Studies of producer self-protection and nisin biosynthesis of *Lactococcus lactis*

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Doctoral dissertation

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Summary

Just before the discovery of penicillin by Fleming, reports in the literature appeared that described potent antimicrobial substances produced by lactic acid bacteria. It was found that these substances were specifically active against a wide range of other gram-positive bacteria. These characteristics meant that these compounds were attractive candidates for application in either food preservation e.g. by preventing food spoilage or by inhibiting growth of food pathogens, or for pharmaceutical use, e.g. to prevent or fight infections in humans or animals.

In the late sixties and early seventies, it was shown that part of the inhibitory activity of some lactic acid bacteria was due to the production of a special class of antimicrobial peptides today called lantibiotics. After the finding that lantibiotics are ribosomally synthesized peptides, a wealth of novel information was generated in the late eighties and early nineties by sequencing several of the gene clusters involved in the biosynthesis of various lantibiotics including the gene clusters for nisin. The biological system behind production, secretion and self-defence of nisin has proven to be both extensive and delicately regulated, requiring the interplay between at least eleven genes, namely *nisA/Z/QBTCIPRKFEG*.

In this work regulation and biosynthesis of nisin was studied and the role of NisI in nisin immunity evaluated. In order to analyse the biosynthesis of nisin, mutations in the *nisZ, nisB and nisP* genes of the *nisZBTCIPRK* operon were made by gene replacement or with the integration of a plasmid. The mutations caused a drastic decrease of the transcription from the promoters upstream of the *nisZBTCIPRK* and *nisFEG* operons resulting in loss of nisin production and nisin immunity. Adding nisin externally to these mutant strains partly restored the transcriptional activity and the tolerance to nisin, but not the ability to produce active nisin. Our results show that the *nisZBTCIPRK* operon and the *nisFEF* operon are positively autoregulated via nisin and are in the same regulon.

The role of NisI protein in the nisin immunity was studied by protein expression, labelling, immuno blotting and *in vitro* interaction methods. The results showed that NisI could exist in two forms i.e. a membrane-bound and a soluble form. NisI can interact *in vitro* with purified nisin and might have two biological functions - as an immunity protein and as an enhancer of the activity of nisin.

The function of NisP for the maturation of nisin was studied by analysing the *nisP* mutant strain. It was shown that NisP is needed to cut the leader part of prenisin to attain the biologically active nisin.

The posttranslational modifications of nisin biosynthesis were studied by analysing the maturation of a His-tagged nisin precursor in *nisB* and *nisC* mutant strains. Mass analysis of the purified precursor showed that no modifications of the nisin precursor occur in the *nisB* mutant strain, whereas dehydration seems to occur in the *nisC* mutant strain, which does contain an intact NisB enzyme. This suggests that NisB is responsible for the first step in nisin maturation, e.g. dehydration of the nisin precursor and NisC is involved in the formation of thioether bridges.

Key words: nisin, lantibiotic, immunity, biosynthesis, bacteriocin, gene function, food preservation

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This dissertation is dedicated to the memory of my parents, Leena and Elmar.

Kuopio, June 2004

Olli Koponen

LIST OF ORIGINAL PUBLICATIONS

This Ph.D thesis is based on the following articles, which are referred to in the text by their Roman numerals

I. Qiao, M., Immonen, T., Koponen, O. and Saris, P. E. 1995. The cellular location and effect on nisin immunity of the NisI protein from *Lactococcus lactis* N8 expressed in *Escherichia coli* and *L. lactis.* FEMS Microbiol. Lett., **131,** 75-80

II. **Qiao, M., Ye, S., Koponen, O., Ra, R., Usabiaga, M., Immonen, T. and Saris, P.E. 1996.** Regulation of the nisin operons in *Lactococcus lactis* N8. J. Appl. Bacteriology **80,** 626-634.

III. Koponen, O., Tolonen, M., Qiao, M., Wahlström, G., Helin, J. and Saris, P. E. 2002. NisB is required for the dehydration and NisC for the lanthionine formation in the post-translational modification of nisin. Microbiology **148**, 3561-3568.

IV. Koponen, O., Takala, T. M., Saarela, U., Qiao, M. and Saris, P. E. 2004. Distribution of the NisI immunity protein and enchancement of nisin activity by the lipid-free NisI. FEMS Microbiol. Lett. **231**, 85-90.

ABBREVIATIONS

3D	three dimensional
ψ	membrane potential
Ap	ampicillin
ATP	adenosine triphosphate
C-terminal	carboxylterminal
Da	dalton
DMSO	dimethyl sulfoxide
DPC	dodecyl phosphocholine
Dha	2,3-didehydroalanine
Dhb	2,3-dehydrobutyrine
DNA	deoxyribonucleic acid
Erm	erythromycin
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobase pairs
kDa	kilodaltons
Km	kanamycin
LAB	lactic acid bacteria
Lan	(2S, 6R)-meso-lanthionine
MeLan	(2S, 3S, 6R)-3methyllanthionine
NMR	nuclear magnetic resonance
N-terminal	aminoterminal
PCR	polymerase chain reaction
PG	phosphatidylglyserol
RNA	ribonucleic acid
SDS-PAGE	sodium dodecyl sulphate - polyacrylamide gel electrophoresis
TFE	trifluoroethanol

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A. REVIEW OF THE LITERATURE

1. Bacteriocins

Pathogenic bacteria permanently threaten the health of animal and human. Microorganisms are the major cause of food-related diseases and spoilage in the production and storage of food and beverages. Antibiotics and food preservatives, such as nitrate, are generally used to combat these pathogens. However, because of the potential danger of selection of antibiotic resistant bacteria and the demand by consumers for purer and safer food, i.e. food with less chemical additives, there is a growing interest to replace these substances by natural products that are easily degradable and harm neither the individual nor the environment. One interesting group of biomolecules in this respect are bacteriocins, which are produced by both gram-positive and gram-negative bacteria (Klaenhammer, 1993; de Vuyst and Vandamme, 1994; Dodd and Gasson, 1994). Bacteriocins are oligo- or polypeptides, which have a bactericidal (bacteria-killing) activity (Tagg et al., 1976).

Two well-known representatives of bacteriocins produced by the gram-negative bacteria are colicins and microcins. First bacteriocin produced by *Escherichia coli* was found about 60 years ago (Gratia, 1925) and later they were named as colicins (Fredericq P, 1948). Colicins have been studied for over six decades and are well characterized (Akutsu et al., 1989; James et al., 1992; James et al., 1996; Pagie and Hogeweg, 1999). They are plasmid-encoded bacteriocins and classified into groups on the basis of the receptor to which they bind. Microcins, produced by the gram-negative bacteria *enterobacteriaceae*, are post-translationally modified. They are active against other gram-negative bacteria and act via inhibition of DNA replication or protein synthesis (Bacquero et al., 1984; Yorgey et al., 1992).

One interesting group of bacteriocins produced by gram-positive bacteria are those produced by lactic acid bacteria (LAB). They are an important group of bacteria due to their usage as starter cultures in the food industry. The first antibacterial polypeptide discovered in lactic acid bacteria was reported by Rogers (1928) who observed an inhibitory substance, later named nisin, from *Streptococcus lactis* strains (classified later as *Lactococcus lactis,* Schleifer et al., 1985). During recent years, a large number of novel bacteriocins have been identified from many different

LAB. Based on their amino acid sequences, stability to heat, size, mode of action, biological activities, secretion mechanisms and the presence of modified amino acids, LAB bacteriocins have been classified into three classes of which the first two classes have further been subtyped (Jung, 1991a, b; Klaenhammer et al., 1992; Klaenhammer, 1993; de Vuyst and Vandamme, 1994; Dodd and Gasson, 1994; Nes et al., 1996).

Class I. Lantibiotics

I a: nisin-like, elongated screw shaped, cationic molecules
I b: duramycin-like, globular molecules with low net negative charge

Class II. Non lantibiotics, small heat stable peptides

II a: pedocin-like bacteriocins with strong antilisterial effects
II b: two-peptide bacteriocins
II c: sec-dependent secreted bacteriocins

Class III. Large heat-labile proteins

The class I and II bacteriocins have been the most intensively studied, since they are the most common ones, and also the most promising candidates for industrial applications (Nes et al., 1996; Parente and Ricciardi, 1999).

2. Lantibiotics

A number of antimicrobial peptides (bacteriocins) that are produced by bacteria have been found to contain, in addition to a few other unusual amino acids, the uncommon thioether-bridged residues lanthionine (Lan) and 3-methyllanthione (Melan). Therefore, these bacteriocins have been called ´lantibiotics´ from lanthionine containing antibiotic (Schnell et al., 1988). These lantibiotics are plasmid-mediated or chromosomally encoded, ribosomally synthesized, peptides that form pores in cellular membranes or inhibit enzymes. They are mainly active against gram-

positive bacteria. Interesting aspects of lantibiotics include (1) their mode of biological action, (2) their biosynthesis, which involves unique enzymes, (3) autoimmunity i.e. how the producer protects itself and (4) their (potential) applications. Examples of lantibiotics described to date are listed in Table 1.

Table 1: Lantibiotics

Lantibiotic	**Producing species**	**Molecular mass (dalton)**	**Modified residues (%)**	**Ring number**
Type-A lantibiotics				
Nisin A	*Lactococcus lactis*	3353	38	5
Nisin B	*Lactococcus lactis*	3350	38	5
Nisin Q	*Lactococcus lactis*	3327	38	5
Subtilin	*Bacillus subtilis*	3317	40	5
Epidermin	*Staphylococcus epidermidis*	2164	41	4
Gallidermin	*Staphylococcus gallinarum*	2164	41	4
Mutacin B-Ny266	*Streptococcus mutans*	2270	41	4
Pep5	*Staphylococcus epidermidis*	3488	26	3
Epicidin 280	*Staphylococcus epidermidis*	3133	27	3
Epilancin K7	*Staphylococcus epidermidis*	3022	32	3
Lactocin S	*Lactobacillus sake*	3764	24	2
SA-FF22	*Streptococcus pyogenes*	2795	27	3
Lacticin 481	*Lactococcus lactis*	2901	26	3
Salivaricin A	*Streptococcus salivarius*	2315	27	3
Variacin	*Micrococcus varians*	2658	28	3
Type-B lantibiotics				
Cinnamycin	*Streptomyces cinnamoneus*	2042	47	4
Duramycin	*Streptomyces cinnamoneus*	2014	44	4
Duramycin B	*Streptoverticillium sp.*	1951	47	4
Duramycin C	*Streptomyces griseoluteus*	2008	47	4
Ancovenin	*Streptomyces sp.*	1959	37	3
Mersacidin	*Bacillus sp.*	1825	42	4
Actagardine	*Actinoplanes sp.*	1890	45	4

Based upon their structure and biological function, the lantibiotics have been subdivided into two subclasses: type-A and type-B (see Table 1) (Jung, 1991; Jung and Sahl, 1991; Bierbaum and Sahl, 1993; Entian and Klein, 1993; Hansen, 1993; Sahl et al., 1995). Type-A lantibiotics are elongated cationic polypeptides, which exert their bactericidal funtion primarily via membrane perturbation. Well-known members of this group are nisin (Gross and Morell, 1971), subtilin (Gross et al., 1973, Pep5 (Kellner et all., 1989),) epidermin (Allgaier et al., 1986), gallidermin (Kellner et al., 1988) and epilancin K7 (van de Kamp et al., 1995). There are three variants of nisin – nisinA, nisinZ and recently discovered nisinQ (Graeffe et al., 1991; Mulders et al., 1991; Zendo et al., 2003). NisinA and nisinZ differ only with one amino acid; nisinA has histidine and nisinZ asparagine at the position 27 of the amino acid sequence (Graeffe et al., 1991; Mulders et al., 1991). NisinQ and nisinA differ with four amino acids in the mature peptide: valine instead of alanine at the position 15, leucine instead of methionine at the position 21, asparagine instead of histidine at the position 27 and valine instead of isoleucine at the position 30, respectively. NisinQ and nisinZ differ from each other similarly, except that they both have asparagine at the position 27 of the amino acid sequence (Zendo et al., 2003). Also epidermin and gallidermin are variants; epidermin contains isoleucine and gallidermin leucine at position 6 of their amino acid sequence (Sahl, 1994).

Type-B lantibiotics are globular, have a low net charge, and are enzyme inhibitors. Representatives of this group are the duramycins (Fredenhagen et al., 1991), cinnamycin (Benedict et al., 1952), ancovenin (Wakamiya et al., 1985), mersacidin (Kogler et al., 1991) and actagardine (Kettenring et al., 1990; Zimmermann et al., 1995). The amino acid sequences of the duramycins, cinnamycin and ancovenin are almost identical. They thus can be regarded as natural variants. The grouping of actagardine and mersacidin together with the duramycin-like lantibiotics as belonging to type-B is based on similarities between their prepeptide sequences (Bierbaum et al., 1995). Model structure of type-A lantibiotic and type-B lantibiotic is presented in the figure 1.

