HEINRICH HEINE
UNIVERSITÄT DÜSSELDORF

Characterization of the nisin immunity system

NisI and NisFEG from *Lactococcus lactis*

Inaugural dissertation

For the attainment of the title of doctor in the Faculty of Mathematics and Natural Sciences at the Heinrich Heine University Düsseldorf

Presented by

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ABSTRACT

Nisin is one of the natural antimicrobial peptides, more specifically lantibiotic produced by some *Lactococcus lactis* strains as part of their defense system against other Gram–positive bacteria. Nisin is considered a model for this peptide family, which has a nanomolar bactericidal activity due to the presence of five specific lanthionine rings. This activity has been thoroughly studied and it was shown that nisin has different modes of action of which the most prominent one is the ability to form stable pores in the target membrane, with a ratio of 4 Lipid II to 8 Nisin molecules. The producer strain expresses an immunity system against nisin consisting of a lipoprotein, NisI, and an ABC transporter, NisFEG (Chapter I).

The aim of this thesis was the investigation of the function of each part of the immunity system by studying the interaction between NisI or NisFEG with nisin.

NisI and NisFEG provide immunity against nisin when expressed separately in *L. lactis* (Chapter III and IV). When NisI is present, cells stop growing at nisin concentration above 70 nM. Still, the NisI protein showed a unique ability to inhibit pore formation by nisin even at concentrations of up to 1000 nM. This inhibition was mediated by the C-terminus since its deletion resulted in cells, which were not able to cope with such high concentrations (Chapter III). The NisI-expressing cells showed a special morphology, where the cells appear to arrange themselves in long chains rather than the normal double cocci observed for *L. lactis* and they stopped growing.

In contrast the ABC transporter NisFEG was able to protect the cells from nisin up to 60 nM. At higher concentration the NisFEG-expressing cells suffered from pore formation (Chapter IV). Furthermore NisFEG depends on the last six amino acids as well as the last lanthionine ring of nisin to confer its maximal immunity. When both, NisI and NisFEG, are expressed together the full immunity is revealed (Chapter V)

Based on these data, a model of the immunity system against nisin was proposed, where initially NisFEG confers immunity at low levels of nisin. When the concentration of nisin rises, NisI is able to confer even higher immunity by shielding Lipid II and changing the morphology of the cells.

ZUSAMMENFASSUNG

Nisin wird von einigen Stämmen des Milchsäure Bakteriums *Lactococcus lactis* produziert und gehört zur Klasse der Lantibiotika. Diese antimikrobiellen Peptide werden als Bestandteil der bakteriellen Abwehr gegen fremde, Gram-positive Bakterien produziert. Die antimikrobielle Aktivität von Nisin wurde in der Vergangenheit intensiv untersucht und Nisin fungiert daher als das Modelsystem für diese Peptidfamilie. Nisin beinhaltet fünf charakteristische Lanthioninringe, welche für die antimikrobielle Aktivität verantwortlich sind.

Der Reaktionsmechanismus von Nisin umfasst unter anderem die Komplexbildung mit dem Zielmolekül Lipid II in einem Verhältnis von vier Lipid II Molekülen und acht Nisin Molekülen. Dieser Nisin-Lipid II Komplex bildet Poren in der Plasmamembran, welche zum Zelltod führen. Um sich gegen das selbst produzierte Lantibiotika zu schützen, besitzen Nisin produzierenden Bakterienstämme ein Immunsystem bestehend aus einem Lipoprotein NisI und einem ABC-Transporter NisFEG (Kapitel 1).

Das Ziel dieser Doktorarbeit war die Untersuchung dieses Zwei-Komponenten-Immunsystems im Hinblick auf die Interaktion mit Nisin.

In Kapitel III wurde der Anteil des Proteins NisI im Hinblick auf die Immunität gegenüber Nisin untersucht. *L. lactis* Zellen, welche das Protein NisI produzieren, zeigen ungehindertes Zellwachstum bis zu einer Nisin-Konzentration von 70 nM. Darüber hinaus wird die Porenformation von Nisin bis zu einer Konzentration von 1000 nM verhindert. Diese Inhibierung wird durch den C-terminus von NisI vermittelt, da eine Deletion des C-Terminus zu einem verminderten Zellwachstum unter hohen Nisin Konzentrationen führt. Eine Kombination aus simultaner Anwesenheit von NisI und Nisin führt zusätzlich zu einer Änderung der Zellmorphologie, wodurch lange, kettenförmige Bakterienansammlungen charakterisiert sind. Dieser Phänotyp weicht deutlich von der typischen Diplokokken Morphologie ab. Außerdem wird das Zellwachstum eingestellt.

