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Protein Engineering and Biosynthesis of Nisin and Regulation of Transcription of the Structural *nisA* Gene

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ABSTRACT

The lantibiotic nisin, produced by Lactococcus lactis, *is an antimicrobial peptide characterized by the presence of three unsaturated amino acid side chains (two dehydroalanines and one dehydrobutyrine) and five (β-methyl)lanthionine rings, which are formed post-translationally. Nisin is widely used in the food industry as a preservative, since it inhibits the growth of unwanted gram-positive bacteria. One of the objectives of our research is to get insight in the complex biosynthesis and regulation of production of nisin. The structure and function of several biosynthetic genes were studied by making gene disruptions and by subsequently investigating their effects on nisin gene regulation, biosynthesis, secretion and immunity. An exciting finding is that nisin itself, when added to the culture medium, can induce the transcription of its own structural gene. Another goal is to design and produce altered nisin molecules with desirable properties by protein engineering. In addition to previously reported mutant nisins with improved stability, solubility or activity, recent results on the protein engineering of residues Ile1, Dhb2, AlaS3, Lys12, AbuS13, Met17, Asn20 and Met21 indicate that (i) residue 1 can be replaced without dramatic loss of activity; (ii) the presence of a Thr residue at position 2 significantly lowers the antimicrobial potency, whereas the presence of a Dha residue at position 2 improves activity; (iii) the replacement of AlaS3 by AbuS leads to a dramatic loss of activity, probably due to a conformational change in the first lanthionine ring; (iv) the integrity and hydrophobicity of ring 3 are important for antimicrobial activity; and (v) the hinge region between rings 3 and 4 is important but not essential for antimicrobial activity.*

INTRODUCTION

Nisin belongs to the rapidly expanding family of lantibiotics, a group of anti-microbial peptides characterized by their low molecular weight and by extensive

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posttranslational modifications (Gross & Morell, 1991; Hurst, 1981; Jung, 1991). Modified residues in nisin comprise 13 out of 34 amino acids. These modifications include dehydration of serine and threonine residues, resulting in dehydroalanine and dehydrobutyrine residues, respectively. Five out of eight of these dehydrated residues subsequently link their C_β atom to the sulfur of five cysteine residues, resulting in the characteristic (β-methyl)lanthionine rings. These modification reactions are probably enzyme-catalysed and likely candidates for these enzymic reactions have been proposed (Engelke *et al.*, 1992; Kuipers *et al.*, 1993a; Van der Meer *et al.*, 1993; Engelke *et al.*, 1994). Figure 1 shows a schematic representation of the modification events taking place to yield nisin Z, a natural variant of nisin A that contains a histidine residue at position 27 instead of Asn (Mulders *et al.*, 1991; De Vos *et al.*, 1993). The structural genes for nisin A and Z have been cloned and sequenced (Buchmann *et al.*, 1988; Mulders *et al.*, 1991), and both genes are wide spread in nature (De Vos *et al.*, 1993). Both peptides have very similar antimicrobial activities, although nisin Z forms larger halo's in bioassays using solid media, probably caused by better diffusion.

Nisin is widely used as a food-preservative in over 45 countries throughout the world (Delves-Broughton, 1990; Vandenburgh, 1993; De Vos, 1993). There are several reasons for this broad application: (i) nisin was the first antimicrobial peptide to be discovered and has a long history of safe use; (ii) nisin is produced by several strains of the food-grade organism *L. lactis*, which allows the application of both the (partly) purified peptide as well as the whole bacterium; (iii) nisin has a wide host-range towards gram-positive bacteria, albeit that the sensitivity of individual species can vary within three orders of magnitude; (iv) nisin has a high antimicrobial activity compared to other bacteriocins, having a minimal inhibitory concentration (MIC) value of 10 μg L^{-1}, corresponding to a concentration of 3 nM against the most sensitive organisms (De Vos *et al.*, 1993); and (v) it is one of the best characterized antimicrobial substances, with respect to the biochemical, genetic, microbiological and biophysical properties.