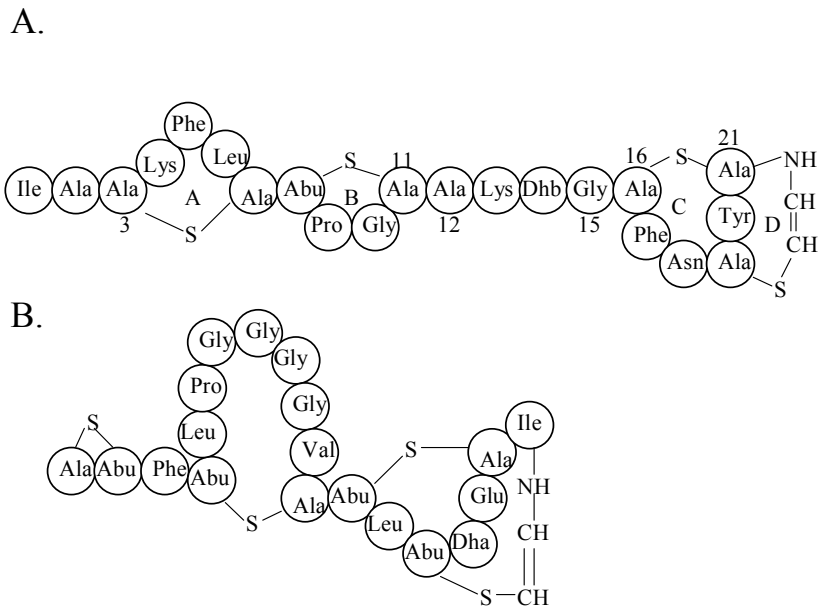

Fig.1 : Model structures of type-A and type-B lantibiotics. A: type-A lantibiotic gallidermin; B: type-B lantibiotic mersacidin. Special structure – thioether bridge (-S-) and some typical amino acid modifications (Dha = dehydroalanine; Dhb = dehydrobutyrine) for lantibiotics are seen. Typical ring structures with the essential thioether bridges, Ala-S-Ala = lanthionine and Abu-S-Ala = β-methyllanthionine, are also shown.

2.1. Genes and biosynthesis of type-A lantibiotics

2.1.1. Organisation of the gene clusters

In the following section the genes for the type-A lantibiotics and how these genes are organized will be briefly discussed. Two classes of genetic organisation have been identified: nisin, epidermin, subtilin and Pep5 (nisin group) are grouped on the basis that they are modified by separate LanB and LanC enzymes, whereas this function is performed by a single LanM enzyme

in the subclass containing lacticin 481, lactocin S, and cytolysin (lacticin group) (McAuliffe et al., 2001).

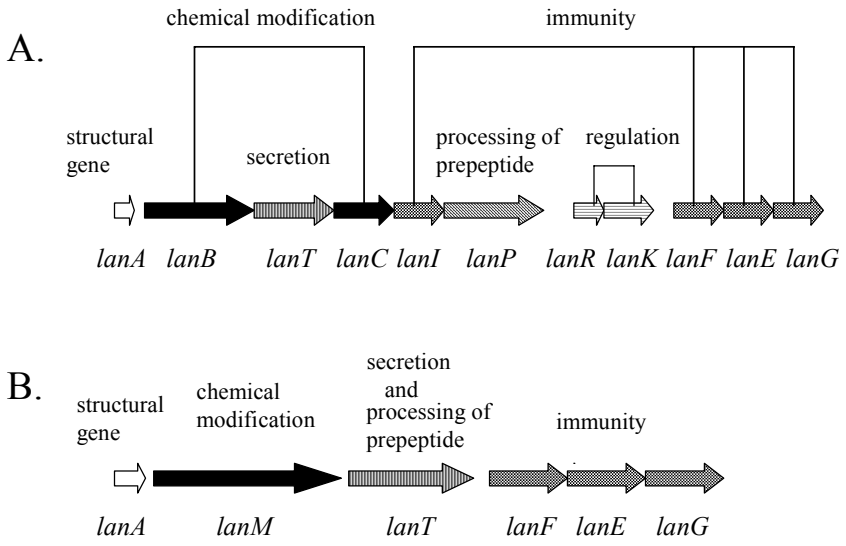

Figure 2: Genomic organization of the two classes of lantibiotic-A described in the text. A: nisin group;B: lacticin group. Putative function of the genes is also signed. *lan* is an abbreviation of the word lantibiotic and the letter to certain gene is according the general nomenclature for lantibiotics (de Vos et al., 1995).

According to a common nomenclature the genes for lantibiotics (lan) is denoted with a *lan* added with a symbol for the gene in question (de Vos et al., 1995). Thus the gene for the immature precursor peptide is called *lanA*. Nisin (*nis*), subtilin (*spa*), epidermin (*epi*) and Pep5 (*pep*) gene clusters contain *lanB* and *lanC* genes that are presumed to code for two types of enzymes that have been implicated in the modification reactions characteristic to all lantibiotics, i.e. dehydration and thioether-ring formation. Cytolysin (*cyl*), lactocin S(*las*) and lacticin 481(*lct*) clusters do not have these genes, but they do contain a much larger *lanM* gene which is the *lanC* gene homologue. Most lantibiotic gene clusters contain a *lanP* gene encoding a serine protease

that is presumably involved in the proteolytic processing of the prelantibiotics. All clusters contain a *lanT* gene encoding an ABC transporter likely to be involved in the export of the lantibiotics. The *lanE*, *lanF* and *lanG* genes encode another transport system that is possibly involved in self-protection. Also, the *lanI* gene, that encodes a lipoprotein, is involved in immunity – self-protection. In the nisin and subtilin gene clusters two tandem genes, *lanR* and *lanK* have been located. These code for a two-component regulatory system. Finally, non-homologous genes are found in some lantibiotic gene clusters such as *epiD* that encodes an enzyme involved in a post-translational modification forming a special kind of ringstructure, unsaturated (S)-[(Z)-2-aminovinyl]-D-cysteine at the C-terminus of the mature epidermin (Kupke and Götz , 1997).

2.1.2. Biosynthesis

All the lantibiotics for which the structural genes have been sequenced so far, are ribosomally synthesized as so called prepeptides (Jung, 1991a; Sahl et al., 1995). These prepeptides consist of an N-terminal leader peptide and a C-terminal propeptide domain, which is modified to become the mature peptide. The propeptide undergoes post-translational modifications including dehydration of specific hydroxyl amino acids and formation of thioether bridges via addition of neighboring cysteines to didehydro amino acids (Sahl et al., 1995). It was proposed (Schnell et al., 1988) and subsequently proven (Weil et al., 1990) that in the first modification step, the Ser and Thr residues are dehydrated to Dha (didehydroalanine) and Dhb (didehydrobutyrine), respectively. (2S, 6R)-meso-lanthionine and (2S, 3S, 6R)-3-methyllanthionine residues are formed subsequently by addition of the thiol-groups of the Cys residue to the didehydroamino acids.

LanB protein is the putative enzyme that catalyzes the dehydration of the Ser and Thr residues in the propeptide domain of type-A lantibiotics. In certain cases, the serine residue, at position 33 of nisin, does not undergo dehydration to Dha33. This feature of nisin biosynthesis was exploited in an investigation of the role of NisB protein in prenisin maturation. Overexpressing the plasmid-encoded *nisB* gene resulted in a fourfold increase in the level of NisB protein that significantly increased the efficiency of the dehydration reaction at Ser33 (Karakas et al., 1999).

This result represents the first experimental evidence for the direct involvement of the NisB protein in the maturation process of nisin.

LanC is assumed to be the thioether bridge forming enzyme. Deletion of the *pepC* gene in *Staphylococcus epidermis* resulted in production of incorrectly modified Pep5 fragments, which contained only one out of the three expected lanthionine residues (Schnell et al., 1992). Overexpression of NisC protein did not appear to influence dehydration of nisin prepeptides, and therefore may have a role in thioether formation (Karakas et al., 1999).

In gene clusters for biosynthesis of cytolysin, lacticin 481, lacticin S and mutacin II *lanB* gene is missing and instead gene named *lanM* is found (Gilmore et al., 1996; Rince et al., 1994 and Woodruff et al., 1998). The C-terminal part of LanM protein is homologous to LanC proteins (Siezen et al., 1996). It is supposed that LanM is able to catalyze both of the reactions thought to be catalyzed by LanB and LanC.

The *lanT* gene encodes a protein that is involved in transport of the prepeptides across the cellular membrane to the outside of the cell (Kuipers et al., 1993; Qiao et al., 1996). Some type-A lantibiotic gene clusters like lacticin 481, cytolysin and SA-FF22 possess LanT transporters, which cleave the leader peptide concomitant with export (Rince et al., 1994; Dougherty et al., 1998; McLaughlin et al., 1999). All known lantibiotic gene clusters were found to contain a *lanT* gene (Siezen et al., 1996), although some of the proteins such as EpiT (Peschel et al., 1997) and PepT (Meyer et al., 1995) seem to be dispensable. In these cases, host cell transporters probably complement the specific transporter deficiency. LanT proteins share homology with a large family of transport proteins, characterized by the presence of a cytoplasmic ATP-binding domain and a membrane-spanning domain (Fath and Kolter, 1993).

By use of the yeast-two-hybrid system and co-immunoprecipitation techniques, it was shown for nisin (Siegers et al., 1996) and subtilin (Kiesau et al., 1997) that the LanB, LanC and LanT proteins can interact with each other and form a multimeric protein complex located on the cytoplasmic membrane. Thus biosynthesis of lantibiotics might occur via this kind of multimeric protein complexes.

The final step in the biosynthesis of lantibiotics is the removal of the leader part from the prepeptide. LanP, which is a serine protease, is responsible for this step (van de Meer et al., 1993). However, in the biosynthesis of some lantibiotics like lacticin 481, LanT can undertake this proteolytic cleavage as well as secretion of the lantibiotic (Havarstein et al., 1995). NisP, produced in *E. coli*, could cut purified nisin prepeptide and consequently lead to the formation of the active nisin molecule (van de Meer et al., 1993). The location at which processing of the leader peptide occurs, varies with the lantibiotic (Siezen et al., 1996). In the case of nisin, NisP is secreted by the host cells and anchored to the cellular membrane on the outside of the cells (van de Meer et al., 1993). Pep5, epilancin K7 and lacticin 481 were reported to be cleaved intracellularly (Siezen et al., 1996). In contrast to the gene clusters mentioned above, the subtilin gene cluster does not contain a gene encoding a protease, and it is assumed that processing of it occurs by a general serine protease of the host *Bacillus subtilis* (Siezen et al., 1996).

2.1.3 Regulation of biosynthesis

It has been reported that apart from displaying a strong antimicrobial activity, lantibiotics can also play an important role in the regulation of its own biosynthesis (Kuipers et al., 1995; Ra et al., 1996). Regulation is supposed to occur via the two-component regulatory proteins, LanK and LanR. To date, these regulatory proteins have been identified in the gene clusters of nisin, subtilin and SA-FF22 (Engelke et al., 1994; Klein et al., 1993 and McLaughlin et al., 1999). Indeed, nisin and subtilin have been shown to act as a signalling molecule of its own biosynthesis via NisRK and SpaRK proteins, respectively (Ra et al., 1996; Stein et al., 2002). Such lantibiotics can be regarded as peptide pheromones, which are sensed by the histidine kinase LanK, whose input domain resides on the outer side of the membrane, probably by a direct protein-peptide interaction. By analogy with other known two-component regulatory systems (Parkinson et al., 1992; Kleerebezem et al., 1997; Kuipers et al., 1998) LanK will autophosphorylate at a specific histidine residue when it senses a certain threshold lantibiotic concentration in the medium and subsequently transfers the phosphate moiety to the response regulator LanR. The response regulator is thought to become phosphorylated at a specific Asp residue, which is considered to trigger its binding to regulated promoters in the lantibiotic gene clusters thereby activating transcription of the structural gene *lanA* and the downstream genes (Kuipers et al., 1995; Ra et al., 1996; de Ruyter et al., 1996).

Transcription start sites have been identified at three positions in the nisin operon, preceding the *nisA, nisRK* and *nisFEG* genes (Kuipers et al., 1995; de Ruyter et al., 1996). These studies have shown that transcription from the *nisR* promoter is independent of nisin, while the *nisF* promoter is under similar NisRK mediated control as the *nisA* promoter. Correspondingly subtilin operon comprehends three promoters: *spaS, spaBTC* and *spaIFEG* that are controlled by subtilin (Stein et al., 2002). In addition to autoinduction subtilin biosynthesis seems to be controlled via sigma factor H. It was shown to regulate positively transcription of *spaRK* genes (Stein et al., 2002). Also, nisin biosynthesis seems to be controlled independently without nisin autoinduction. It was shown that the *nisA* promoter could be induced independently of NisRK system by lactose and galactose (Chandrapati et al., 1999). Thus there seems to be different ways to regulate the biosynthesis of lantibiotics.

Another known interesting case of regulatory proteins of type-A lantibiotics is EpiQ, which regulates production of epidermin. EpiQ possesses some similarities to LanR proteins at the C-terminus, but lacks the highly conserved phosphoryl acceptor Asp residue (Schnell et al., 1992). In addition, no corresponding LanK protein, histidine kinase, has been identified in the epidermin gene cluster. However it has been proposed that EpiQ may direct epidermin biosynthesis following phosphorylation by an intrinsic histidine kinase (Augustin et al., 1992). The regulator EpiQ activates the expression of most of the genes encoded in the epidermin gene cluster. The genes responsible for the synthesis of the epidermin precursor peptide (*epiA)* and its post-translational modification (*epiBDC),* for producer self-protection (*epiFEG* and *epiH),* and for secretion (*epiH* and *epiT*[5]) are controlled by *EpiQ* (Peschel et al., 1993; Peschel et al., 1997). However, there is no evidence for a potential signal-transduction via epidermin leading to the activation of the EpiQ and to the expression of epidermin (Kies et al., 2003). Instead, it was lately suggested that the activity of epidermin is regulated at the level of precursor peptide processing. It was claimed that the EpiP protease, that cuts the leader part of epidermin, is controlled by the accessory gene regulator quorum sensing system (Kies et al., 2003).