In Kapitel IV wurde der Immunitätsbeitrag des ABC-Transporters NisFEG charakterisiert. NisFEG verleiht *L. lactis* Zellen Immunität gegenüber Nisin bis zu einer Konzentration von 60 nM. Wenn die Nisin Konzentration diesen Schwellenwert überschreitet, bilden sich wiederum Poren in der Plasmamembran. Die Aktivität von NisFEG ist spezifisch von den letzten sechs Aminosäuren des C-Terminus von Nisin als auch von der Anwesenheit des fünften Lanthioninrings abhängig. Die simultane Präsenz beider Immunitätssysteme, NisI und NisFEG, verleiht *L. lactis* Zellen erst die vollständige Immunität (Kapitel V).

Aufbauend auf diesen Daten wurde ein Model, welches die Immunität von Nisin produzierenden *L. lactis* Stämmen erklärt, erarbeitet. In diesem Model verleiht NisFEG eine initiale Grundimmunität gegen niedrige Nisin Konzentrationen. NisI sorgt darüber hinaus für Immunität bei steigenden Nisin Konzentrationen indem das Zielmolekül Lipid II abgeschirmt wird und die Zellen in eine lange, kettenförmige Morphologie übergehen.

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INTRODUCTION

Antibiotics & Antibiotic resistance

Antibiotics are natural compounds produced by a wide range of microorganisms as defense agents against other competitive bacteria to survive in their habitat. The discovery of antibiotics is considered as the start of the golden age for humans in their fight against fatal pathogenic microorganisms. Over decades, scientists have achieved considerable results in the discovery and the characterization of many natural antibiotics, like penicillin or streptomycin. Some of these compounds were used directly after being isolated from their living organisms like the aminoglycosides, while others were used after synthetic modifications like the beta-lactam antibiotics, which include penicillins (produced by fungi of the genus *Penicillium*) the cephalosporins, and the carbapenems. Other antibiotics were produced synthetically like the fluoroquinolones, the quinolones, and the oxazolidinones[1].

Antibiotics are grouped in two major classes depending on their virulence, the bacteriostatic class, which specifically inhibits bacterial cell division, and the bactericidal class, which can directly kill the target bacteria. In both cases, antibiotics attack specific targets on the outside or inside of the bacterial cell. In most cases the target is part of an important biological pathway that are essential for cell division and growing of the bacteria (Figure 1). A wide range of antibiotics attack the cell wall synthesis pathway, like vancomycin, pencillins and teichoplanin. Other groups interfere in some essential protein synthesis pathways, like chloramphenicol and tetracycline, while other antibiotics can interrupt different metabolism pathways, like the sulfonamides [1,2]

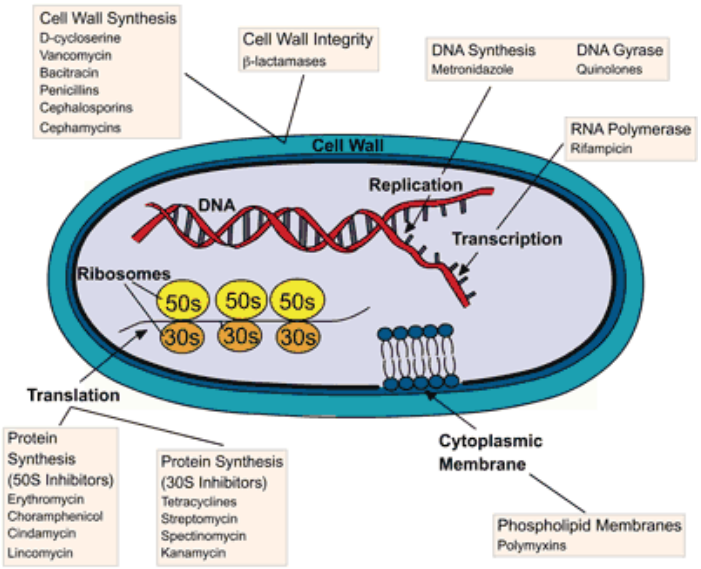

Figure 1. Mode of actions of antibiotics (Taken and adapted from "Essential Biochemistry" www.wiley.com)

However, the major problem that appeared directly after the wide spread usage of antibiotics was the resistance appearing against these compounds by bacteria. Bacteria that were treated with antibiotics developed different mechanisms to become resistance or immune. This problem was the concern of scientists for decades in order to understand the mechanisms of such resistance and thus to find the solutions for it.