In the last few years the organization of the nisin gene cluster, the function of the encoded proteins in biosynthesis, nisin precursor-secretion and immunity, and the way by which the expression of the nisin genes is regulated, have been studied. It is now also feasible to perform protein engineering studies of nisin to study the requirements for the complicated biosynthesis of nisin. By exchanging residues involved in posttranslational modification reactions one can gain insight in the importance of these residues for processes such as modification, secretion and even immunity. Protein engineering can also be applied to reach a more practical goal, i.e. improving relevant properties of the molecule. In this context one can think of improving the specific activity against notorious food-pathogens, increasing the stability of nisin at neutral pH and increasing the solubility of nisin at neutral pH and high salinity. However, without a thorough knowledge of the mode of action at the molecular level, it is hard to deduce which residues are involved in specific interactions with membrane phospholipids and cell-wall components. For this reason further research is required to elucidate the molecular mechanism of action of nisin, the phospholipid composition of membranes of target bacteria, and the composition and thickness of their cell walls. As an alternative for rational design of nisin mutants with improved properties, the use of random mutagenesis of the structural nisin gene can be considered, provided that a good system for phenotypic screening of the desired feature is available.
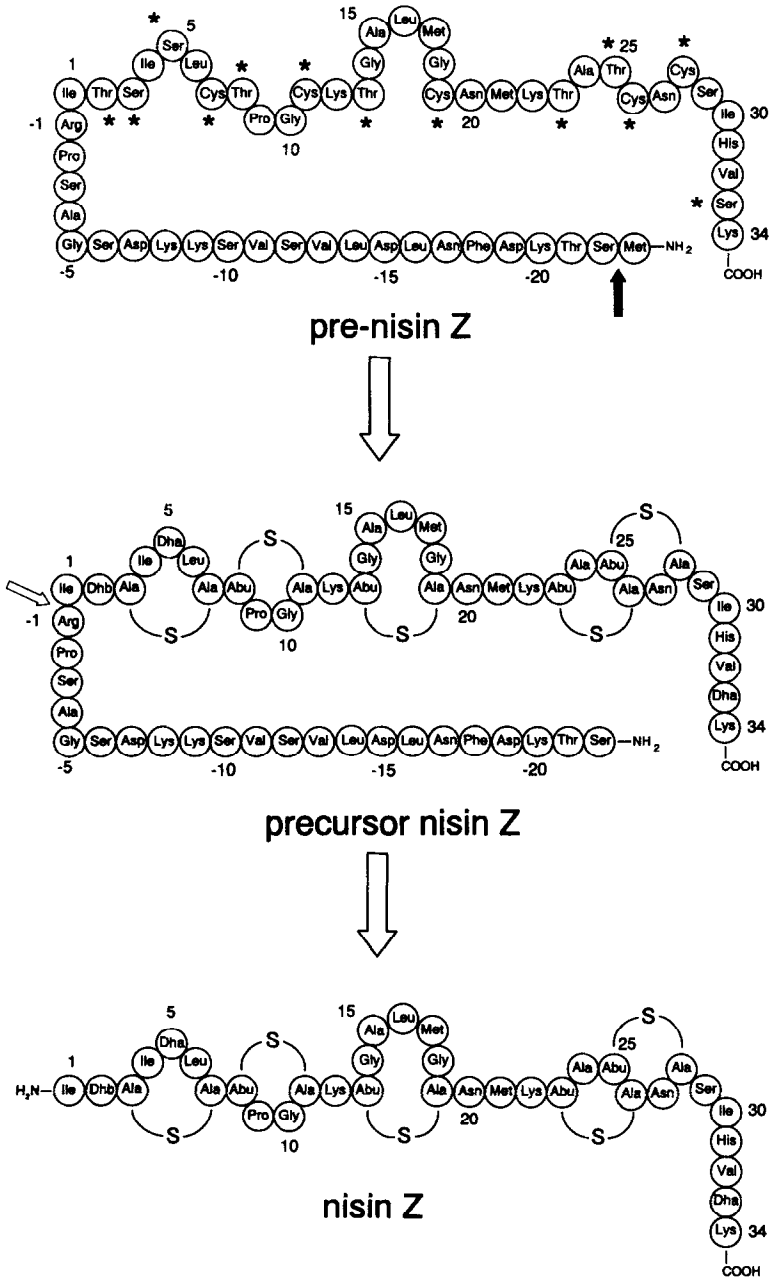
Fig. 1. Intermediates in the biosynthesis of nisin Z: pre-nisin Z, the primary translation product of the *nisZ* structural gene, and precursor nisin, an exported intermediate containing all modifications. Asterisks: residues that are modified; black arrow: cleavage of N-terminal methionine; small open arrow: position of cleavage of the leader peptide by the extracellular protease NisP.