3. Nisin

3.1. Foreword

The best-known example of a lantibiotic is nisin. Not only was nisin the first lantibiotic to be discovered, it also is the only one, which has so far found substantial commercial applications. In addition, nisin is also the most extensively studied and best known of all lantibiotics. It is considered not toxic to mammals including man. The toxicity of orally applied nisin as tested in rats, was estimated to be very low (LD50 7 g/kg of animal body weight), similar to that of common salt (Hurst, 1981). Nisin was the first of these agents to be introduced commercially as a food preservative in the UK, approximately 30 years ago. Its first established use was as a preservative in processed cheese products and since then numerous other applications in foods and beverages have been identified (Table 2). Nisin is currently used as food preservative (food additive number E234) in over 50 countries including the EU, the USA and China. There is also an increasing interest to use nisin for medical applications as an antibiotic, because of the emergence of bacterium resistance against `classical` antibiotics (Hancock, 1997; Hoffmann et al., 2002).

Table 2: Applications of nisin

Food	**Bacteria inhibited by nisin**	**References**
Cheese products		
Processed cheeses	*Clostridium butyricum* *Clostridium tyrobutyricum*	1 2
Hard and semi-hard cheeses	*Clostridium botulinum* *Listeria monocytogenes* *Staphylococcus aureus*	3 4 5
Milk		
Pasteurised milk	*Clostridium botulinum*	6
Canned evaporated milk	Clostridium thermosaccharolyticum	7
Dairy dessert	thermophilic heat-resistance spores	8
Acidic canned food		
Mushrooms, peas, potatoes	Clostridium thermosaccharolyticum	9

Table continued

Beer and wine		
	Lactobacilli sp	10
	Pediococci sp.	11
Meat		
	Listeria monocytogenes	12
	Staphylococcus aureus	13.
Medical therapy		
Peptic ulcer disease	*Helicobacter pyroli*	14.
Atopic dermatis	*Staphylococci* sp.	15.
Bovine mastitis	*Staphylococci* sp.	16.

References within this table 1) Fowler et al., 1979; 2) Fowler et al., 1991 3) Somers et al., 1987 4) Lipiska, 1977 5) Hugenholtz et al., 1991 6) Fowler et al., 1979 7) Gregory et al., 1964 8) Heinemann et al., 1965 9) Vas et al., 1965 10) Ogden et al., 1985 11) Radler et al., 1990 12) Taylor et al., 1985 13) Calderon et al., 1985 14) Hancock et al., 1997 15) Valenta et al., 1996 16) Serieyes and Poutrel, 1993.

3.2 Primary structure of nisin

The molecular weight of nisin is 3353 Da (Jung, 1991). It is both soluble and stable in aqueous solutions at low pH, but at high pH it is inactivated by chemical modification (Liu and Hansen, 1990). It consists of 34 amino acid residues, several of which are modified residues such as lanthionine, 3-methyl lanthionine, dehydroalanine and dehydrobutyrine (Figure 3). Nisin can exist not only as a monomer (3350 Da) but also as dimers (6700 Da) or tetramers (13400 Da), which suggests that the dehydroamino acids and amino groups of two or four nisin molecules can interact (Liu et al., 1990). The lanthionines in positions 3 and 7, 8 and 11, 13 and 19, 23 and 26, and 25 and 28 form five ring structures in the nisin molecule, these rings are designated A, B, C, D and E, respectively. The lanthionine rings D and E are intertwined. D-amino acids are found in positions 3, 8, 13, 23 and 25. The total chemical synthesis of nisin has been published (Fukase et al., 1988). The primary structure of nisin is similar to that of subtilin, the lantibiotic produced by *Bacillus subtilis* ATCC 6633 (Jansen et al., 1944; Gross et al., 1973). The sequence identity between these lantibiotics is 60% and their ring structures are identical.

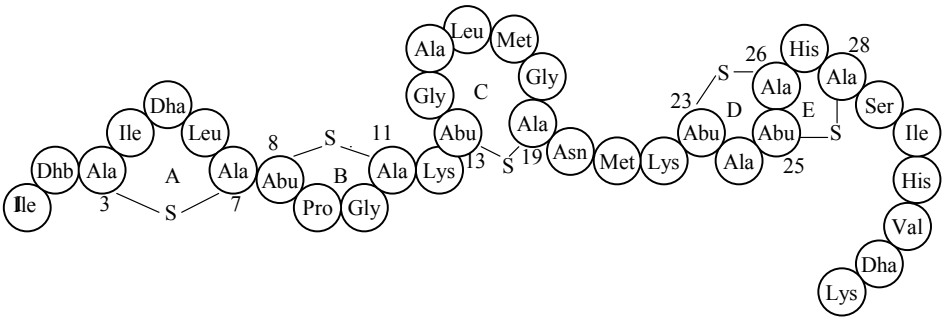

Figure 3: The primary structure of nisin. Special amino acid modifications are marked with Dha and Dhb, five ring structures with thioether bridges (Ala-S-Ala = lanthionine , Abu-S-Ala = β-methyllanthionine) are signed with letters A, B, C, D and E and the amino acids for the start and end of the ring are numbered based on their position in the amino acid sequence of nisin.

3.2.1 Primary structure of epidermin, gallidermin, Pep5 and epilancin K7

Epidermin, Pep5 and epilancin K7 are three lantibiotics that are produced by *Staphylococcus epidermidis* Tu 3298 (Allgaier et al., 1985; Allgaier et al., 1986), *S. epidermidis* 5 (Sahl and Brandis 1981), and *S. epidermidis* K7 (Pulverer and Jeljaszewicz 1976), respectively. Gallidermin, which is 6L-epidermin, was isolated from *S. gallinarum* DSM 4616 (Kellner et al., 1988).

The structure of epidermin was elucidated in 1985 (Allgaier et al., 1985; Allgaier et al., 1986). It is composed of 22 amino acid residues and is the smallest type A lantibiotic. Epidermin has a net charge of +3 with a free N-terminus and two Lys residues. It is a four-ringed peptide, which contains one Melan and two Lan residues. Interestingly, the second ring of epidermin is identical with the second ring of the lantibiotic nisin, which is produced by *L. lactis* (Mattick and Hirsch, 1944). The fourth ring at the C-terminus of epidermin is formed by the unusual amino acid S-

aminovinyl-D-cysteine. Gallidermin has the same structure as epidermin except that there is Leu in position 6 instead of the Ile of epidermin.

Pep5 was first isolated in 1981 (Sahl and Brandis 1981). It has a molecular mass of 3488 Da (34 amino acids) and is the largest of those lantibiotics for which the complete primary structure is known. Pep5 has only three rings and carries 8 positive and one negative charge on its C-terminal carboxy group. The structure elucidation of Pep5 was impeded by the N-terminal dehydrobutyrine residue (Dhb), which spontaneously deaminates into an oxobutyryl residue and prevents Edman degradation (Kellner et al., 1989; Kellner et al., 1991).

Although epilancin K7 had been discovered as early as 1976 (Pulverer and Jeljaszewicz 1976), its structure was elucidated only recently (van de Kamp et al., 1995). Epilancin K7 consists of 31 amino acids (3032 Da) including three ring structures. It contains 6 Lys residues and a free C-terminal carboxy group. The N-terminus of epilancin K7 carries a hydroxypropionyl residue, which derives from a serine in the propeptide (van de Kamp et al., 1995). Though the latter structural features of epilancin K7 are reminiscent of Pep5, the C-terminal double ring of epilancin K7 is very closely related to the C-terminal double rings (ring 4 and 5) of subtilin and nisin. The second ring of epilancin K7 is identical to the fourth ring of subtilin and these features place epilancin K7 in a ” missing link ” position between Pep5 and subtilin (Sahl et al., 1995).

3.3 3D-structure of nisin

Three different groups reported simultaneously of the NMR assignments of nisin. Slijper et al. (1989) focused on nisin in aqueous solution. Chan et al. (1989) used both water and dimethyl sulfoxide (DMSO) as the solvent. The latter was also used by Palmer et al. (1989), who studied the individual rings A and B, obtained via chemical synthesis. The 3D-structure of nisin in aqueous solution was subsequently reported by van de Ven et al. (1991) and Lian et al. (1992). The NMR data did not indicate a well-defined over-all folding of the molecule; rather it appears that nisin is quite flexible in solution. There is a good consensus on the structures of the three small rings B, D and E; these are essentially B-turns, which are fixed by the thioether bond

joining the first and the fourth residue of the rings. The A and C rings show some structural variability and are not so well defined.

Although nisin molecule is quite flexible, two amphipathic structured domains can be found in aqueous solution. The first domain consists of residues Ala3 – Ala19 containing the first three lanthionine rings, A, B, C with the hydrophobic side chains of Ile4, Dha5, Leu6, Ala15, Leu16 and Met17 on one face and lanthionines and hydrophilic Lys12 on the opposite face. The second domain consists of residues Ala23 – Ala28, including the intertwined lanthionine rings D and E, and the hydrophobic face is formed by the residues Met21 and Ala 24, while the hydrophilic, positively charged side chains of Lys22 and His27 protrudes from the opposite face. The N- and C-termini, as well as the ” hinge ” region around Met 21, which joins the two domains ” ABC ” and “ DE “, appears to be quite flexible. The molecule is also amphipathic from another point of view; the hydrophilic and charges of nisin are mainly located in the C-terminal half of the molecule, whereas the majority of the residues in the N-terminal half are hydrophobic and only a single charged residue, Lys 12, is present (Figure 4).

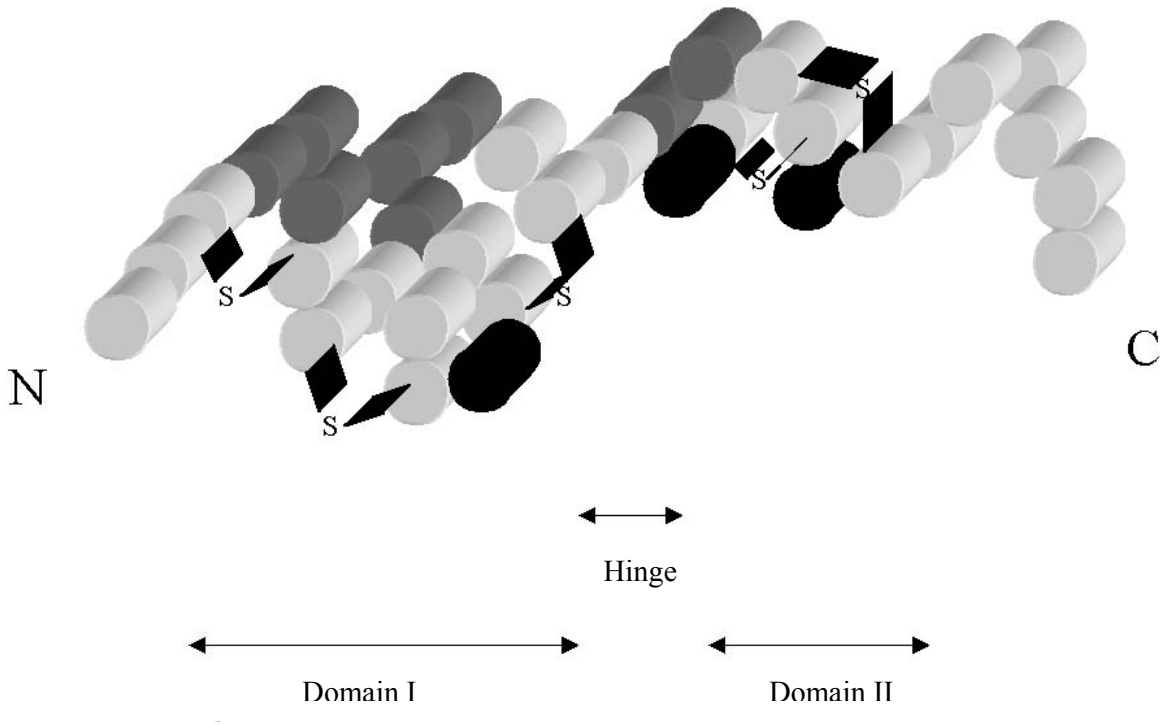

Figure 4. Drawing of the 3D-features of nisin molecule. Grey balls demonstrate hydrophobic and black ones hydrophilic amino acids. It can be seen that hydrophobic amino acids form one face of the molecule and hydrophilic another on the opposite side of the molecule. Structured domains I and II with the thioether rings and the flexible hinge region of the molecule are signed.

Van den Hooven et al. (1993) proceeded to determine the structure of nisin when it was bound to micelles of sodium dodecyl sulphate (SDS) or dodecyl phosphocholine (DPC). The binding to micelles is considered to model the binding of nisin to the cytoplasmic membrane, which is the first step in the execution of its antimicrobial activity. These studies showed that the amphipathicity is retained when nisin binds to the micelles, but that these membrane mimetics induced a conformational change in a ring A. Upon elucidation of the 3D-structure, it was found that this conformational change entailed the concerted "flipping" of the two peptide bonds flanking Dha5 (van den Hooven et al., 1995).