One of the early explanations of the occurring resistance, was found for the penicillin resistance. *Staphylococcus aureus,* a major human pathogen, was able to inactivate penicillin by producing penicillinases. This resistance is mediated by β-lactamase which cleaves the beta lactam ring of the penicillin molecule thereby inactivating the molecule [3]. A different type of resistance appeared after using vancomycin, which was isolated in 1956 from *Amycolatopsis orientalis*[4], and used in the treatment against methicillin-resistant *staphylococcal* infections [5]. In this case, the bacteria were able to change the binding target of vancomycin on its own peptidoglycan precursor from D-Ala-D-Lac into D-Ala-D-Ala. This became possible via small changes in the protein sequence of the involved proteins. As a result vancomycin was binding with lower affinity to the new D-Ala-D-Ala moiety and therefor the bacterial cells expressing this modified precursor were less or even not sensitive to vancomycin anymore [6 7].

Another mechanism of resistance against antibiotics is the chemical modification. Here, bacteria have developed specific enzymes which can inactivate antibiotics and thereby lower their biological activity. One known example for such resistance is the action of already mentioned β-lactamase enzymes that cleave the β-lactam ring of penicillin and cephalosporin[8], as well as chloramphenicol resistance where chloramphenicol is inactivated acetylation[9].

Multidrug-resistant (MDR) bacteria are the most severe type of gained resistance. Bacteria, which developed MDR, acquired resistance to several antibiotics at the same time. As a consequence, a lot of the used antibiotics were less effective to treat such predominantly fatal pathogens. The methicillin resistant *Staphylococcus aureus* (MRSA) is one of the prominent examples. MRSA strains are resistant to all β-lactams as well as to other groups of antibiotics like erythromycin and tetracycline. The resistance against β-lactam derivatives is mediated by penicillin-binding protein 2a (PBP2a), which has a low affinity to β-lactams and this leads to the tolerance of higher concentrations of β-lactams. Therefore, MRSA is also resistant against methicillin, which is not inactivated by β-lactamases [10,11]

The focus of much research was to solve the problem of resistance by investigating the mechanisms by which resistance occurred and how drugs were modified, as well as searching for alternatives of the used antibiotics, especially among the natural products, which was the better choice in most cases.

Antimicrobial peptides (AMPs) were considered to be very strong candidates as alternatives, because of their high antimicrobial activity and the low tendency to generate resistance against them [12]. AMPs showed to have the ability to inhibit growth of clinical pathogenic

strains like *staphylococci*, *streptococci*, *enterococci* and *clostridia*[12]. Interestingly, they share the same target molecule as clinically used antibiotics, namely the cell wall precursor Lipid II although with differences in the exact binding site. Whereas antibiotics like vancomycin and teichoplanin complex the D-Ala-D-Alanyl group, most of the AMPs bind other parts of Lipid II i.e. the pyrophosphate-moiety.

Antimicrobial peptides

Antimicrobial peptides (AMPs) are ubiquitous and natural antibiotics produced by various organisms including mammals, plants, and bacteria as a defense mechanism against other organisms. AMPs are small biological molecules <10 kDa that present anti-bactericidal, anti-fungal, anti-parasitic, or antiviral activity [13], which enables them to survive in the surrounding habitat. They are active against a wide range of species including a big group of human pathogens. AMPs are able to kill the target almost instant upon addition and are synergistic with some antibiotics [14]. For all these reasons, they were identified as a possible class of pharmaceuticals, which could be used to treat the antibiotic-resistant bacterial infections [15].

Until today, more than 880 different antimicrobial peptides have been identified [16]. Based on the differences in their three-dimensional structures, they were classified into 4 groups as shown in figure 2, (1) β-sheet like tachyplesin (2A); (2) α-helical peptides like magainin2 and cecropins P1 from *Ascaris nematodes* [17] (2B); (3) extended like indolicidin (2C); and (4) loop like thanatin (2D). [18]

