deletion of C-terminal residues only slightly influences the antimicrobial activity [91]. Moreover, the alteration of C-terminal residues [94] suggests that large changes in that part of molecule are tolerated. The flexible hinge region of nisin was also subjected to intense mutagenesis [89, 93, 94]. A variety of mutagenic changes introduced in the hinge region, which is postulated to be important in pore formation, showed only a minor influence on the antimicrobial activity of nisin [93], but had a severe impact when the flexibility was reduced by introducing proline residues. In conclusion, a wide variety of nisin mutants has been obtained to date and their in-depth characterization has shed more light not only on the mode of action and properties of the peptide but also revealed substrate specificities of the nisin modification and transport machinery.

Use of lantibiotic enzymes for the production of stabilized bioactive peptides

It has been demonstrated that for modified peptide design different lantibiotic enzyme combinations can be exploited. For a long time, the NisBTC enzymes have been considered specific since many mutated nisin genes apparently did not lead to production of the corresponding mutant nisin peptides. Strains

Table 1. Nisin A/Z mutants and their characteristics.

Mutation	Gene name	Biological activity (relative to the wild type)	Physical properties	Characteristics	Ref.
S5T	*nisZ*	2–10-fold lower	NT	Dhb present in the final product instead of Dha	77
S5A	*nisZ*	NT	NT	No production/secretion	77
M17Q/G18T	*nisZ*	2–4-fold lower	Similar stability	This mutation resulted in production of two different species with different properties. Additional dehydrated amino acid was introduced	77
M17Q/G18Dhb	*nisZ*	Similar	Similar stability		77
S5A	*nisA*	2-fold lower	NT	Altering dehydrated residue	90
S33A	*nisA*	100-fold lower	NT	Altering dehydrated residue	90
S5A/S33A	*nisA*	100-fold lower	NT	Altering dehydrated residues	90
N27K	*nisZ*	Similar	Improved solubility	Charge alteration	103
H31K	*nisZ*	Similar	Improved solubility	Charge alteration	103
I1W	*nisZ*	Similar	NT	Fluorescent label	89
T2S	*nisZ*	Increased activity	NT	Dha present in the final product instead of Dhb	89
S3T	*nisZ*	Very low	NT	Alteration of dehydratable residue which takes part in the ring formation	89
K12P	*nisZ*	Similar	NT	Positive charge reduction	89
T13C	*nisZ*	Reduced	Reduction possible	Alteration of dehydratable residue which takes part in the ring formation	89
M17W	*nisZ*	Reduced	NT	Fluorescent label	89
M17K	*nisZ*	Reduced	Improved solubility	Lysine in ring 3	89
$NisA^{1-29}$	*nisA*	10-fold lower	NT	Proteolytically cleaved. All lanthionine ring present	91
$NisA^{1-20}$	*nisA*	100-fold lower	NT	Proteolytically cleaved. Ring D and E removed	91
$NisA^{1-12}$	*nisA*	Inactive, antagonistic growth inhibitory effects on nisin	NT	Proteolytically cleaved. Ring C, D and E removed	91
S5A	*nisA*	Similar activity to wild type in inhibiting vegetative cells; abolished activity to inhibit the spores outgrowth	NT	Altering dehydrated residue	92
V32E	*nisZ*	3–5-fold lower	NT	Influence the charge of the C-terminal part of nisin	94
V32K	*nisZ*	Similar	NT	Influence the charge of the C-terminal part of nisin	94
V32W	*nisZ*	3–5-fold lower	NT	Influence the charge of the C-terminal part of nisin	94
$NisZ^{1-32}$V32E	*nisZ*	3–5-fold lower	NT	Influence the charge of the C-terminal part of nisin	94
Residues 1, 4, 5, 6, 17, 21, 31, 32, 33, 34 were change to C	*nisZ*	Inactive or not produced/ secreted	NT	Introduction of additional cysteines residues	94
T13C	*nisZ*	100-fold reduced but only when reducing conditions are applied	NT	Introduction of additional cysteines residues	94
N20K	*nisZ*	Slightly decreased activity against Gram-positive species. Activity against Gram negative species	Improved solubility	Altering the hinge region by introducing positive charge	93
M21K	*nisZ*	Slightly decreased activity against Gram-positive species. Activity against Gram negative species	Improved solubility	Altering the hinge region by introducing positive charge	93