This paper describes the recent advances at NIZO on the characterization of the nisin biosynthetic gene cluster, the role of a two-component regulatory system and of nisin itself in transcriptional activation of the nisin genes, the development of expression systems for (mutant) nisin genes, the application of site-directed and random-mutagenesis and the purification and characterization of engineered nisins.

ORGANIZATION OF THE NISIN GENE CLUSTER: BIOSYNTHESIS AND GENE REGULATION OF NISIN

A cluster of 8 genes (*nisABTCIPRK*), located on transposon Tn5276 (Rauch & de Vos, 1992) of *L. lactis* NIZO R5, encodes proteins involved in biosynthesis, regulation, secretion and immunity (Engelke *et al.*, 1992; Kuipers *et al.*, 1993a; Van der Meer *et al.*, 1993; Engelke *et al.*, 1994) (Fig. 2). The NisB and NisC proteins are likely to be involved in dehydration and thioether bridge formation, while the NisT protein belongs to the ABC-family of transport proteins. NisI has the characteristics of an extracellular lipoprotein and was shown to be involved in producer immunity against nisin (Kuipers *et al.*, 1993a; Engelke *et al.*, 1994). NisP is a leader peptidase responsible for extracellular processing of precursor nisin (Van der Meer *et al.*, 1993; Siezen *et al.*, 1995), while NisR and NisK are putative regulators of expression of the gene cluster (Van der Meer *et al.*, 1993; Engelke *et al.*, 1994). Recently, it was found that a mutation in the structural nisin gene (*nisA*) abolished its transcription. This was observed in *L. lactis* NZ9800, a plasmid-free strain harboring Tn5276 with a disrupted *nisA* gene (this gene has a 4 bp internal deletion and is named Δ*nisA*), which cannot produce nisin. Remarkably, Δ*nisA* transcription could be restored by adding nisin to the growth medium, suggesting that the presence of nisin is a signal to be transduced via specific regulator proteins to activate transcription of its own structural gene (Kuipers *et al.*, 1995). Likely candidates for the signal transduction are the proteins encoded by *nisR* and *nisK*, which belong to the family of two-component regulatory proteins. NisR could act as the response regulator, which actually activates transcription, while NisK could act as the sensor for (mutant) nisin and is a histidine kinase.

Gene disruptions were made in most of these genes, i.e. *nisA*, *nisB*, *nisT*, *nisI*, *nisAIP* and *nisK* (Kuipers *et al.*, 1995). In all cases nisin production and transcription of the *nisA* gene were abolished, showing the involvement of these genes in nisin biosynthesis. An exception was the case of *nisI* disruption, which yielded bacteria which were still able to produce nisin, although the amount of nisin
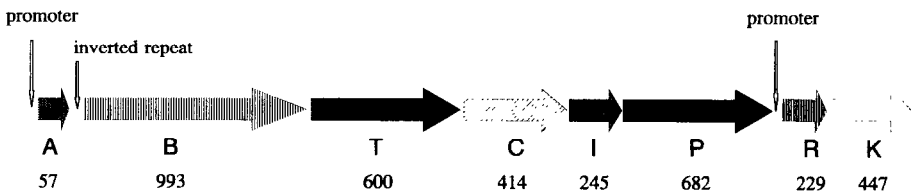