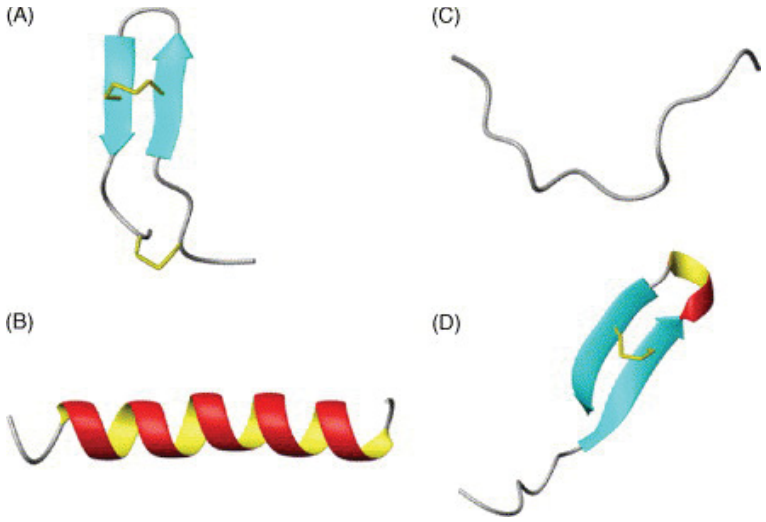

Figure.2, Structural classes of antimicrobial peptides: (A) β-sheet, tachyplesin I [19]; (B) α-helical, magainin 2 [20]; (C) extended, indolicidin [21]; (D) loop, thanatin [22] Disulfide bonds are shown in yellow. Figure is taken from [23].

While depending on their net charges, they are grouped as cationic peptides including nisin, produced by *Lactococcus lactis*, anionic peptides like dermicidin from humans[24].

Furthermore, there are other classifications based on their targets, which include bacterial AMPs (bacteriocins), fungal AMPs, plant AMPs or animal AMPs.

The group of bacterial AMPs are called bacteriocins and they are small, heat-stable peptides produced by bacteria species to compete with other members of the same species (narrow spectrum) or with a wide range of other genera (broad spectrum) [25]. They can be divided into two subgroups. The lantibiotic class, which contains all lanthionine-containing bacteriocins (Class I) i.e. nisin, and the non-lantibiotics, including the non-lanthionine-containing bacteriocins (Class II) i.e. sakacin (Figure 3). The class I bacteriocins are called lantibiotics as they contain the lanthionine or methyl-lanthionine residues, which are formed during some post-translational modifications [26] [27]. In contrast, Class II bacteriocins are not subjected to these modifications and thus they have no lanthionine rings as shown in Figure 3.

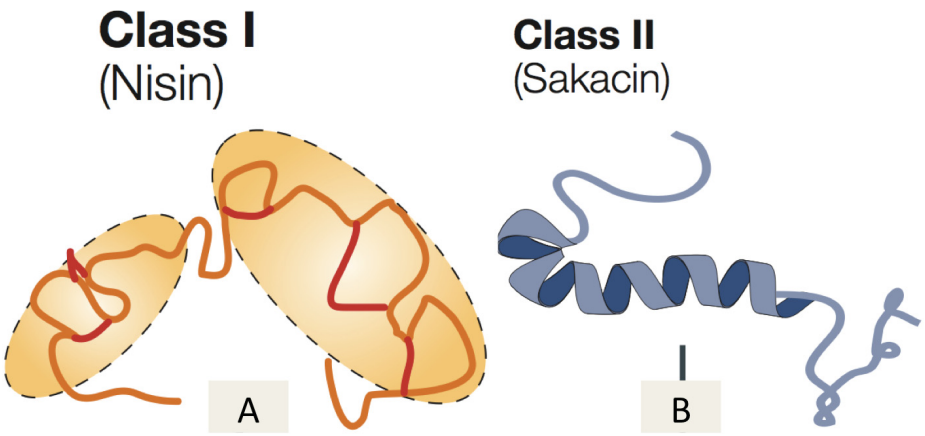

Figure 3. Classes of bacteriocins. (A) Lanthionine-containing bacteriocins (Class I), (B) Non-lanthionine-containing bacteriocins (Class II) [26]

Although different AMPs target different parts of the membrane of the microbial cells, they share almost the same steps in their mode of actions against their targets. Those steps can be summarized here as following:

(1) Attraction:

The interaction between the AMPs and the cell surface determines the ability of this AMP to kill the target bacteria. The electrostatic bonding resulting from the charges of the cationic peptides and the negative charges, exposed on the cell surfaces, is the most common mechanism [28]. Most of the antimicrobial peptides are composed of 12–100 amino acid residues, with a net positive charge that facilitates the interaction with the negatively charged microbial membranes [29]. This attraction is considered as the first step in the killing mechanism of most of AMPs.

2) Attachment:

After successful attraction of the antimicrobial peptide towards the microbe, the peptide has the ability to interact with the lipid bilayer. It was demonstrated *in vitro*, when the antimicrobial peptides were incubated with lipids in membranes or vesicles [30], that the peptides tend to interact in two physically distinct states; when the peptide/lipid ratio is low, the peptides are adsorbed and embedded into the lipid head group region [31], while at high peptide/lipid ratios, the peptides are able to be inserted into the bilayer to form stable trans-membrane pores, leading to cell leakage and consequently cell death [18].