Table 1 *(Continued)*

Mutation	Gene name	Biological activity (relative to the wild type)	Physical properties	Characteristics	Ref.
N20E	*nisZ*	Inactive (low production)	NT	Altering the hinge region by introducing negative charge	93
N21E	*nisZ*	Inactive (low production)	NT	Altering the hinge region by introducing negative charge	93
N20V	*nisZ*	Very reduced	Heat and pH resistance comparable to the wt	Altering the hinge region	93
N20A	*nisZ*	Very reduced	Heat and pH resistance comparable to the wt	Altering the hinge region	93
M21K/Dhb/ K22G	*nisZ*	Very reduced	Heat and pH resistance comparable to the wt	Altering the hinge region	93
N20Q	*nisZ*	Slightly decreased	Improved stability in higher temperature or alkaline pH	Altering the hinge region	93
M21G	*nisZ*	Slightly decreased	Improved stability in higher temperature or alkaline pH	Altering the hinge region	93
N20H	*nisZ*	Slightly decreased	Heat and pH resistance comparable to the wt	Altering the hinge region	93
M21H	*nisZ*	Slightly decreased	Heat and pH resistance comparable to the wt	Altering the hinge region	93
K22G	*nisZ*	Slightly decreased	Heat and pH resistance comparable to the wt	Altering the hinge region	93
K22H	*nisZ*	Slightly decreased	Heat and pH resistance comparable to the wt	Altering the hinge region	93
N20K/M21K	*nisZ*	Slightly decreased	Heat and pH resistance comparable to the wt	Altering the hinge region	93
N20F/M21L/ K22Q	*nisZ*	Slightly decreased	Heat and pH resistance comparable to the wt	Altering the hinge region	93

Note: recently an array of mutants in ring A and ring B was reported [88b], which are not listed in above table.

containing disrupted *nisB* or insertion-inactivated *nisC* failed not only to produce nisin [31], but also to produce partially modified nisin precursors. Furthermore, NisT appeared to be unable to transport an unmodified His-tagged prenisin [28]. Taken together, these data suggested that the transporter NisT could only transport fully modified prenisin. It could, however, not be excluded that interaction of the unmodified prenisin with the remnant of disrupted or inactivated modification enzymes blocked export. Subsequent research has demonstrated that NisT and NisB have a broad substrate specificity [30, 39, 41] and that NisB, NisC, NisT and NisP can all function independently (Fig. 7) and this opened the way to design the modification and production of peptides by selected enzyme combinations.

Using plasmid-encoded enzyme combinations and designed leader peptide fusions, information has been reported on the substrate specificity of NisB, NisC, NisT and NisP. A leader-angiotensin (1–7) variant peptide was the first NisB-dehydrated, non-lantibiotic peptide that was not at all related to nisin [30]. This demonstrated that neither an identical peptide length of 34 amino acids nor a specific amino acid sequence was required for NisB to perform its enzymatic function on a leader peptide fusion. For the dehydratase of nisin, NisB, guidelines extracted from the *in silico* studies could be largely validated by experi-

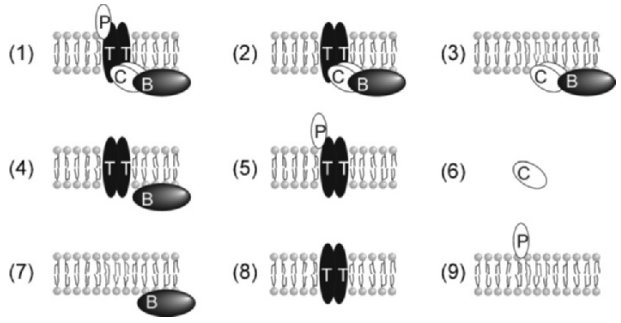

Figure 7. Dissection of the nisin modification enzyme complex into active subcomplexes and components. Case 1 is based on Siegers et al [25]; cases 2, 4, 5, 8, and 9 are based on Kuipers et al. [30]; case 3 and 7 are based on Kuipers [27]; case 6 is based on Li et al. [26].