Fig. 2. Organization of genes involved in nisin biosynthesis, secretion, immunity and gene regulation. The size of the encoded proteins is given.

produced had decreased significantly. Interestingly, also the maximum level of immunity was reduced in the strain carrying a deleted *nisI* gene, which indicates that the presence of NisI is necessary for maximum producer self protection. In all other cases the immunity level and transcription of the structural gene could be restored to almost wild-type level by the addition of small amounts of nisin, except in the case of Δ*nisK*, whose Δ*nisA* transcription and immunity were not inducible anymore by nisin (Kuipers *et al.*, 1995). This suggests that NisK is the regulator protein that can sense the presence of nisin in the extracellular medium, and can transduce the signal, probably to the intracellular response regulator NisR, which in turn would activate transcription of the structural *nisA* gene. This system has been exploited to achieve heterologous gene expression in a strictly controlled way, by development of expression vectors in which the *nisA* promoter fragment is cloned in front of the gene of interest and using *L. lactis* NZ9800 as a host. Expression of the gene is negligible without the presence of nisin, whereas upon addition of small amounts of nisin to the culture medium overproduction of the desired protein is obtained.

PROTEIN ENGINEERING OF NISIN

Expression systems for structural nisin genes

Several expression systems for wild-type and mutated nis genes have been reported. The first system makes use of the modification and translocation machinery present in a plasmid-free nisin A producing *L. lactis* strain named NZ9700, harboring a single intact copy of *Tn5276* (Kuipers *et al.*, 1991; Kuipers *et al.*, 1992; Kuipers *et al.*, 1993a). Simultaneous production of nisin A and the other (mutant) nisin is observed when this strain is complemented with a plasmid harboring nis genes differing from *nisA* under control of the efficient *lac* promoter of the *lac* operon of *L. lactis* (Van Rooijen *et al.*, 1992). The shuttle vector pNZ123 was used and examples of nisin expressing constructs are pNZ9010 (*nisA*), pNZ9013 (*nisZ*) and pNZ9019 (M17Q/G18T nisZ) (Kuipers *et al.*, 1991a, Kuipers *et al.*, 1992). In almost every case the two nisin species produced can be separated by reversed-phase HPLC, yielding the desired mutant peptide (Kuipers *et al.*, 1991a, Kuipers *et al.*, 1992). The second system uses a similar strain as described above, with the distinction that strain NZ9800 is used, in which the chromosomal *nisA* gene is disrupted by a 4 bp deletion. This strain cannot produce nisin, but can be complemented by the introduction of plasmids carrying a (mutant) nisin gene (Kuipers *et al.*, 1993a). This system offers the advantage of producing nisin mutants without the simultaneous production of native nisin A. A third system was developed in which the *nisABTCI* genes are present on plasmid pNZ9000 (Kuipers *et al.*, 1991a), a pIL253 (Simon & Chopin, 1988) derivative. This plasmid is able to complement strain NZ9800 as well as the nisin-deficient strain *L. lactis* NIZO R520, a mutant of NIZO R5 (Kuipers *et al.*, 1991a). However, because of the large size of the plasmid, and because it cannot replicate in *E. coli* this plasmid is less suitable for manipulation than the pNZ123 derivatives. Independently, another expression system for nis genes was developed, based on a disruption of *nisA* on the chromosome by introduction on the chromosome of the insertion element IS905 (Dodd *et al.*, 1992). Due to promoter

activity of a region in the IS element the expression of genes located downstream of the inactivated nisA was secured. Recovery of nisin production was achieved by using a plasmid-encoded (mutant) *nisA* gene (Dodd *et al.*, 1992).