(3) Peptide insertion and membrane permeability:

There are many proposed models that explain the mechanism of membrane permeabilization induced by the antimicrobial peptide. In the 'barrel-stave model', the attached peptide is inserted into the membrane bilayer where the hydrophobic peptide regions align with the lipid core region to form the interior region of the pore (Figure 4A), while in the 'carpet model', the peptides are oriented parallel to the surface of the lipid bilayer and thus they disrupt the membrane forming the carpet (Figure 4B). In the third model, the so-called 'toroidal-pore model', the antimicrobial peptides induce the formation of pores caused by the bending of the monolayers (Figure 4C). In all these models, the peptide molecules are able to disrupt the target membrane.

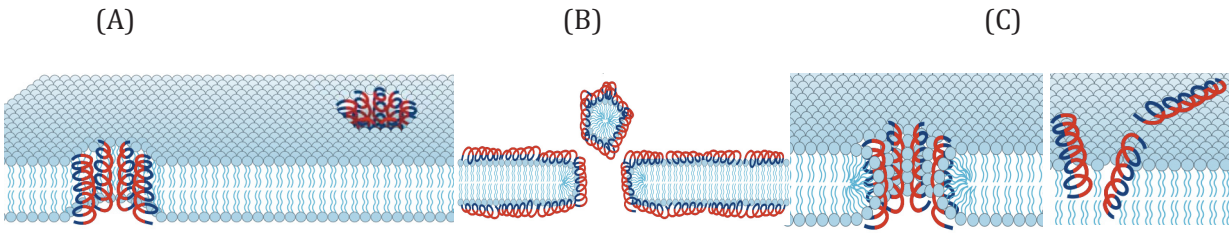

Figure 4. Models of AMP-induced cell killing. Barrel-stave model (A), carpet model (B) and toroidal-pore model (C) [18]

(4) Intracellular killing:

After the insertion of antimicrobials into the target bacteria, the cell is lysed after the formation of pores. However studies revealed that the final target of some antimicrobials is not the membrane, rather they have intracellular targets for their activities. For examples, some antimicrobial peptides bind the nucleic acids, like in case of buforin II [32]or even inhibit nucleic-acid synthesis like pleurocidin[33]. Other AMPs inhibit protein synthesis like dermaseptin and some inhibit enzymatic activity like histatins[34]. More details about the mode of action of inhibition of the cell wall synthesis is described later.

Lantibiotics & its classification

Lantibiotics are antimicrobial peptides produced by mainly Gram-positive bacteria. They are generally sub-grouped considering their mode of action, while they are classified, depending on biosynthesis pathway, under a recently added family called "Ribosomally synthesized and post-translationally modified peptides (RiPP) [35]" as they are ribosomally synthesized, post-translationally modified peptides. The most common biosynthesis pathway is shown in the figure 5. The precursor peptide is ribosomally synthesized as a core peptide with an attached signal and a recognition sequence at, named as prepeptide.

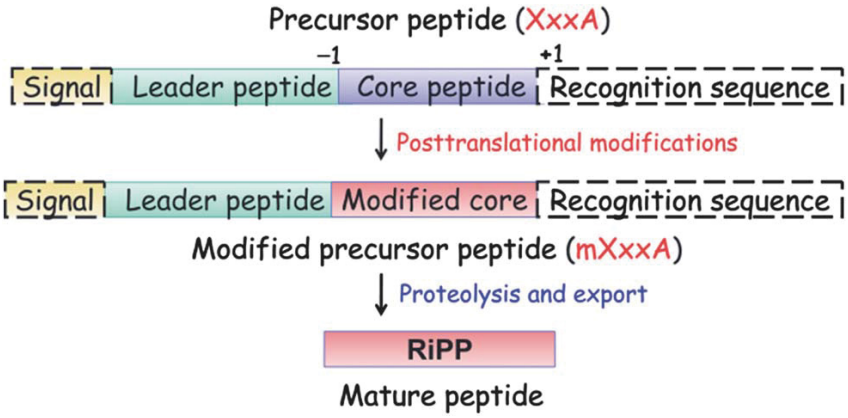

Figure 5. Biosynthesis pathway for most of RiPPs. Precursor peptide is composed of the core peptide attached to the leader peptide, signal and recognition sequence. After the modifications, secretion and cleaving processes, the mature and active peptide is produced [35].