3.3.1. 3D-structure of subtilin, gallidermin and Pep5

Of the type-A lantibiotics, subtilin is most closely related to nisin. A detailed description of the subtilin 3D-structure is not yet available, but the first results (Chan et al., 1992) indicate that the similarity of a primary structure to nisin also extends to the tertiary structure.

The solution structure of gallidermin has been reported (Freund et al., 1991). The 3D-structure was calculated on the basis of data obtained in a mixture of trifluoroethanol (TFE)/water (95:5). Thus, the conformation obtained could represent the membrane-bound state, rather than the one in aqueous solution. Gallidermin consists of two structured domains, the first domain being formed by the two lanthionine rings in the segment 3-11, and the second domain consists of the intertwined rings constituting the segment 16-21. A more flexible region ranging from Ala 12 to Gly 15 connects the two domains. Just how flexible this region is, remains somewhat unclear; the molecular dynamics protocol used in the calculations yielded a rather elongated screw-like overall shape for this molecule. This motif, of two domains fixed by lanthionine rings joined by a flexible ” hinge ” also occurs in nisin (van de Ven et al., 1991), and might be common to type-A lantibiotics. Gallidermin has a similar amphipathic character as nisin, except that the charged C-terminal fragment is absent.

A preliminary account of NMR studies, leading to a structure determination of Pep5 has been presented (Freund et al., 1991). The data indicate a transition from a rather flexible, unordered structure in water, to a more helical conformation in TFE. A similar observation has been made for epilancin K7. This molecule has been studied, both in aqueous solution and complexed to DPC micelles. This complexation with DPC micelles indicated a generally more ordered, possibly helical structure, for the DPC bound state.

Thus, rather little is still known about the 3D-structure of the type-A lantibiotics. Nisin and gallidermin are the only two examples for which more detailed 3D-structure do exist currently.

3.4. Mechanism of the biological action

Nisin exerts its bactericidal activity primarily via its interaction with the membrane of sensitive cells. It has been postulated that the membrane function is disturbed by pore formation and not by general destabilization of the bilayer. Addition of nisin to cells of several gram-positive bacteria leads to a rapid efflux of amino acids, ATP and monovalent cations (Ruhr and Sahl, 1985; Kordel and Sahl, 1986). The efflux velocity was for example as rapid for Rb+ as for larger positively and negatively charged amino acids, indicating that there is no selectivity for charge and size (up to 500 dalton) in the nisin mediated efflux (Ruhr and Sahl, 1985).

Furthermore it has been found that the addition of nisin leads to a rapid decrease of the membrane potential (ψ) in sensitive cells (Ruhr and Sahl, 1985) and to a dissipation of the pH gradient (pH) in artificial liposomes (Gao et al., 1991; Garcia Gancera et al., 1993). In fact, nisin-induced dissipation of the membrane potential and loss of the pH gradient in liposomes both display the same concentration dependence, which suggests that the two components of the protonmotive force are equally effective in promoting insertion and pore formation of nisin in the membrane (Gao et al., 1991). It has been hypothesized that bacteriocins from lactic acid bacteria share a common mechanism of action, this being the dissipation of the protonmotive force of the target bacterium (Montville and Bruno, 1994). The activity of nisin depends on a sufficient electrical transmembrane potential (Sahl et al., 1987). Energy is required for both formation and opening of the pores. The threshold potential for intact cells is between -50 and -80 mV at pH 7.5 and below -50 mV at pH 5.5 (Sahl et al., 1987). The membrane potential has to be inside of the cell negative (transnegative) and the pH gradient inside of the cell alkaline (Sahl et al., 1987; Gao et al., 1991; Garcia Garcera et al., 1993). Single channel recordings on black lipid membranes demonstrated the presence of transient multistate pores with diameters of 0.2 to 1 nm with lifetimes of a few to several hundred milliseconds (Sahl et al., 1987).

A variety of other experiments has been performed, the results of which are all consistent with the conclusion that the membrane is the target site of nisin. The gram-negative bacteria *E. coli* becomes sensitive to nisin when the outer membrane is made permeable by osmotic shock (Kordel and Sahl, 1986; Stevens et al., 1991). The disruption of the membrane function helps to explain the general inhibition of macromolecule synthesis noted in *Micrococcus luteus* (Henning

et al., 1986). The antimicrobial activity of nisin is dependent on the phospholipid composition of the liposomal membrane (Gao et al., 1991; Garcia Garcera et al., 1993; Driessen et al., 1995). Nisin associates with the anionic surface of PG liposomes and disturbs the lipid dynamics near the phospholipid polar head-group water interface. Anionic liposomes are affected to a greater extent than neutral membranes, reflecting the primarily electrostatic nature of the interactions of nisin with negatively charged phospholipid head-groups (Kordel et al., 1989). Recent studies have confirmed that nisin inserted into lipid monolayers in an anionic lipid-dependent way (Demell et al., 1996). Thus data above suggests that nisin exerts its antimicrobial activity via an interaction with the phospholipid components of the cytoplasmic membrane followed by pore formation and interference with membrane function.

However, very current results have changed the picture of the mechanism of nisin activity. It was shown that nisin binds to the so-called docking molecule, lipid II (Brötz et al, 1998; Breukink et al., 1999). Lipid II is a precursor of the bacterium cell wall synthesis. It seems that nisin at micro molar concentrations disrupts the membrane in a non-targeted fashion, described in the earlier research, but at nano molar concentrations nisin acts via target-mediated, i.e.via lipid II pore formation (Hsu et al.; 2002; Heusden et al., 2002). Recently it has even been proposed that nisin might have two killing mechanisms: one is pore formation into the membrane and the other inhibition of cell wall biosynthesis by preventing incorporation of lipid II into the peptidoglycan chain (Wiedemann et al., 2001).

In addition to the inhibition of bacterial growth, nisin also inhibits the outgrowth of bacterial spores. Evidence has been obtained that nisin interferes with the membranes of germinated spores. It has been speculated that nisin becomes covalently attached to the membrane sulfhydryl groups by reacting with one or more dehydro residues in the nisin molecule, although no covalent adduct has been identified (Morris et al., 1984). The inhibition of growth of vegetative cells and outgrowth of bacterial spores most likely occurs via different mechanisms. A mutant of the peptide antibiotic nisin in which the dehydroalanine residue at position 5 has been replaced by an alanine has been produced and structurally characterized (Chan et al., 1996). It was shown to have activity very similar to that of wild-type nisin in inhibiting growth of *L. lactis* and *M. luteus* but was very much less active than nisin as an inhibitor of the outgrowth of spores of *B. subtilis*.

Although the primary structure of several type-A lantibiotics has been known for some years, the mechanism of their action has only recently been clearly elucidated. Type-A lantibiotics seem to form potential dependent, ion-permeable channels in energised bacterial membranes. This action leads to the loss of membrane potential, concomitant cessation of energy production and efflux of essential low molecular mass, cytoplasmic components into the surrounding media. Thus the action of other type-A lantibiotics is quite similar to that of nisin.

However, there are differences in the details how the different type-A lantibiotics act to kill cells. Subtilin, epidermin and gallidermin have been found to produce relatively large (1-2 nm in the case of subtilin), stable pores with a lifetime up to tens of seconds. Nisin and Pep5 form relatively unstable pores (hundreds of milliseconds) and with slightly smaller diameters (1.2 nm). SA-FF22 produces pores approximately 0.5-0.6 nm in diameter and with lifetimes of only milliseconds, considerably smaller than those of other type-A lantibiotics and perhaps of shorter duration. SA-FF22 like the other type-A lantibiotics requires a certain threshold membrane potential that gives energy for pore formation. However, the threshold potential for SA-FF22 (> 100 mV) is somewhat higher than that required for pore formation by nisin or Pep5 (-70 to -80 mV), subtilin (70 to 80 mV) and epidermin or gallidermin (40 to 50 mV) (Benz et al., 1991). Depolarisation of the membrane is not so severe following SA-FF22 treatment as that observed for other type-A lantibiotics (Sahl et al., 1987; Kordel et al., 1988; Schuller et al., 1989). There is also a difference in the speed of efflux of amino acids, ATP and ions. The efflux of those molecules is more rapid with other A-lantibiotics than SA-FF22. Therefore, it has been suggested that death of SA-FF22 affected cells results not so much from immediate loss of metabolites, as is the case with other type-A lantibiotics, but from the disruption of the membrane potential which would lead to energy exhaustion (Jack et al., 1994). It has been shown previously that nisin (Sahl et al., 1987) and Pep5 (Kordel et al., 1988) form pores only with the application of *trans*-negative membrane potentials, while subtilin (Schuller et al., 1989), epidermin and gallidermin (Bentz et al., 1991) can act with voltages in either orientation. In this feature, SA-FF22 apparently resembles the latter substances since it can affect membranes regardless of the orientation of the potential (Jack et al., 1994).

The discovery of lipid II as a docking molecule of nisin and as an essential part of the targeted nisin bioactivity will probably also change the view of the action of other type-A lantibiotics. It

has been already shown that epidermin interacts with lipid II (Brötz et al.; 1998). In the future most probably similar kind docking molecules will be find also for other type-A lantibiotics.

3.4.1. Models for pore-formation

The small size of the 34 residue peptide nisin, with a length of maximally 5 nm, excludes the possibility that one molecule could span a membrane several times, which would be needed for the formation of a channel from one single molecule. Therefore one has to assume that several molecules participate in channel formation, for instance forming a ” barrel-stave-pore ” or some other structure in order to form the channel.

Several models have been proposed to explain the pore-forming ability of nisin. The ” insertion model ” assumes that the molecules are initially bound at the lipid surface, and subsequently in the presence of ψ, they may flip into a membrane-spanning orientation. The membrane-inserted molecules may form a cluster around a central pore as in the above mentioned ” barrel-stave model ”. In all likelihood, the order of events will be first insertion and subsequently aggregation (Sahl, 1991; Benz et al., 1991). Another model called the ” wedge model ” assumes that nisin molecules bind tightly to the anionic membrane surface leading to a high local concentration and disturbance of the lipid dynamics. In the presence of a membrane potential (-100 mV), the nisin molecules insert into the membrane, whereas anionic phospholipids result in bending of the lipid surface forming a wedge-like, non-specific pore (Driessen et al., 1995). An important aspect of this model is that the actual pore is formed by an array of nisin molecules that temporarily force the lipids into a thermodynamically unfavourable non-bilayer conformation. Such pores are intrinsically unstable, since the lipid will try to rearrange into a bilayer structure. Moreover, since the association and dissociation of nisin oligomers is likely to be a dynamic event, lowering of the magnitude of ψ below the threshold of insertion, will result in a relaxation of the nisin molecules to the surface-bound state and disassembly of the pore (Driessen et al., 1995). Figure 5 shows the wedge-like model of nisin induced pore-formation. The latest feature for this model comes from results of van Kraaij et al. (1998), which showed that at least the C-terminus of nisin translocates across the membrane and actually reaches the inner leaflet of the membrane. It is also possible that the entire nisin molecule might translocate across the membrane as has been proposed for the mechanism of action of α-helical peptides like magainin (Matsuzaki et al., 1995).

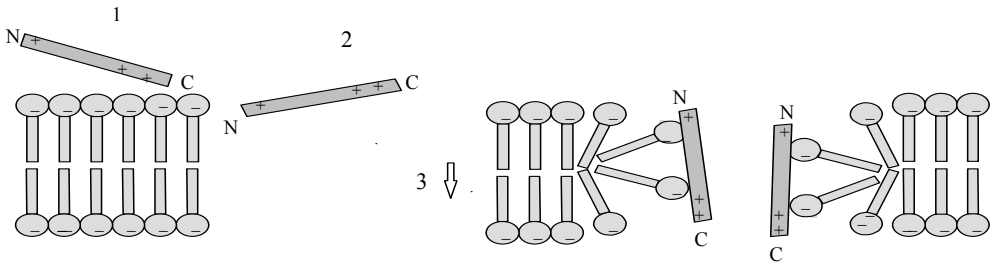

Figure 5: Wedge-model for nisin pore-forming. (1) Binding of nisin to the membrane. Positive charges in the C-terminal part of nisin electrostatically interact with negatively charged phosphor lipids. (2) Insertion into the membrane. Nisin takes an orientation parallel to the membrane surface, with the N-terminus slightly deeper inserted than the C-terminus. (3) The pore is formed, when the interaction of nisin with the anionic phospholipids results in bending of the lipid surface forming a wedge-like, non-specific pore. It is likely that this step is preceded by aggregation of nisin molecules. The lifetime of the pore is short, milliseconds and the nisin rapidly flips to the orientation parallel to the membrane (Driessen et al., 1995).