Site-directed mutagenesis and random mutagenesis methods

Several methods for site-directed mutagenesis exist, but in the last 5 years muta-genesis using PCR has become the method of choice, since it obviates the use of *in vivo* amplification steps, e.g. by using phage M13, and because PCR is fast, reli-able, cheap and yields a high frequency of desired recombinants. The preferred method to generate nisin mutants is the so-called megaprimer method (Landt *et al.*, 1990). During the course of this work it became apparent that frequently untemplated addition to the 3' end of the mutagenic primer occurred, mostly an A nucleotide, that gave unwanted mutations after use of this fragment as a megaprimer. This problem was efficiently solved by designing the mutagenic primer in such a way that the nucleotide preceding the ultimate 5' residue in the corresponding template sequence was a T residue, which results in a wild-type sequence even when an A nucleotide is added in the complementary strand during amplification (Kuipers *et al.*, 1991a). Another group (Dodd *et al.*, 1992) has succesfully used PCR-mediated overlap extension (Ho *et al.*, 1989) for site-direc-ted mutagenesis of the nisin structural gene.

For random mutagenesis essentially two methods are very suitable. First, one can make use of doped oligonucleotides either as PCR primer or for direct clon-ing as a cassette. The other method is based on the intrinsic error frequency of *Taq*-polymerase in PCR, which can be increased by several factors, e.g. Mg^{2+} concentration or addition of Mn^{2+} to the reaction mixture. This mutation frequency can further be enlarged by using dITP in four different PCR reactions, each limited for one of the usual nucleotides, to enforce incorporation of dITP at random positions (Spee *et al.*, 1993). This will then give rise to mutations in the next PCR cycles. This method was developed using the *nisZ* gene as a template and resulted, after cloning of the PCR fragments in suitable vectors, in lacto-coccal colonies of which more than 50% contained nisin fragments that had one or more point mutations in the DNA.

Purification and characterization of mutant nisins

Wild-type and mutant nisins can be purified after production in a pH-regulated 10 L fermentor, by a two-step procedure involving hydrophobic interaction chromatography and reversed-phase HPLC (Kuipers *et al.*, 1992). This procedure typically yields 20–200 mg of mutant nisin, depending of the characteristics of the mutant and the efficiency of biosynthesis. In some cases the mutant nisin-produ-cing *L. lactis* has a markedly decreased growth rate, which could be caused by inefficient modification reactions resulting in accumulation of precursor nisin in the cells, by inefficient secretion, or by reduced immunity to the mutant nisin.

The first engineered nisins reported were nisin Z species in which dehydrated residues (or their precursors Ser or Thr) were either introduced (M17Q/G18T and M17Q/G18Dhb) or replaced (Dha5Dhb) (Kuipers *et al.*, 1991b, Kuipers *et al.*, 1992). In all cases a 2-to 10-fold reduction in antimicrobial activity against three indicator strains was observed, except for M17Q/G18T nisin Z, which had

twice the activity of wild-type nisin Z against *Micrococcus flavus*, but not against *Bacillus cereus* and *Streptococcus thermophilus*, indicating that a change in specificity occurred in this mutant. Mutant Dha5Dhb nisin Z was shown to possess a higher resistance against chemical degradation at low pH values than wild-type nisin Z (Rollema *et al.*, 1995). The primary structures including the modified residues of all three mutants were confirmed by ^{1}H NMR (Kuipers *et al.*, 1991b).

Using a different expression system, interesting nisin mutants were also obtained by Dodd *et al.* (1992), i.e. H27Q, H27Q/V32I, H27Q/T23S and Δ21-34 nisin A. The strains producing the first three mutant nisins were shown to exhibit some antimicrobial activity using a plate-diffusion or a colony-overlay assay, although the amount of mutant nisin was not quantified and hence the specific activity could not be determined.