The post-translational modifications are summarized by the dehydration of Ser and Thr residues in the prepeptide to produce 2,3-didehydroalanine (Dha) and (Z)-2,3-didehydrobutyrine (Dhb), respectively. Afterwards, the lanthionine rings are formed by coupling of the dehydrated residues to C-terminal cysteine residues [36] leading to a modified core peptide, with the attached leader peptide. It is then secreted out of the cell in an inactive form. The mature and active lantibiotic is obtained after cleavage off the leader peptide catalyzed by anchored cell-membrane proteases.

Lantibiotic biosynthesis pathways are arranged in a "gene cluster" called operon. Each operon contains all the genes responsible for the synthesis, modification and secretion of the lantibiotic and the genes on the operon are induced by their own product. Although these genes have been given the generic locus symbol "lan", each member of this family has its own designation (e.g. nis for nisin, epi for epidermin) [36]. These clusters may be found on transposon (e.g. nisin), or on the chromosome of the host (e.g. subtilin), or on a plasmid (e.g. epidermin)[37] [38]. Studies have mentioned that there is no uniform orientation or order similarity in the genes of different clusters [39] with some exceptions including *nis* and *sub* operons, which show a high similarity, and *epi* and *gdm* clusters which are identical.

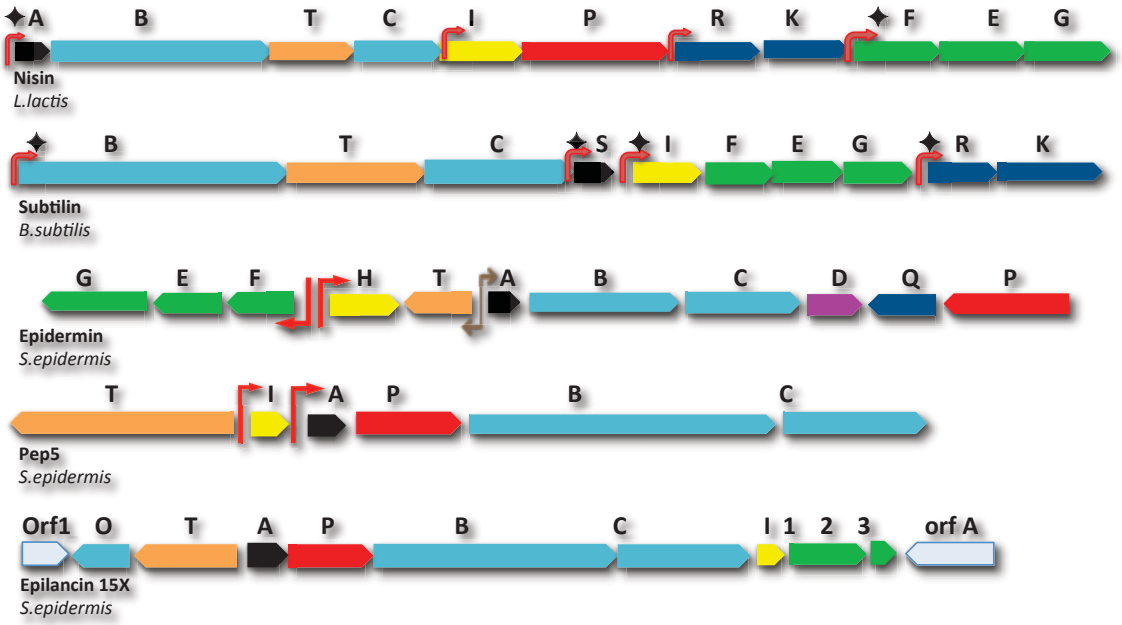

Figure 6. Representative biosynthesis gene clusters of some selected lantibiotics. Nisin, subtilin, epidermin, Pep5, and epilancin. Genes with the same function have identical colors. For biosynthesis and maturation (turquoise), transport (orange), processing (red), expression (black), regulation (dark blue) and immunity (yellow and green). Operons are associated with the transcriptional promoters A red Arrow indicates a constitutive promoter and arrow with ✦ refers to a regulated promoter" [40].

The clusters of genes encoding for the expression of some lantibiotics produced by bacteria are shown in the Figure 6 [40]. To highlight their similarities, genes are color-coded according to the function of the resulting protein. Thereby, the similarity in the operon structure as well as their encoded proteins are visualized according to their involvement in biosynthesis and maturation (turquoise), transport (orange), processing (red), expression (black), regulation (dark blue), as well as immunity (yellow and green).