Models described above do not take into account specific interactions with an integral membrane component. There have been contradictions between the observed nisin activity results *in vitro* and *in vivo*. First, *in vivo* nisin is active at nM concentrations, which is 1000-fold more active than its effects on membranes composed of solely phospholipids. Second, it was observed for many mutant species that their activity on model membrane systems was almost equal to wild-type nisin, while their antimicrobial activity towards bacterial strains was significantly reduced. Third, the analysis of the phospholipid composition of bacterial strains showed that nisin sensitive and resistant strains both contain high amounts of negatively charged phospholipids. The small differences in the level of these lipids are unlikely to account for the large variations in their nisin sensitivity. Current research has indeed shown that nisin interacts with a precursor component of bacterium cell wall

synthesis, namely lipid II (Breukink et al., 1999; Wiedemann et al., 2001; Hsu et al.; 2002). So the model of nisin pore forming has been revised and lipid II has been taken as an essential factor to it. Very recent report suggested that lipid II is not only a docking molecule for nisin, but might even be part of the nisin pore; lipid II would most likely be situated at the outer boundaries of the pore complex (Breukink et al., 2003). It was also suggested that the pore is composed of five to eight nisin molecules and an identical number of lipid II (Breukink et al.; 2003). Figure 6 shows the latest pore-forming model of nisin.

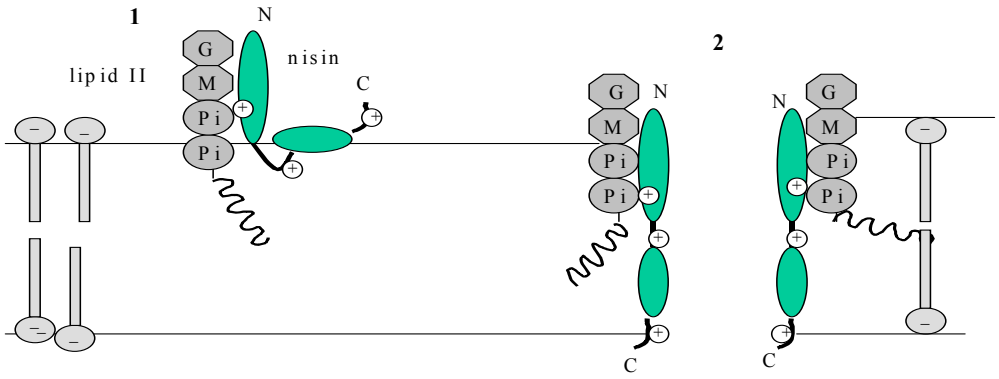

Figure 6: The lipid II model for the nisin pore-forming. (1) Nisin first binds to the outwardly orientated carbohydrate moiety of lipid II in a 1:1 stoichiometry. The N-terminal segment of the nisin is essential for the binding and a negative surface charge is not necessary. (2) The C-terminal part of nisin is then assumed to translocate across the membrane in accordance with the translocation found in the absence of lipid II (Kraaij et al., 1998). Several nisin/lipid II complexes are presumed to assemble the pore. Recent preliminary results with lipid II-doped artificial lipid bilayers indicated that the pore stability increases from the milliseconds range to several seconds and that the pore formation process becomes voltage-independent (Wiedemann et al., 2001).

While the overall mechanism of the cell death induced by a type-A lantibiotics has been characterised, details of the mechanism involved in the formation of the pores remain still

enigmatic. The model for nisin presented in the figure 6 is the latest and best view for the mechanism of pore-formation of the type-A lantibiotics. Studies made with Pep5 have shown that the mechanism of non-targeted pore-formation by nisin and Pep5 are very similar (Sahl et al., 1987). Thus it seems likely that the mechanism of pore-formation for other type A-lantibiotics will prove to be similar to that of nisin; that is including also the docking molecule as it already has been shown for epidermin (Brötz et al.; 1998).

3.5. Immunity

Immunity is here defined as the ability of the bacterial cell to protect itself from a particular bacteriocin that it produces itself. Immunity might be achieved by several strategies against the pore-forming bacteriocins like nisin. Adsorption of the bacteriocin to the membrane could be inhibited, membrane adsorbed bacteriocins could be translocated back to the environment or taken into the cytoplasm for degradation or transport. Several molecules of the bacteriocins are required to form a pore into the membrane of the target bacteria. This assembly process of the pore could be inhibited by specific interactions of the bacteriocin with the membrane associated immunity proteins. Secondly the assembled pore could be destabilized or the pore could be blocked by an immunity protein (Saris et al.; 1996)

There is no cross immunity between producers of lantibiotics. Nisin producers are sensitive to subtilin and vice versa, even though the structures of these lantibiotics are rather similar. This reveals that the interactions resulting in immunity are very specific. Cross immunity has been observed between strains producing natural variants such as nisin A and nisin Z (de Vos et al., 1993) or epidermin and gallidermin (Peschel and Götz, unpublished).

Inhibition by protein interactions at the surface of the cytoplasmic membrane seems to be a potential immunity mechanism of nisin producers. Recently it has been shown that nisin interacted with NisI (Stein et al.; 2003). This supports the idea that NisI intercepts nisin at the membrane surface giving immunity to the producer against nisin. In the case of nisin it was shown first that the peptide NisI, a putative hydrophobic lipoprotein that is considered to be attached to the outside of the membrane, is involved in the immunity process (Kuipers et al., 1993 ; Engelke et al., 1994). Overexpression of *nisI* in the cells that do not possess the lantibiotic biosynthesis machinery gave protection against nisin, although the protection levels was quite

low; only 1-4 % of that of the immunity level of the natural nisin producer (Kuipers et al., 1993; Engelke et al., 1994). A *nisI* mutant strain (no NisI production) still produced a significant amount of nisin (Siegers et al., 1995) and an in-frame disruption of *nisI* in *Lactococcus lactis* yielded a strain that could still produce nisin, albeit at levels five times lower than wild-type (Ra et al., 1999). Thus those *nisI* mutant strains still tolerated nisin indicating that additional factors are required for full self-protection. Also, the result that even very high expression of NisI in *L. lactis* gave only 25 % of the wild type immunity level against nisin supports the idea that there must be other immunity determinants (Takala et al., 2002).

The *nisFEG* genes downstream to the *nisZBTCIPRK* operon have been cloned and sequenced (Siegers et al., 1995; Immonen et al., 1998). The hydrophilic protein NisF and the hydrophobic proteins NisE and NisG show a very strong similarity to the ATP-binding cassette (ABC) transporters. These NisE and NisG proteins contain six potential transmembrane domains with structural homology to importers and exporters. Disruption of genes *nisF, nisE* and *nisG* respectively resulted in a decrease of nisin production and an increase in the sensitivity of the cells to the addition of nisin. Inhibition of translation of *nisEG*-mRNA and *nisG*-mRNA by antisense RNA produced from expression plasmids, also lowered the level of nisin immunity (Immonen and Saris, 1998). It has been also shown that a *L. lactis* strain initially sensitive to nisin can obtain about 20 % resistance of the wild type when it received a plasmid containing the *nisRKFEG* genes (Duan et al., 1996). Recently it was shown that NisF/E/G give immunity against nisin when expressed in the surrogate host *B. subtilis.* It was suggested that NisF/E/G system transported nisin back into the environment (Stein et al., 2003). In the same report it was indicated that maximum immunity level against nisin, about 30 % of that of the nisin producer, was achieved when expressing both NisI and NisF/E/G proteins (Stein et al.; 2003). These results imply that the NisF/G/E, a putative ABC-transporter, is involved in immunity to nisin. In Figure 7, a model of nisin immunity is presented.

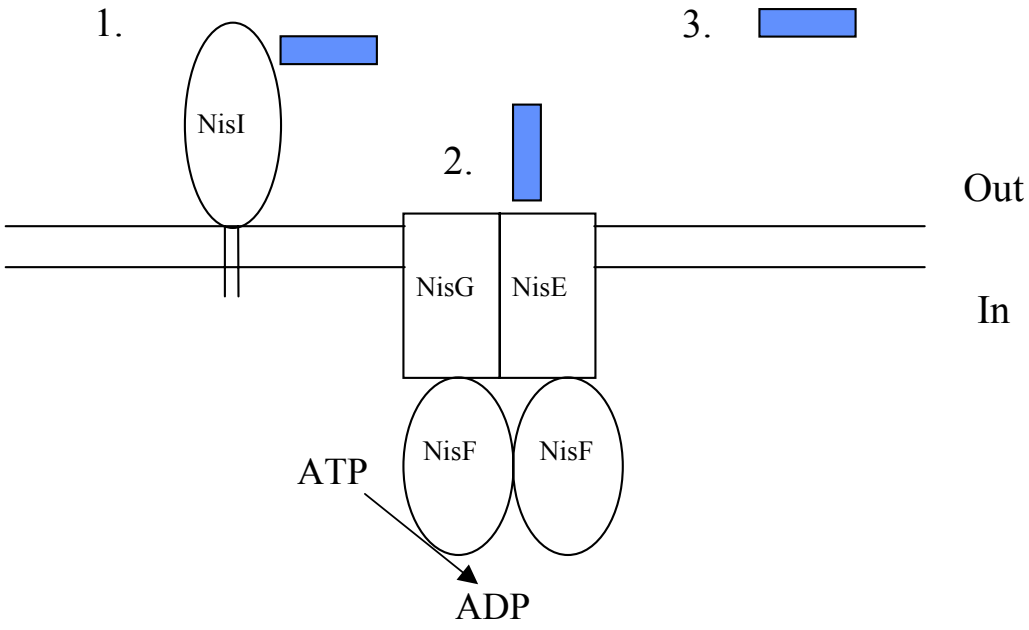

Figure 7: Mechanism of nisin immunity. According to the binding-protein hypothesis, external nisin is bound to the lipoprotein NisI (1), which might promote subsequent expel of nisin via NisFEG, the ABC-translocator (2) back to the environment (3). (Out = outside of the cell; In = inside of the cell.)

Inhibition of pore formation by protein interactions at the surface of the cytoplasmic membrane also seems to be a potential immunity mechanism of subtilin producers like that of nisin producers, as suggested by the location of the lipoprotein SpaI. The difference between these lantibiotics is that the SpaI lipoprotein is encoded in the same operon as the ABC transporter system. Lipoproteins encoded in the same operon as ABC transporters (Gilson et al., 1988; Russell et al., 1992, Sutcliffe et al., 1993; Tynkkynen et al., 1993) are gram-positive bacteria counterparts of the periplasmic binding proteins present in gram-negative import systems. By analogy to that one might speculate that the transporter system encoded by *spaFEG* genes is an importer. However, the results of Stein et al. (2003) suggest that that SpaFEG might be an exporter. The strains with the non-functional regulatory SpaR and SpaK proteins were also

sensitive to subtilin, indicating that they are needed in the signal transduction system to turn on the immunity genes. Thus the expression of immunity genes, in the case of subtilin and nisin, is also regulated by the concentration of the lantibiotic in the medium, which means that by sensing low (subinhibitory) amounts of the antimicrobial peptide in the medium, cells can rapidly increase their immunity level, concomitant with or even faster than the biosynthesis rate (de Ruyter et al., 1996). Recently it was shown that the *spaRK* two-component regulatory system, and hence subtilin biosynthesis and immunity, is under dual control of two independent regulatory systems; autoinduction via subtilin and positive transcriptional regulation via sigma factor H (Stein et al., 2002). Thus subtilin immunity seems to be more complex with respect to the regulation of the spaRK two-component regulatory system than that of nisin.

So far, there is no evidence for a lipoprotein-mediated immunity for epidermin or gallidermin producers. Therefore their producers appear to protect themselves with the putative ABC transporters like nisin and subtilin producers. It has been shown for epidermin that the ABC transporter acts by expelling the lantibiotic from the cytoplasmic membrane into the surrounding medium (Otto et al., 1998). The biosynthetic gene clusters of epidermin and gallidermin are distinguished by the presence of the unique genes *epiH* and *gdmH,* respectively. They encode accessory factors for the ATP-binding cassette transporters that mediate secretion of the peptides. Currently it was shown that GdmH protein of gallidermin gene cluster improved the immunity against gallidermin and was suggested to act synergistically with the GdmFEG system (Hille et al., 2000). Thus, like the nisin and subtilin gene clusters, the epidermin and gallidermin systems also seem to contain a second specific immunity system, H proteins. In contrast to the H proteins, however, NisI and SpaI are lipoproteins, and there is no evidence that they are involved in the secretion of nisin and subtilin, respectively (Klein et al., 1994; Kuipers et al., 1993).

The mechanism of Pep5 immunity seems to differ from that of nisin, subtilin and epidermin. It involves an interaction of the PepI protein with Pep5 at the outer surface of the cytoplasmic membrane. The structure of PepI does not indicate that any transport event would be involved in Pep5 immunity. Rather it is thought that the mechanism involves inhibition of pore formation by interactions on the outer surface of the cytoplasmic membrane. The elements needed for the immunity of Pep5 seem to be the *pepI* immunity gene and the *pepA* structural gene (Reis and Sahl, 1991). One striking feature of the Pep5 system seems to be the apparent coupling of the

immunity phenotype with the production of Pep5. However, Pag et al. (1999) have now demonstrated that *pepI* is sufficient for expression of Pep5 immunity. Coupling to Pep5 production is achieved at the transcriptional level through the stabilization of *pepI*-containing transcripts by means of an inverted repeat, which is located downstream of *pepA* . The presence of the terminator element rather than its position in the transcript was found to be important for mRNA stabilization, permitting the construction of hyper immune and eventually hyper producer strains (Pag et al., 1999).