Recently, we have produced new nisin mutants, which include I1W, T2S, S3T, K12P, T13C, M17W, N27K and H31K nisin Z, and N20P/M21P and ΔN20M21 nisin A. An overview of the position and type of the mutations in nisin Z is given in Fig. 3. Introduction of the bulky Trp residue at position 1 reduces the activity of nisin but not dramatically. This mutant will be useful for studying nisin-membrane interactions by fluorescence spectroscopy. Mutant T2S gave rise to a Dha residue at position 2 and interestingly this mutant nisin displayed a twofold higher antimicrobial activity against several target organisms, e.g. *Micrococcus flavus* and *Streptococcus thermophilus*. In contrast, the S3T mutation led to a β-methyllanthionine residue in ring 1, instead of the original lanthionine, and a dramatically decreased antimicrobial activity. Mutant K12P nisin Z showed similar antimicrobial properties as wild-type, indicating that a positive charge in the first half of nisin is not essential for nisin action, although it could play a role in enhancing the solubility of nisin. A mutant in which Thr13 was replaced by Cys gave rise to a nisin species with a disulfide bond between residues 13 and 19, significantly lowering the antimicrobial activity. Upon reduction of this disulfide bond by DTT, almost complete loss of activity was observed. This indicates that the integrity of the third ring structure contributes to the antimicrobial activity. Mutant M17W nisin Z was produced with the purpose of studying the binding affinity of nisin to lipid–water interfaces using Trp as a fluorescent probe. Pre-
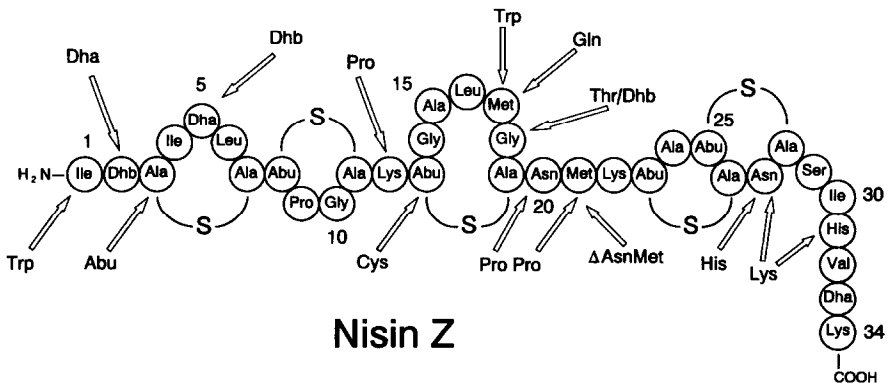

Fig. 3. Position and type of mutations in nisin Z.

liminary results indicate that this nisin is less active than wild-type nisin, but that it can efficiently bind to micelles of *n*-dodecylphosphocholine (DPC) and to small unilamellar vesicles composed of different zwitterionic and negatively charged phospholipids (Gallay & Kuipers, unpublished results). The two mutations at positions 20 and 21 were made to restrict the mobility of the flexible hinge region (Van de Ven *et al.*, 1991) between rings 3 and 4. Preliminary characterization showed that the two prolines indeed affected the activity of nisin, while a deletion of the two residues severely lowered the antimicrobial activity. A possible explanation for the latter phenomenon is that rings 4 and 5 are in another orientation relative to the first half of nisin, thereby destroying the amphipathic nature of the peptide. Two mutants were made with the objective of increasing solubility at neutral and higher pH, i.e. N27K and H31K. Both mutants indeed showed a 4-to 8-fold higher solubility at pH 7, which can be important when nisin is being applied at this pH in the presence of high salt concentrations (Rollema *et al.*, 1995). Moreover, mutants were produced by random mutagenesis (Spee *et al.*, 1993) and include L16P, M17V, M21L, T25A and I30T. These mutant nisins await further detailed characterization, but preliminary results indicate that mutants L16P, M17V, M21L and I30T are produced, secreted and active (Kuipers *et al.*, unpublished results).