The gene operon of nisin serves as a model for the biosynthesis pathways of lantibiotics. It consists of 11 genes encoding the following proteins, NisA is the nisin precursor, NisB is a dehydratase catalyzing the dehydration of serine and threonine residues in the prepeptide of prenisin, which is the nisin precursor [41]. This dehydrated prenisin is then modified by NisC which catalyzes the condensation of dehydrated residues to C-terminal located cysteine residues to form methyl-lanthionine or lanthionine rings[42]. Subsequently, NisT, the ABC transporter, exports the modified prenisin, which is still inactive until the leader is cleaved off by the cell-membrane anchored protease, NisP. The nisin biosynthesis is auto-regulated by a two-component regulatory system, NisR and NisK [43].

Additionally, the nisin operon contains four other genes *nisF, nisE, and nisG*, highlighted in green, encoding an ABC transporter with an immunity function against nisin [44], and *nisI*, highlighted in yellow in the figure 6, which encodes for NisI lipoprotein that also contributes to the protection against nisin[39]. More details about this immunity system are provided below.

The other gene clusters display different percentages of conservation of the genes. For examples, the subtilin gene operon shares a high similarity with the nisin operon with the exception of the absence of the *nisP* gene. It has recently been shown that pre-subtilin is cleaved by an non subtilin related serine protease during maturation in *B. subtilis* [45]. On the other hand, epidermin has additional genes, like *epiD* which encodes an enzyme that is responsible for special post-translational modifications during epidermin biosynthesis [46]. The *elxO* gene in Epilancin codes for a protein that catalyzes the formation of N-terminal D-lactate ([R]-2-hydroxyprpionate) in the N-terminal lactate group and results in a unique mechanism of interaction with bacteria [47], which is different from the usual binding to lipid II of the other lantibiotics.

Since the discovery of the first lantibiotic, nisin, in the 1920s, almost 50 different lantibiotics have been characterized from about 30 different bacteria, including lactic-acid-producing bacteria and *Streptomyces spp.*[48]. New lantibiotics are continuously been identified, like subtilomycin produced by *Bacillus subtilis* MMA7, which was identified last year [49] and NAI-802, which is produced by *Actinoplanes* [50].

There are different classification schemes for lantibiotics based on different characteristics. They were first divided depending on their structures into type A (linear peptides) and type B (globular peptides) [51]. However, Bierbaum and Sahl [52], modified this scheme to include the newly discovered lantibiotics and lantibiotics were divided into three groups. Type A includes the lantibiotics with flexible elongated peptides with strong antibacterial activity like nisin, epidermin, pep5 and lacticin 481 and type B includes peptides which have more globular structure with net negative or with no net charge, like mersacidin and cinnamycin and the two-peptides lantibiotics [53], as a third group, which includes the lantibiotics containing of two parts, one is similar to Type B peptides and one resembled Type A peptides.

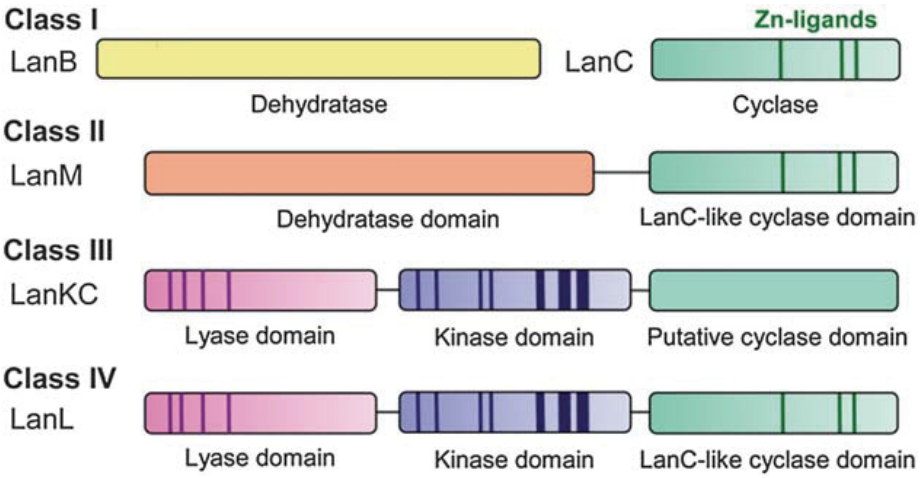

Figure 7. Scheme of four different classes of lantibiotics showing the different enzymes responsible for the post-translational modifications of lantibiotics. [35]

Recently, another classification scheme was proposed. Here lantibiotics are divided into four different classes depending on the enzymes involved in their modifications [54]. This approach is considered very simple and more flexible to include new discovered peptides [27] [55] and it was later adopted as the formal classification of lantibiotics.