ments on the modification of designed hexapeptides. For example, the peptide leader-ATVECK was dehydrated well by NisB, whereas leader-DTRICK was not [41]. In addition, it was found that serines were less readily dehydrated than threonines. A subsequent study, which was not based on all lantibiotic structures, but only on NisB-dehydrated substrates, demonstrated that hydrophobic amino acids as flanking residues of serines and threonines favored NisB-mediated dehydration [40]. Two and also one hydrophobic flanking residue(s) allowed in most cases NisB-mediated dehydration, but simultaneous flanking at both sides by hydrophilic residues appeared to preclude dehydration. Furthermore, negatively charged amino acids at the N-side appeared to preclude dehydration. Several therapeutic peptides of varying length and amino acid composition have been dehydrated by NisB [39]. A series of multiple threonines were also dehydrated as well as serines and threonines further away from the leader peptide than position 33. The feasibility of generating a library of dehydroresidue-containing peptides has been demonstrated [40]. An overview of dehydrated peptides is presented in Table 2.

NisC catalyzes the coupling of cysteines to the dehydroresidues formed in prenisin. In dehydrated prenisin, NisC regio- and stereospecifically catalyzes the cyclization of five rings. Rings A, B and C have different sizes, while the intertwined rings D and E have a similar size as ring B. Furthermore, the distance between each ring and the leader peptide, which is necessary for cyclization [26] probably because of a targeting and/or activation function, is different. In one study a truncated nisin fragment appeared to spontaneously cyclize forming a bridge between Dha5 with Cys11 [95].

Thioether rings have been introduced at different distances from the leader peptide than the distances found in prenisin [40]. These data on NisC-mediated cyclization were to a large extent in agreement with *in silico* analyses involving all lantibiotics that report abundance of specific amino acids at the N- and C-side of cysteines. In nisin cyclization reactions all occur by coupling of N-sided dehydroresidues to C-sided cysteines. Peptide sequences of ring B and ring E alone appear to contain sufficient information to direct stereospecific ring closure [96]. In the case of ring A, a precursor peptide, which contained two dehydroamino acids, underwent cyclization that was totally regioselective, although not totally stereoselective. For ring A

Table 2. (Semi)-designed model peptides and analogs of therapeutic peptides that are modified by the modification enzyme(s) of nisin A, provided that N-terminally the nisin A leader peptide is present. Serines and threonines that are dehydrated by the dehydratase NisB are in bold. Sequences that are analogs of therapeutic peptides are underlined.

Sequence	Reference
NR**S**YICP	30
A**T**VECK	41
A**S**VECK	41
A**T**VWCE	41
A**S**VWCE	41
I**T**PGCK	41
ICPG**T**K	41
I**T**RICK	41
DSRWARVALIDSQKAAVDKAI**T**DIAEKL	41
V**T**LR	40
I**S**ARAD	40
N**T**LRAS	40
V**S**LLAR	40
L**T**AEAR	40
P**T**NVAG	40
P**T**RPAW	40
P**T**RDAL	40
R**T**WPAK	40
F**T**V**S**AR	40
A**TT**LAL	40
P**ST**IAI	40
A**T**KGLP**S**RHVLL	40
A**ST**PAW	40
V**TT**HAI	40
C**TS**VAF	40
R**TS**HAA	40
L**S**LPAA	40
L**S**ANAG	40
V**S**NRAS	40
G**T**VRAS	40
R**T**VAAV	40
R**T**VAAG	40
W**S**ELAG	40
I**S**REAF	40
H**T**DLAD	40
K**S**HYAM	40
I**TTTTT**	40
SY**S**MECFRWG	39
A**T**FQCAPRG	39
AY**T**QNCPRG	39
I**TS**I**S**RA**S**VA	39
IAAIARY**T**GFC	39
QHW**S**YGCRPG	39
DRV**T**IHC	39
YA**S**HFGPLGWVCK	39

there was still some degree of stereospecificity since the isomers were produced in a three to one ratio. The amino acid sequence of ring A might contribute to the cyclization by having a spontaneous tendency to fold in a conformation that facilitates cyclization [96]. This might be a phenomenon crucial in the NisC-mediated formation of thioether rings with more than two residues under the sulfur bridge. Molecular modeling might constitute an important tool in the prediction of sequences that might be cyclized by NisC.