Other mechanisms of immunity to type-A lantibiotics are also possible. An immunity mechanism involving inhibition of membrane adsorption or pore assembly of the lantibiotic might also contribute to the total level of immunity. Potentially all of the proteins in the biosynthetic machinery have affinity for the lantibiotic produced by the biosynthetic pathway in question and could therefore have direct interactions with the corresponding lantibiotic. The biosynthetic proteins form most likely a complex located in the membrane as in the case with NisBTC proteins, which have been shown to form a complex (Siegers et al., 1996; Kiesau et al., 1997). A wild type level of immunity might require a functional biosynthetic complex. This could be the situation in nisin immunity, because a wild type level of nisin immunity has been achieved only when all of the components of the biosynthetic machinery are present and production is evident. Additional proteins taking part in immunity might still be found. However, Ra et al. (1999) showed, by using a consensus sequence of nisin inducible promoter as a probe, that no other potentially nisin inducible promoters other than *nisA* and *nisF* appear to exist in *L. lactis*.

3.6. Structure/function relationships

Interest for detailed structure/function of lantibiotics comes from the possibility that knowledge of it would give tools for protein engineering. Thus the properties of lantibiotics could be improved or even artificially transported to other proteins that could be then used commercially. Structure/function relationships of lantibiotics can be evaluated from (1) the mode of action, (2) immunity, (3) biosynthetic pathway and (4) induction of operons. Almost nothing is known yet

about the structures on the molecular level affecting immunity whereas some data exists about those structures, which can affect induction. It is known that the first three lanthionine rings A, B and C are essential for induction (Kuipers et al., 1995; Dodd et al., 1996). Knowledge about the structures involved in the mode of action and the biosynthetic pathway are also in their infancy. Although membrane depolarization is generally accepted as the primary mode of action of nisin, little is known about the structural features responsible, and the contribution of individual amino acids is even less understood. Not much is known, except which amino acids are modified, about the structural signals that are essential for the nisin biosynthetic machinery in order to modify nisin.

Recent studies concerning the structures of the biological activity of nisin have indicated that primarily the N-terminal part (residues 1-22) penetrates into the lipid phase. Reduction of the flexibility at positions 20 - 21 has a negative effect on monolayer interaction and activity, and the C-terminal part is probably responsible for ionic interactions of nisin in a monomeric or oligomeric form with anionic lipids (Demel et al., 1996; Giffard et al., 1997; Breukink et al., 1997). In the N-terminal portion, the amphiphilicity seems to be an important aspect. Thus MeLan and Lan structures, which are ring structures, could be important in stabilizing the spatial structure of nisin (Sahl et al., 1995). The hydrophobic residues in this part of the molecule appear to interact with the membrane, while the hydrophilic residues are orientated outwards (van den Hooven et al., 1996). The importance of ring A of nisin for the biological activity has been most clearly shown. The integrity of ring A and the modified amino acid Dha5 as a hydrophobic moiety in an amphipathic region seem to be essential factors (Rollema et al., 1996). Recently, it has also been demonstrated that the ring C is important for the biological activity of nisin by replacing this Lan ring with a disulfide bond (van Kraaij et al., 2000). A study examining the interaction of lipid II and a number of mutant nisin species to identify structural elements of the nisin molecule involved found that mutations affecting the conformation of rings A through C led to reduced binding of lipid II and increased the concentration needed for pore formation (Wiedemann et al., 2000). The positive charges of the C-terminus (Lys 22, Lys 34, His 27, His 31) are important for the initial interaction of nisin with the target membrane, i.e. binding. The N-terminal portion charges, i.e. the terminus itself and the lysine at position 12, seems to have a minor effect on the initial binding of nisin (Breukink et al., 1997). The flexible hinge structure between rings C and D has been shown to be essential for the biological activity of nisin (Bierbaum et al., 1996). Two mutations at positions 20 and 21 (N20P/M21P) have been

made to restrict the mobility of the hinge and the results showed that those two prolines indeed reduced the activity of nisin (van de Ven et al., 1991). Recent studies have given hints for the function of individual amino acids, namely the lysines, in nisin pores (pores mean here holes that nisin makes into the bacterium membrane.) Those studies indicated that when the nisin molecule is in the pore-forming state, the positively charged lysines are probably situated such that this results in anion selectivity (Breukink et al., 1997). Furthermore lysine-12 appears to have a role in gating (i.e. regulating) the flow of charge through a nisin pore (Giffard et al., 1997).

The biosynthesis of lantibiotics of type-A seems to be a complex process. It is quite difficult to make changes in the sequence of the prepeptide that do not effect on the biosynthetic pathway; i.e. residues important for the interaction with the modification, export and processing systems and as well as in the regulation of expression. For example, the Phe-Asx-Leu-Asp/Glu motif in the leader peptide of nisin has been identified as being essential for nisin biosynthesis (van der Meer et al., 1994). However, not only the leader peptide, but also the propeptide part of nisin influences expression and/or maturation of the peptide. The Ser5 - Thr8 mutant of nisin Z was not expressed by a strain of *L. lactis* that did not contain an intact nisin gene (van de Meer et al., 1994; Kuipers et al., 1992, 1993). The same was found for mutants in the protease cleavage site of the leader peptide (Asp^{-4})nisinZ and (Gln^{-1})nisin Z; although mRNA transcripts of these peptides were present in the cell, the prepeptides could not be found. As the latter two peptides and (Thr^{5})nisin Z are recognized and processed by the modification system when the nisin A gene is present, one could speculate that biosynthesis of the modification system is regulated to some extent by a characteristic structural feature of the nisin precursor peptide (van de Meer et al., 1994).

Even less is known about the structures of other type-A lantibiotics than nisin concerning the detailed knowledge of structure/function relationships. Some examples are reviewed in the following text, which refer to the biological activity or the biosynthesis of the lantibiotics. Partially deleted epilancin K7 (4-33) is almost as active as the complete peptide indicating that the first three amino acids of the N-terminus might not be crucially important for the activity of epilancin K7 (van de Kemp et al., 1995). In contrast, a naturally occurring fragment of bacteriocin SA-FF22, devoid of the first four amino acids (i.e.SA-FF22 5-27), had no demonstrable biological activity (Jack and Tagg, 1991), suggesting that the N-terminal region of streptococcin SA-FF22 is necessary for activity. Naturally occurring subtilin with a succinylated N-terminus has also been reported to be less active (Chan et al., 1993). Also the succinylated

Pep5 showed reduced activity against intact gram-positive bacteria (Kordel et al., 1988). The experiments have focused so far on the importance of the dehydroamino acids and it has been suggested that these residues might interact with the sulfhydryl groups of the cell envelope of susceptible bacteria (Liu and Hansen, 1993). This interaction seems to be essential at least for sporicidal activity (ability to kill bacterium spores; spores are the resting stage of the bacterium) of subtilin, which might be mediated by an interaction with the sulfhydryl groups of the spore coat (Liu and Hansen, 1992). In subtilin the exchange of Dha5 to Ala led to the total loss of the inhibition of the outgrowth of *B. subtilis* spores, but the bactericidal activity, i.e. pore-formation in the cell membrane of vegetative cells of the same strain, seems to be independent of the dehydro residue (Liu and Hansen, 1993). Thus it seems that no generalizations can be made of the importance of certain structures for the function of all type A lantibiotics. However the flexible hinge between the amphiphilic N- and C-terminus, does appear to be important for the biological activity of all type A- lantibiotics. A mutant in the hinge region of Pep5 (K18P) and also a mutant of gallidermin (Dhb14P) showed decreased activity against their indicator strains (Bierbaum et al., 1994; Freund et al., 1991).

There are also examples of the effects of individual amino acids or structures on the biosynthesis of other type-A lantibiotics. The cysteine residues have been replaced in the primary sequence of Pep5 in order to prevent ring formation. The mutant C33A was overproduced, although the main peptide produced consisted only of amino acids 1-29, probably generated by proteolytic degradation behind position 29 (Bierbaum et al., 1994). One can speculate that one of the functions of the third ring is to protect the peptide against this proteolytic degradation. The B ring of this peptide was shown to be present. Another mutant of Pep5, C27A, was devoid of ring C in addition to the expected lack of ring B, which indicated that formation of ring C is hampered in the absence of ring B (Bierbaum et al., 1994 b). The results obtained by the study of mutants of epidermin indicated that formation of rings A and B is important for completion of epidermin biosynthesis and production. Two epidermin EMS-mutants, i.e. S3N and G10E could not be produced, probably because the mutations prevented the correct formation of ring A and ring B, respectively (Augustin et al., 1992). Also the amino acid changes that affect the thioether bridge formation at rings C and D, such as the deletion of the last two cysteine residues as well as the change of the Ser-19 residue into Ala-19 residue, resulted in the complete loss of epidermin production (Ottenwälder et al., 1994). These results

strongly indicate that formation of all thioether amino acids is important for the completion of type-A lantibiotic biosynthesis and secretion.

B. AIMS OF THE STUDY

The biological system behind nisin production, secretion and producer self-protection has proven to be both extensive and delicately regulated, requiring the interplay between at least eleven genes, namely *nisA/Z/QBTCIPRKFEG*. In spite of the wealth of novel information the exact function of the individual genes is so far not very well known. Therefore, the main goal of this study was to characterize the function of certain nisin genes by analysing their effects on the protein level and secondly the regulation of nisin operons.

1. to characterize the role of NisI in producer self-immunity, its distribution and interaction with nisin
2. to characterize the function of *nisP, nisB and nisC* genes in the biosynthetic pathway of nisin
3. to characterize the regulation of the nisin operons

C. MATERIALS AND METHODS

1. Bacterial strains, plasmids, media and growth conditions (I-IV)

The bacterial strains and plasmids used in this doctoral thesis are listed in the following tables. *Escherichia coli* strains were used as the primary gene cloning and expression host and were cultured at 37 °C in LB broth with shaking or on LB agar plates (I-IV). *Lactococcus lactis* strains were incubated at 30 °C in M17GS medium without shaking or on M17GS agar plates (I-IV). *Micrococcus luteus* was used as a nisin sensitive indicator and was grown at 37 °C in LB broth with shaking or on LB agar plates (I-IV). Bacterium strains used in the work IV on a nisin bioassay test were grown in LB medium at 37 °C or on LB agar plates except *Lactobacillus plantarum*, which was grown at 30° in MRS medium or on MRS agar plates. The media were supplemented with antibiotics, if needed, at the following concentrations: ampicillin 30 μg/ml or 100 μg/ml (for *E. coli)*, erythromycin 200 μg/ml (for *E. coli*) and 5 μg/ml (for *L. lactis*), kanamycin 50 μg/ml (for *E. coli*) and chloramphenicol 10 μg/ml (for *E.coli*) and 5 μg/ml (for *L. lactis*) (I-IV).

Strains used in this work.

Strain	**Relevant phenotype/genotype**	**References**	**Used in**
L. lactis N8	Nis^+ $Immunity^+$	1)	I-IV
L. lactis MG1614	Nis^- $Immunity^-$	2)	I-IV
L. lactis LAC34	Producing NisI		IV
E. coli Eco395	Producing GST-NisI fusion		IV
E. coli BL21 (DE3)	*Hsd*R17 *rec*A1 *gyr* A96 *thi*-1 *rel*A1 F^+ *opmT* r^-B m^-B(DE3)	4)	I -II
M. luteus *AL* NCIMB 86166	Nisin sensitivity	National collection of industrial and marine bacteria	IV
S. faecalis strain N	Nisin sensitivity	ARS Culture Collection	IV
B. subtilis *BRB1*	Nisin sensitivity	American Type culture collection	IV

Table continued

B. megaterium ATCC 13632	Nisin sensitivity	American Type culture collection	IV
B. stearothermophi*lus* ATCC 12980	Nisin sensitivity	Collection of the Finnish state Technological center	IV
B. amyloliguefacie*ns VTTE 18*	Nisin sensitivity	Collection of the Finnish state Technological center	IV
B. coagulans DMS 459	Nisin sensitivity	Deutsche Sammlung von Mikroorganismen	IV
B. natto BGSC27A1	Nisin sensitivity	Bacillus Genetic Stock Center	IV
Mutations in *L. Lactis*			
LAC53	*nisB* mutation	This thesis	II
LAC67	*nisZ* mutation	This thesis	II
LAC71	*nisP* mutation	This thesis	II
LAC104	*nisC* mutation	This thesis	III
LAC212	*nisC* mutation	This thesis	III
LAC214	*nisB* mutation	This thesis	III
NZ8940	*nisA, nisI* and *nisP* mutation	5)	IV

References within this table 1) Graeffe et al., 1991 2) Gasson, 1983 3) Hanahan, 1983 4) Studier & Moffatt, 1986. 5) Ra et al., 1999

The plasmid DNA of *E. coli* strains was isolated by alkaline lysis and purified further using the Magic miniprep kit (Promega, Madison, WI, USA) or silica particles (Carter et al., 1993) (I-II) and *L. lactis* plasmid DNA was isolated by the method of O`Sullivan (1993) (I-II). The molecular cloning protocols used were essentially according to Maniatis et al. (1982) (I-II). Plasmids were electroporated into *L. lactis* using the method of Holo et al. (1989) (I-II):

Plasmids used in this work.