Protein engineering was also applied to investigate the function of residues in the leader peptide of nisin (Van der Meer *et al.*, 1994). More than ten mutations were generated at different positions in the leader peptide, with the purpose of altering the character of these residues. Mutations were applied both in the conserved region occurring in the leader peptides of various lantibiotics between positions to 18 and to 14 (consensus FNLDV), at or near the processing site (residues at positions to 4, to 2 and to 1) and in other non-conserved residues. Mutations in non-conserved residues did not affect nisin biosynthesis or export, whereas mutations in the consensus sequence strongly affected either nisin biosynthesis or export. Mutations near the processing site (residues to 4 and to 1, but not residue to 2) did not affect nisin biosynthesis or export, but they inhibited processing by the leader peptidase NisP, resulting in the secretion of a fully modified nisin precursor (Van der Meer *et al.*, 1994). These results show that the leader peptide indeed is important for nisin biosynthesis, especially the conserved residues located between positions to 18 and to 15. A mutant in which the whole leader peptide was exchanged by the subtilin leader peptide, was also fully modified and secreted as a precursor, indicating that the 43% of non-identical residues in the two leader peptides are not crucial for nisin biosynthesis (Kuipers *et al.*, 1993b).

DISCUSSION AND PERSPECTIVES

The genes involved in the complex biosynthesis of nisin have been identified and their (in some cases putative) functions have been determined. Interestingly, nisin biosynthesis appears to be autoregulated via signal transduction, which is a completely new mechanism in both prokaryotes and eukaryotes (Kuipers *et al.*, 1995a). The expression of the genes downstream of *nisA* is probably co-regulated by limited read-through. The nisin promoter is able to direct the expression of homologous and heterologous genes to a high level, provided that *nisR* and *nisK* are present. Expression of these genes is fully dependent on the presence of nisin

in the culture medium. This stringently controlled system has great advantages for the controlled expression of many genes because it is induced by a food-grade molecule. Other advantages of the system are that small amounts of inducer are sufficient (<0.01 mg L^{-1}) and that a simple fermented product containing nisin can be used as inducing material, which makes it inexpensive (Kuipers *et al.*, 1995a).

More than 30 mutants of nisin have already been generated and characterized by us. Although almost every mutation has resulted in a decreased antimicrobial activity and only a few (T2S, M17Q/G18T) show increased activity against some target strains, these studies have provided a wealth of information on fundamental aspects of nisin biosynthesis, secretion and processing. Mutations in non-standard residues have been produced, thereby expanding the possibilities of protein engineering. Mutant nisins with enhanced solubility (H27K and H31K) have been obtained as well as more stable ones (Dha5Dhb) (Rollema *et al.*, 1995). Moreover, information on the importance of certain characteristics of specific residues for activity has been gained, such as the importance of Dha5 and the flexibility of the hinge region for activity. What lacks at the moment is a detailed insight in the molecular interactions governing the action of nisin. Especially the characterization of membranes of target organisms in relation to nisin sensitivity is an urgent requirement for optimization of the action of nisin. Another problem is related to the limited knowledge about the molecular mechanism underlying immunity: several mutants might be lethal to the producing organism. Development of an *in vitro* modification system could provide a solution for this problem. Another alternative to circumvent the problem of reduced immunity to mutant nisins would be to express the mutants with an additional mutation at residue -4, in which case the mutant will be secreted as an inactive precursor protein that can be processed *in vitro* with trypsin.

Random mutagenesis seems a powerful approach in generating nisins with desired properties, provided that an adequate and sensitive phenotypic screening method is available. More futuristic goals to be reached are the development of newly designed peptides with posttranslational modifications for pharmaceutical use, the use of nisin as a vehicle for the secretion of other (non-lantibiotic) peptides, and combining functional domains of different lantibiotics into one peptide to broaden the antimicrobial spectrum.

ACKNOWLEDGEMENTS

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