Design of peptides in such a way that they are efficiently transported cannot yet be based on a large body of information. Interestingly, significant differences in the transport efficiency of a series of designed hexapeptides were observed [41]. The peptide leader-ASVECK appeared to be much less efficiently exported than leader-ITRICK. It is tempting to speculate on a favorable effect of positively charged amino acids on the transport efficiency. Some leader fusion peptides, like a 13-amino acid erythropoietin fragment [39] are clearly inefficiently transported, while a peptide of 28 amino acids, obtained by coincidence, reached well-detectable extracellular levels [41].

Interestingly, NisB-catalyzed dehydration of serine and threonine residues in peptide sequences can still take place even when the nisin leader was preceded by a Sec or Tat signal sequence [27]. Using a construct composed of a Sec signal sequence followed by the nisin leader peptide and a peptide of interest, export *via* the Sec-translocon was observed in a strain lacking the lantibiotic transporter NisT. Some peptides were even secreted to higher levels than observed with NisT-mediated transport. In view of the observation that serines and threonines that are further away from the nisin leader than position 33 can also be modified, the export of modified peptides and possibly even modified proteins *via* the Sec system or Tat system opens large possibilities for the design of a broad range of (poly)peptides that are both modifiable and exportable.

NisC could cyclize the dehydrated prepeptide NisA when preceded by the Sec signal sequence as evidenced by the generation of antimicrobial activity. As a result of NisC-mediated modification, secretion of (methyl)lanthionines-containing prenisin *via* the Sec pathway appeared to be impossible. The dimensions of the solvent accessible surface of a completely modified nisin are about $2.2 \times 2.7 \times 4.2$ nm, whereas molecular dynamics simulations suggest that the monomeric SecY pore has a maximal pore diameter of about 1.6 nm [97]. Hence the completely modified nisin molecule might be too large to fit in the SecY pore. Translocation of peptides with a single lanthionine *via* the Sec pathway could, however, not be excluded. Since prenisin can be modified by NisBC when preceded by a Tat signal sequence it might be that fully modified prenisin and other designed thioether-ring-containing NisBC-modified peptides can be translocated *via* the Tat system. To verify this idea, it will be required to reconstitute the Tat pathway in *L. lactis* by heterologously introducing a system from another bacterial host or express the nisin modification enzymes and leader constructs in an alternative host with an endogenous Tat system. Alternatively, NisB-modified peptides can be produced *via* either the Sec or Tat system, and subsequently cyclized by *in vitro* action of NisC. Taken together, these findings open large possibilities for the design of peptides with respect to both modification and export [27].

Prospects

Nisin is applied extensively for food preservation. Using the increased knowledge on the mechanisms of action of nisin and utilizing the improved expression systems, mutants with enhanced efficacy against pathogenic or food spoiling bacteria can be obtained by engineering. The recently developed two-plasmid expression system [41] allows significant production levels, while maintaining the leader peptide attached to the modified peptide. Since the leader peptide keeps nisin inactive, nisin mutants can be generated using this expression system without limitations by autotoxicity to the producer. Indeed, enormous possibilities for engineering nisin exist [88b]. Alternatively, heterologous expression may raise the yield of nisin variants and/or facilitate engineering. In this respect it is interesting to note that the nisin gene cluster has been successfully inserted in the chromosome of *Bacillus subtilis* 168 [98].

Generally, it is not recommended to use the same compound for both food conservation and for antibiotic treatment. Strictly separate from the application of food preservation, sufficiently different engineered nisin variants may have potential as novel antibiotics. Due to the spread and increase of resistance to classical antibiotics there is a need for new antibiotics. As nisin's highest stability is at around pH 2.5, its ability to act against the Gram-negative *Helicobacter pylori*, which causes gastric ulcers, received attention for many years. Engineering nisin may now allow sufficient efficacy against this pathogen.

The obtained insight on the distinct mechanisms of action of nisin by Breukink and co-workers [85] may allow the development of nisin variants that are optimized with respect to only one of the two

mechanisms. Indeed, one nisin hinge mutant (N20P M21P)-nisin [84, 99] was still bactericidal despite its incapability to form pores in the target cells. Such selective optimization may further extend the spectrum of activity or enhance the activity against selected target organisms.