Name of plasmids	**Resistance marker**	**Relevant feature**	**References/ used in**
***E. coli* plasmids**			
pBluescript	Amp	*LacZ,* T7, T3	Stratagene
pBAT-1	Amp	T7 promoter	1)
pCR II	Amp, Km	T/A vector	Invitrogen
pGX-2T	Amp, Km	GST	Pharmacia LKB
pGB301	Cm	*cat* gene	2)
pPUC6S	Ap	vector	3)
pLEB21	Ap, Em	*ery* gene	4)
pLEB379	Ap	*nisI* expression (sense)	I
pLEB381	Ap	*nisI* expression (antisense)	I
pLEB423	Ap	*nisI-gst* fusion gene	I, IV
***E. coli* and *L. lactis* plasmids**			
pLEB384	Em, Cm	*nisZ* expression vector	II
pLEB415	Em	*nisI* expression vector	I
pLEB561	Em	*nisZ* with His-tag	III
pLEB563	Em, Cm	*nisZ*with His-tag	III
pLEB 124	Em	expression vector P_{46} promotor	III
pT*Clux*Hb	Em	*nisC* complementation plasmid	III
pLEB507	Em	*nisC* complementation plasmid	III
pLEB544	Em	His-tagged *nisZ*	III
pKTH1984	Em	*nisZ*	III
***E. coli* and *L. lactis* mutant constructions**			
pLEB281	Em	*nisP* gene mutation	II
pLEB320	Em	*nisZ* gene mutation	II
pLEB329	Em	*nisB* gene mutation	II, III
pLEB406	Em	*nisC* gene mutation	III

References within this table 1) Peränen et al., 1987 2) Behnke et al., 1981 3) Viera and Messing , 1991 4) Axelsson et al. 1988

2. Methods

The methods used in this thesis are listed in the table with the reference to the work in which they have been used.

Methods used in this work.

Method	**Purpose**	**Used in**
DNA and RNA techniques:		
Cloning	Plasmid constructions	I-IV
PCR	Amplification of DNA	I, II
Southern blotting	Analyses of DNA	II
Northern blotting	Analyses of RNA	II
In vitro transcription	Producing of mRNA	I
In vitro translation	Production of protein	I
Protein techniques:		
SDS-PAGE	Analyses of protein	I-IV
Tris-Tricine SDS-PAGE	Analyses of protein	II-IV
Western blotting	Analyses of protein	I-IV
HPLC-RP	Purification of nisin	I-IV
HPLC-MonoQ	Purification of NisI	IV
Hydrophobic chromatography	Purification of nisin	IV
His Trap chromatography	Purification of His-Tag nisin	III
Gel filtration chromatography	Purification of NisI	I, IV
Affinity chromatography	Purification of GST-NisI protein	I, IV
Lipoprotein labelling	NisI	I
N-terminal sequencing	NisI	II, III
MS-spectrometry	NisI	III
Nisin bioassays:		
Nisin activity test	Nisin activity	II-IV
Immunity test	Nisin immunity of bacterial strains	I, II, IV

D. RESULTS AND DISCUSSION

1. Characterization of the NisI polypeptide

1.1. Localization of the NisI

1.1.1 The cellular location of NisI lipoprotein

Since the amino acid sequence of NisI contains a consensus lipoprotein-processing site (von Heijne 1989), it is assumed to be located on the outer surface of the bacterial membrane. There were no experimental data of the location of NisI. Therefore, experiments were done in order to show that NisI is truly located on the outer surface of cell membranes.

The expression plasmid pLEB379 containing the *nisI* gene in sense orientation under the inducible T7 promoter was used for *in vitro* transcription and translation of the *nisI* gene, and for labeling of the lipoproteins with [^{3}H] palmitic acid (I). Analysis of the proteins produced by *in vitro* translation using SDS-PAGE and autoradiography showed two bands (32 kDa and 33 kDa). Kuipers et al. (1993) have reported bands of similar size for NisI produced and labeled *in vivo* in *E. coli.* Labeling of the lipoproteins in *E. coli* strain ECO395 under IPTG induction also showed two bands. Since the labeling was done in palmitic acid, only those proteins with the fatty acid tail attached to the cytoplasmic membrane could be detected (I). One explanation for the two sizes of the NisI lipoprotein is that one represents the form with the signal sequence still attached. Western analysis of the amount of NisI in the cytoplasmic membranes of ECO395 and LAC34 harboring the *nisI* gene expression plasmid showed that the NisI protein was located in the cytoplasmic membrane both in the *E. coli* ECO395 cells grown with IPTG induction and in the *L. lactis* strain LAC34 (I). These results demonstrated that the NisI protein is a lipoprotein and that it is located on the cytoplasmic membrane.

1.1.2. The soluble NisI protein

If the signal sequence of a polypeptide aimed for lipid modification is cleaved before the lipid modification, then the polypeptide can escape the lipid modification and be secreted out into the growth medium. Recently it has been shown that in the heterologous bacterium, *B. subtilis,* where the *nisI* gene was removed under the control of the inducible promoter P_{spac}, half of the produced NisI was secreted into the medium (Stein et al., 2003). The extent to which this occurs in the nisin producer, *L. lactis* N8 was analysed. The location of NisI in different growth stages of the *L. lactis* N8 was analysed by growing the cells, taking samples from different growth stages and analysing the amount of NisI in the cells and the cell-free growth medium by Western analysis (IV). The results showed that during the first hours of growth almost all of the produced NisI was cell membrane associated but in the later stages of growth an increasing amount of NisI was released and found in the growth medium. After growth for seven hours, there were almost equal amounts of the membrane-bound and soluble NisI. Thus it was demonstrated that NisI is secreted similarly in the natural nisin producer N8 as in the heterologous *B. subtilis.* This would refer that the secretion of NisI is a biological phenomenon and not only a result of over expression as it could be in the *B. subtilis* host. Thus we have shown that NisI can exist in two forms; i.e. membrane-bound lipoprotein and secreted, lipid-free soluble protein, named here LF-NisI (I, IV).

1.2. The biological function of NisI protein

1.2.1 Nisin immunity

Bacterial strains producing antibiotic substances have to protect themselves from their own product. Among bacteriocin producers, the protection mechanism is typically based on dedicated peptides or proteins, so called immunity proteins, which specifically antagonise the bacteriocin (Konisky, 1982; James et al., 1992). NisI has been proposed to be such an immunity protein for nisin (Kuipers et al., 1993; Saris et al., 1996; Stein et al., 2003).

We studied the effect of NisI on nisin immunity by expressing it in two different bacterial strains - *E. coli* ECO395 and *L. lactis* LAC34. These strains produce NisI but none of the other proteins encoded by the nisin operons. The results of the Western analysis showed that the amounts of

NisI in the cytoplasmic membrane were comparable to that of the nisin producer *L. lactis* N8 (I). Expression of NisI was not able to protect the cells against the lethal effect of nisin in *E. coli* ECO395 and only 1 - 2 % of the immunity level of the wild type strain N8 could be achieved by expressing NisI in the strain *L. lactis* LAC34 (I). Kuipers et al. (1993) reported that the *L. lactis* MG1614 strain harbouring *nisI* expression plasmids showed only 1-4 % of wild type nisin immunity. Engelke et al. (1994) also reported an increase in the immunity level of the MG1614 strain containing the *nisI* gene under the control of the constitutive promoter P_{32}. In those reports the level of expression and the cellular location of the NisI protein was not studied. Recently Takala et al. (2002) achieved 25% immunity level of that of nisin producer N8 while expressing high levels of NisI in nisin sensitive *lactococci.* These results are showing clearly that additional immunity determinants are needed for wild-type level of nisin immunity.

Stein et al. (2003) have showed that NisI and nisin interacted physically with and without the lipid anchor of NisI. They suggest that membrane bound NisI would intercept nisin at the surface of the cytoplasmic membrane and by sequestering nisin, prevent it from inserting into the membrane and/or prevent high local density of nisin molecules necessary for pore formation. We have also shown that lipid-free NisI interacted with nisin using CD-spectroscopy and surface plasmon resonance analysis (Qiao, 1996). According to surface plasmon analysis the interaction is rather weak and NisI seems to interact with nisin aggregates. Interestingly the molecular ratio of NisI:nisin on the cell membrane of *L. lactis* N8 is during the growth of bacterium about 1:10 (IV). This result supports the assumption that NisI interacts with nisin aggregates. Thus the immunity mechanism could be that NisI intercepts rather with nisin aggregates than single nisin molecules on the membrane surface.

We have shown that nisin producer N8 secreted considerably amounts of NisI into the growth medium (IV). Thus it could be postulated that secretion of NisI would be part of the immunity mechanism. LF-NisI had no affinity to cells, nor self-aggregated to such extent that it would have precipitated in the conditions used for precipitation of the cells (IV). Therefore, one potential immunity mechanism of LF-NisI could be to bind nisin after secretion through the membrane and transport nisin away from the cellular surfaces.

However, even so, soluble and membrane bound NisI are clearly not enough for development of full immunity. Therefore, other determinants are needed for the wild type level of nisin

immunity. One possible candidate for NisI to co-operate with is the putative transport system encoded by the *nisFEG* genes (Siegers and Entian, 1995; Immonen et al, 1998; Ra et al., 1999), which has been shown to give a certain degree of resistance to nisin in the initially nisin sensitive *L. lactis* strain (Duan et al., 1996). Most probably both NisI and NisFEG proteins are needed for development of full nisin immunity. Recent report of Stein et al. (2003) supports this hypothesis. They achieved maximum immunity, about 30 % of the nisin immunity level of nisin producer *L. lactis* 6F3, when they expressed together NisI and NisFEG in the surrogate host *Bacillus subtilis*. However, it is possible that to achieve the wild type level of immunity, co-operation of NisI and NisFEG with other proteins encoded by nisin operons is needed. Thus the challenge still exists to unravel the complete mechanism of immunity at the molecular level.

1.2.2. Enhancement of the activity of nisin

Since NisI is an immunity protein we analysed if the activity of nisin could be inhibited with the purified LF-NisI polypeptide. Therefore, nisin and LF-NisI were incubated together and the nisin activity of the mixture was measured using the nisin sensitive indicator strain *Micrococcus luteus*. Remarkably incubation of soluble LF-NisI with nisin actually enhanced the effect of nisin against indicator bacteria instead of inhibiting the bacteriocidal effect of nisin (IV). This was a totally new and unexpected observation. Not only did LF-NisI stimulate activity of nisin against *M. luteus* and *L. lactis* but also against several other gram-positive indicator bacteria. Addition of LF-NisI to nisin resulted at best in a twenty-fold increase of the activity of nisin. However, LF-NisI could not stimulate the killing effect of nisin on gram-negative bacteria. These bacteria are sensitive to nisin only if their outer membrane has been destabilized, for example by EDTA (Stevens et al., 1991; Stevens et al., 1992). The NisI-nisin complex might be too large to pass through even a deformed outer membrane. Interestingly enhancement of the nisin activity was also not seen against *L. lactis* strain NZ9840, which contains active NisFEG immunity system (IV). Thus it might be concluded that NisFEG can handle similarly nisin and possible complex of nisin and NisI. If the LF-NisI:nisin ratio in the mixture was lower than 0.2, no stimulation of nisin activity could be observed and on the other hand the stimulation was not enhanced further by increasing the LF-NisI: nisin ratio above 2 (IV). This could imply that one molecule of soluble LF-NisI could bind up to five nisin molecules closely together and that might then enhance the nisin activity, e.g. by promoting the aggregation of nisin molecules

which is potentially one step of the mechanism of nisin action. A high salt concentration and addition of detergent decreased the interaction of nisin and LF-NisI, as judged from loss of stimulatory effect of NisI on nisin activity (IV). This indicates that the nisin-NisI interaction involves both electrostatic forces and an interaction with the hydrophobic parts of the amphiphilic nisin polypeptide.

In order to further study the biological significance of the LF-NisI enhancement for the nisin activity we determined the ratio of NisI:nisin in the growth medium. The ratio was on the avarage 1:50 NisI molecules per nisin. Thus in the biological system the ratio is quite low and so the significance of NisI enhancement of nisin activity might be minor. At least the amount of nisin has to be locally decreased; for example nisin can be adsorbed to the particles of the growth medium. Nisin is known to be a very sticky molecule. Depending on concentration, volume and pH up to 75 % of nisin content in the plastic tube can be lost because of the adsorption to the surface (Joosteen and Nunez; 1995).

However, our result could indicate that secretion of NisI by the nisin producer has a biological function and that NisI has a dual role as a membrane bound immunity protein and in the soluble form as a factor that enhances the nisin activity. LF-NisI might also take part into the nisin immunity. The mechanism by which NisI stimulates nisin activity is unknown and requires further study. As it has been shown LF-NisI can interact with nisin and thus it might concentrate nisin molecules *in vivo* and this way enhances the bioactivity of nisin.

2. The biosynthetic pathway of nisin

2.1. The nisin operons

The biosynthesis of nisin is encoded by the operons *nisA/ZBTCIPRK* and *nisFEG* (Kuipers et al., 1993; van der Meer et al., 1993; Engelke et al., 1994; Immonen et al., 1995; Siegers and Entian 1995; Immonen et al., 1998). In order to evaluate the biological function of the polypeptides

encoded by the genes *nisZ, nisB, nisC* and *nisP,* mutations in those genes of the biosynthetic *nisZBTCIPRK* nisin operon were made by gene replacement or integration of a plasmid.

The *nisZ* mutation in the *nisin Z* operon of the nisin producer strain *L. lactis* N8 was made by replacing the 3`-part of the *nisZ* gene with the erythromycin resistance marker (Axelsson et al., 1988) of plasmid pLEB320 (II). In this plasmid, the *erm* gene was flanked by a DNA region upstream (-970 to -27 bp) and downstream (+57 to +162 bp) of the *nisZ* gene (Immonen et al., 1995) in order to provide homology for recombination. The *nisZ* mutant strain was named LAC67 (II).

The *nisB*, *nisC* and *nisP* mutations in the nisin Z operon of *L. lactis* N8 were constructed by integration of the plasmids pLEB329, pLEB406 and pLEB281 respectively, which contain a replicon from gram-negative bacteria (not replicating in *L. lactis*), an erythromycin resistance marker and an internal fragment of the gene to be knocked out as the homology region for intergration (II). The *nisB*, *nisC* and *nisP* mutant strains were named LAC53 (II), LAC104 (III) and LAC71 (II).

The results showed that mutant strains did not secrete active nisin (II, III). This was quite expected and supported the idea that all of these four genes are truly needed for the production of active nisin. The nisin immunity level of the LAC67, LAC53, LAC104 and LAC71 strains with mutations in the nisin biosynthetic operon was strongly reduced (II). In addition, immunodetection with NisI antisera showed that the level of the lipoprotein NisI located on the cytoplasmic membrane of these mutant strains was low (II, III). Thus the mutation in the genes of *nisZ, nisB*, *nisC* and *nisP* seemed to have an effect on the expression of NisI, which is one factor of the nisin immunity system of *L. lactis*. Northern analysis was carried out in order to evaluate potential polar effects. Northern analysis of total RNA from these strains showed that the transcription level of the nisin operons *nisZBTCIPRK* and *nisFEG* was reduced (II). Thus this result as well as the results of Ra et al. (1996 and 1999) indicated that mutations that knock out nisin synthesis, i.e. maturation, modification or translocation of nisin also affect negatively the transcription of the nisin operons. Therefore, it is likely that the decrease in the immunity level of these strains was due to a lowered transcriptional activity and that the regulatory circuit was broken, resulting in a low expression level of the nisin immunity proteins.

2.2. Induction of the nisin operons

In the presence of externally added nisin, the nisin immunity level of our mutant strains was increased (II). Western analysis using the anti-NisI antisera KH 1422 showed that the level of NisI protein located on the cytoplasmic membrane was also increased compared to the situation when no external nisin was added to the growth medium of mutant strains (II). Northern blot analysis using total RNA from various mutant strains using *nisZ*, *nisI* and *nisE* gene fragments as probes showed that transcription of the nisin operon was activated when external nisin was provided (II). The size of the detected transcripts indicated that the *nisZBTCIPRK* gene cluster forms one transcriptional unit and the *nisFEG* gene cluster forms another. These results suggested that the nisin operons are positively autoregulated by the external nisin and that they create a single regulon. Kuipers et al., (1996) have shown independently that the *nisA* promoter is also autoregulated by nisin. This autoregulation is probably mediated by the two-component regulatory pair NisR/NisK encoded by the two last genes in the biosynthetic nisin operon as shown by several studies (van de Meer et al., 1993; Engelke et al., 1994; Immonen et al., 1995). In two-component signal transduction, the histidine kinase recognizes an environmental signal and phosphorylates a cytosolic factor, which stimulates the transcription of specific genes by binding to specific sequences close to the promoter to be activated (Albright et al., 1989). The results presented here suggest that nisin is the environmental signal recognized by NisK, thus regulating its own production.

2.3. The function of NisP

The *nisP* gene encodes a putative protein of 682 amino acids with a molecular mass of 74.7 kDa. NisP belongs to the group of lantibiotic proteins that show strong homology to an exocellular membrane bound subtilisin-like serine protease (van de Meer et al., 1993; Engelke et al., 1994). Furthermore, when compared to other serine proteases, the enzyme is characterised by a C-terminal extension, which might act as a membrane anchor. This suggests that NisP may be

transported out of the cell and then anchored in the cytoplasmic membrane (van de Meer et al., 1993). NisP is a protease and most likely cuts off the nisin leader of the prepeptide as the last step of biosynthesis producing active, mature nisin (van der Meer et al., 1993).

Van der Meer et al. (1993) showed that a cell extract of *E. coli* overproducing NisP could cleave the nisin leader from the precursor but that the expression of the *nisABTCP* genes in *L. lactis* MG 1614 resulted in the secretion of a 6 kDa nisin precursor with the leader still attached in spite of the precence of the *nisP* gene in the plasmid. This raised the question if some other protease was responsible for the cleavage of the nisin leader in nisin producers. In order to verify the function of the *nisP* gene, we constructed a *nisP* mutant strain, LAC 71(II). The strain with a mutation in the *nisP* gene secreted fully modified nisin with the leader peptide still at the N-terminus. The lack of NisP activity, however, could be compensated by treatment with trypsin or an incubation of the growth culture of LAC71 with heat-killed cells of the wild type strain N8 (II). The LAC71 was cured from the pLEB281 plasmid by growing the cells for 100 generations without selection. Curing of the plasmid resulted in wild type nisin production and immunity (II). This result indicated that the phenotype of strain LAC71 was not due to any secondary mutation elsewhere in the chromosome (II). Our result strongly suggested that NisP is the nisin leader protease and supports van der Meer`s observation (1993) that NisP cleaves the leader of the modified nisin precursor resulting in biologically active nisin.

2.4. The function of NisB and NisC

The *nisA/ZBTCIPRKFEG* gene cluster in *L. lactis* encodes biosynthesis, regulation and immunity of nisin. NisB and NisC are regarded as potential enzymes carrying out the dehydration reactions and lanthionine formation during nisin maturation because polypeptides homologous to NisB and NisC are encoded only by operons required for the biosynthesis of other lantibiotics (Siezen et al., 1996). The NisB protein, which is a protein of 993 amino acid residues in size, is the putative enzyme that catalyzes dehydration of the Ser and Thr residues in the propeptide domain of nisin (Jack et al., 1995). NisC, a 418 amino acid residue protein, is assumed to be the thioether-forming enzyme (Kuipers et al., 1993). It was currently shown that NisC might be a member of a growing family of proteins that utilize zinc for activation of thiol substrates (Okeley et al., 2003). Both NisB and NisC are bound to the membrane (Engelke et al., 1992; Siegers et al., 1996). However, their function is not completely understood and no

conclusive evidence for their putative function has been presented. Knocking out the *nisB* or *nisC* genes results in loss of nisin production (Siegers et al., 1996; Ra et al., 1999). Gene deletions within *spaB* and *spaC* of subtilin totally abolished subtilin synthesis, whereas similar deletions in *pepB* and *pepC* caused an accumulation of unmodified Pep5 prepeptides. The *pepC* mutant secreted completely dehydrated (serine and threonine) Pep5 peptides. Only one of the Pep5 fragments secreted by the *pepC* mutant contained a single lanthionine residue instead of the three expected, whereas the remaining cysteine residues were unmodified. These results are convincing evidence that LanB proteins are responsible for the dehydration reaction, and that the LanC proteins are needed for correct formation of the lanthionine rings (Meyer et al., 1995). Recently, Karakas et al. (1999) showed that the dehydration level of a mutant nisin could be increased by overexpressing NisB, supporting the proposal that NisB seems to be involved in the dehydration process.

In order to study the function of NisB and NisC gene products of the nisin Z producer *L. lactis* N8, a plasmid encoding a His-tagged nisin precursor was constructed (III) and introduced to *nisB, nisC* and *nis*A mutant strain resulting in strains LAC214, LAC212 and LAC208, respectively. The LAC208 (*nisA*) strain did not produce any nisin activity, but if the cells were induced with native nisin, then nisin activity could be detected from the growth supernatant (III). This suggested that the C-terminal His-tag affected the inductive capacity of the fusion protein. An intact C-terminus seems to be important for nisin to be able to cause induction. The fact that nisin activity could be produced into the growth medium indicated that even if the His-tag impaired the inductive potential, the His-tagged precursor was still a functional substrate for the NisB and NisC enzymes and the His-tag did also not inhibit transport of the modified precursor. Therefore, the His-tagged nisin precursor can be used to monitor the effects of the *nisB* and *nisC* mutations on nisin modification. For this purpose, the His-tagged precursor was purified from nisin induced LAC214 and LAC212 cells, since no His-tagged prenisin could be detected in the growth medium. This indicated that in contrary to the effect of *pepB* and *pepC* mutations, which resulted in secretion of partially modified Pep5 polypeptides and fragments, secretion of prenisin was inhibited by a lack of either NisB or NisC activity. The purified His-tagged prenisin was analysed by N-terminal sequencing and SDS-PAGE (III). The result of these studies showed that the leader sequence was not cleaved and that no modification of serine, threonine and cysteine residues in the leader sequence had occurred. In normal nisin biosynthesis, no modification of these residues in the leader that potentially could be modified, occur. Clearly, knocking out the function of either one

of the putative modification enzymes does not upset the system, since at least the specificity of the putative dehydration still remains. Dehydration of serine and threonine residues results in a decrease of the molecular weight by 18 Da per dehydration. Since SDS-PAGE analysis cannot discriminate such small differences in the molecular mass, the purified His-tagged nisin precursors were analysed using a mass spectrometer. The molecular mass of the His-tagged nisin precursor isolated from the *nisB* mutant LAC214 corresponded to the calculated mass of an unmodified His-tagged nisin precursor (III), whereas the molecular mass of the majority of the His-tagged nisin precursor from the *nisC* mutant LAC212 corresponded to an almost completely dehydrated (all Ser and Thr residues except for those residues in the leader) His-tagged nisin precursor. Part of the polypeptides analysed from strain LAC212 had a slightly larger molecular mass (18 Da, 36 Da, 54 Da, etc) potentially representing partly dehydrated His-tagged nisin precursors. These results strongly suggest that NisB is the enzyme required for dehydration of serine and threonine residues in nisin maturation. Lanthionine formation does not change the mass (condensation). Therefore, other methods have to be used in order to determine if the modified His-tagged nisin precursors isolated from the LAC214 strain also contain lanthionine residues. Fully modified nisin with the leader has almost no activity and cleavage of the leader by trypsin activates nisin (II). The His-tagged nisin precursor from the LAC214 strain was treated with trypsin and potential nisin activity was analysed. Trypsin treatment did not activate the potentially dehydrated nisin precursor suggesting that lanthionine formation in the LAC214 strain is affected in a way that at least wild type lanthionine formation does not occur. We cannot exclude the possibility that some of the lanthionines are formed as in the case of the *pepC* mutant (Meyer et al., 1995). Additional experiments are needed for analysis of the extent of lanthionine formation in the His-tagged nisin precursor isolated from strain LAC214.

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E. Concluding remarks

The role of NisI protein as a determinant in the self-immunity of nisin producers was studied. It was shown that NisI cannot confer alone the wild type level of immunity and that other factors are also needed. Most probably, the lipoprotein NisI together with the transporter composed of proteins NisFEG assembles the immunity system of nisin producers. Expression of only NisI and NisFEG proteins in a nisin sensitive *L. lactis* in a wild type level would be needed in order to see if they can together produce a wild type level of nisin immunity. However, before such an experiment is done the possibility of unknown factors affecting nisin immunity level still exist, for example complete immunity against nisin might need ongoing nisin biosynthesis.

Based on sequence information NisI had been proposed to be a membrane-bound lipoprotein. Our results proved that this assumption was true. In addition, it was also shown that there is a soluble form of NisI, which is secreted out into the bacterium growth medium. Taking together our results indicated that NisI can exist in two forms e.g. a membrane-bound form and a soluble form and that these two forms of NisI seem to have different functions – the membrane-bound NisI is a factor involved in the self-immunity of nisin producers whereas the soluble NisI can act as an enhancer of the activity of nisin. In our *in vitro* assays it was shown that the biological activity against indicator bacteria of the incubation mixture of purified soluble NisI and nisin was enhanced compared to that of pure nisin alone. This was an unexpected discovery, as an immunity protein should potentially have inhibited the biological activity of nisin.

A gene cluster, *nisZBTCIPRKFEG*, encodes nisin production. It is known that the *nisRK* gene products are essential for regulation of nisin biosynthesis, but the nature of the environmental stimulus activating the regulatory pathway was not known. Mutations in the *nisZ, nisB, nisC and nisP* genes of the biosynthetic *nisZBTCIPRK* nisin operon were made by gene replacement or integration of a plasmid. The mutations caused a drastic decrease of the transcription from the promoters upstream of the *nisZBTCIPRK* and *nisFEG* operons resulting in loss of nisin production and nisin immunity. The transcription of the nisin operons and nisin immunity could be partially restored by adding nisin to the growth medium of the bacterium. Thus our results

indicated that nisin is the environmental stimulus and the nisin operons are positively autoregulated via nisin and are in the same regulon.

The functions of *nisP, nisB* and *nisC* in the biosynthetic pathway of nisin were studied. The *nisP* mutant strain, LAC71 secreted inactive nisin in which the leader part of the molecule was still attached. This result supported information in the literature that the role of the NisP protein is to cleave the leader part of nisin to produce active nisin as the final step of nisin maturation.

Analysis of a His-tagged nisin precursor isolated from the *nisB* and *nisC* knock-out strains showed that no dehydration occurred if NisB was lacking whereas if NisB was functional but NisC was missing, then dehydration occurred but normal lanthionine formation was disturbed. This clearly suggested that NisB is responsible for dehydration and NisC for lanthionine formation in nisin maturation.

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ORIGINAL PUBLICATIONS

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