The demonstration that NisB, NisC, NisT and NisP can function independently, is of great importance for further characterizion of these unique enzymes and for unraveling the detailed features of their mechanism of action. The obtained crystal structure and *in vitro* reconstitution of cyclase activity of NisC is an important step forward for further detailed mechanistic characterization [26]. For NisB no information is as yet available about its mechanism. *In vitro* reconstitution of NisB activity and crystallization of NisB are presently major goals to reach. The recent finding that NisB can act in the absence of NisC and NisT may animate renewed efforts to reach *in vitro* activity of this intriguing dehydratase. A possible mechanism might involve kinase activity of NisB followed by dehydration. Yet neither phosphorylated substrate intermediates nor an ATP binding site in NisB have been found thus far. NisB, NisC, NisT and NisP, being able to act independently, largely facilitate effective engineering to ultimately modulate the substrate specificities.

Intriguing questions concerning the role of the leader peptide in the modification processes remain to be answered. In view of the variety of substrates of leader peptide fusions for NisB, NisC and NisT, it seems that the leader peptide interacts with these enzymes, but interactions of the leader peptide with the substrate other than being peptide-bonded to it, might not be required in the modification and transport processes.

Various applications of the nisin modification enzymes can be expected. NisP appears to be specific for thioether-ring-containing prenisin. It might be an option to engineer a NisP variant that recognizes a specific cleavage site. Contrary to what has been thought for a long time, NisT has a broad specificity. Many peptides are efficiently exported. NisT might be useful for the production of unmodified peptides. Peptides that have been dehydrated by the action of NisB can be exported *via* NisT but also *via* the Sec system and likely other non-lantibiotic export systems. Dehydroalanine and dehydrobutyrine, which are formed by NisB, are two new peptide constituents, which expand the range of peptide building blocks. (Poly)dehydrobutyrine sequences can be utilized as starting points for synthesis of other non-natural amino acid residues, or serve as coupling sites in further organic synthesis. Due to their particular planar shape and reactivity, dehydroresidues can be relevant constituents of biologically active peptides. Therefore, it can be envisaged that a dehydroresidue-containing peptide library could be screened for peptides with desired properties.

Many applications of designed peptides that are modified by NisB and NisC are possible. The recent development of an expression system, involving the pIL-plasmid encoded *nisBTC* genes and the pNZ8048-encoded leader peptide construct, allows for the efficient production of thioether ring containing (therapeutic) peptides. Thioether bonds are significantly more stable than disulfide bonds and peptide bonds [100]. At present some therapeutic peptides are known into which a thioether ring has been chemically introduced. In the case of thioether enkephalin, this has led to a dramatic increase in resistance against proteolytic degradation. In a defined protease mix enkephalin without a thioether ring was broken down by 50 % within 7 min, whereas after incubation for 24 h, no breakdown of thioether-ring-containing enkephalin was observed. The *in vivo* efficacy of thioether enkephalin turned out to be 10 000 times higher than morphine [101]. Enhanced *in vivo* stability was also observed for a thioether-ring-containing somatostatin analog. Furthermore, the specificity of the receptor interaction of the thioether-ring-containing variant was strongly enhanced.

Within prenisin NisC cyclizes five different thioether rings. NisC can catalyze the cyclization of many peptides that are not related to nisin [102]. NisC can also introduce multiple thioether rings in peptides that are not related to nisin. In the absence of NisC, peptides containing dehydroalanines can easily cyclize by spontaneous reaction of these residues with cysteines. This might be of some use in peptides that escape NisC-mediated cyclization, for instance in the case of strongly constrained thioether rings that would have only one or too many amino acid(s) under the sulfur bridge. However, the latter process might in many cases yield racemic mixtures rather than a pure stereoisomer. The introduction of thioether rings by the consecutive action of NisBC will generally lead to strongly enhanced resistance against proteolytic degradation (Biomade Foundation). This opens up the perspective of the stabilization of many therapeutic peptides by the introduction of thioether rings. Such stabilization will strongly enhance the therapeutic potential of the peptide as a result of enhanced intrinsic stability and increased half-life time *in vivo*. The thioether-ring-enhanced intrinsic stability suggests the possibility of using lower doses and/or a reduced frequency of administration of stabilized therapeutic peptides. Oral delivery may become possible for some thioether-ring-containing peptides. Oral uptake is less invasive and more patient-friendly than injection. Hence, in view of the number of

therapeutic peptides and their economic relevance, the stabilization of these peptides by the consecutive action of NisB and NisC may have a major impact.

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