Under this scheme, illustrated in Figure 7, Class I lantibiotics contains all peptides, which are modified by two enzymes, the dehydratase LanB and cyclyase LanC like nisin, subtilin and epidermin. In contrast, Class II peptides are modified by a single LanM enzyme, which is responsible for both, dehydration and cyclization of the prepeptide, like lacticin 481, mutacin mersacidin and lacticin 3147. Class III, was first proposed by van der Donk [27] and describes lanthionine-containing peptides that lack antimicrobial activity, but may have another function in the bacterial cell like SpaB secreted from *S. coelicolor* [55]. However the cyclase domain of class III enzymes has a significant homology with the other cyclase enzymes but lacks the three zinc ligands. Class IV was recently discovered from *Streptomyces venezuelae* and contains lantibiotics modified by a synthetases termed LanL [56]. One example of this class is venezuelin (Figure 8). For both, class III and IV, the dehydration is carried out by a central kinase domain and an N-terminal phosphoSer/phosphoThr lyase domain [57]. As a result of these post-translational modifications, lantibiotics have a very unique general structure, as shown in Figure 8, presented in "lanthionine rings". The number and localization of these rings differ intra classes as well as inter class. Some lantibiotics, like nisin, has 5 lanthionine rings, while mersacidin has 4 rings.

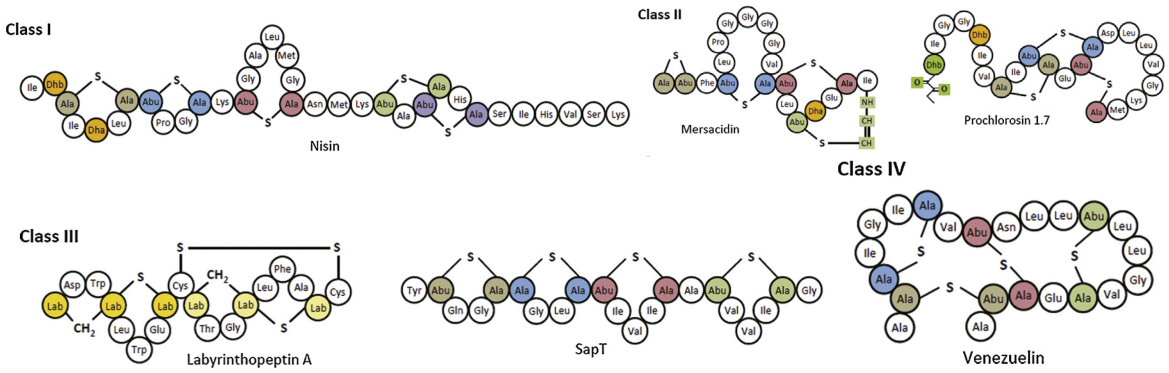

Figure 8. lantibiotics with different structures are shown.

Mode of action of lantibiotics

Lantibiotics display antimicrobial activity against a wide range of Gram-positive bacteria and also few effect Gram-negative bacteria. The mechanism by which the lantibiotics act against other bacteria has been the concern of the researchers since decades. In 1973, Linnet and Strominger gave the first evidence that the activity of both nisin and subtilin is related to cell wall synthesis in an *in vitro* system. Later many studies showed that many lantibiotics like nisin, subtilin, Pep5 and others attack the cell wall synthesis

pathway as a target for their activity in similar way like many antibiotics, e.g. vancomycin.

The bacterial cell wall is the first defense line that protects the cell from the mechanical damage and osmotic lysis. It has a rigid but flexible structure, which is built via network of peptidoglycan (Figure 9a). The peptidoglycan subunit is assembled in various steps. First, Lipid I is produced by coupling N-acetylmuramic acid MurNAc, which has already bound to a pentapeptide, with bactoprenyle-phosphate. Then the amino sugars N-acetylglucosamine GlcNAc is coupled to lipid I by the peripherally membrane-associated protein MurG to produce Lipid II that is linked to the membrane via pyrophosphate (Figure 9b). Subsequently, Lipid II is translocated to the outer surface of the cell probably via FtsW, as recently published [58], and incorporated into the peptidoglycan through transglycolysation and transpeptidation reactions by pencilin-bindin proteins (PBPs) [59]. This highly designed network is rigid, to give the shape of cell, but also elastic as it enables the cells to be dynamic during both growth and division and to protect it from lysing due to the high internal osmotic pressure [60 61]. This dynamics of the cell wall has been described also as "morphological changes" of the bacteria as a response to the environmental conditions, like the spiral-shaped pathogen *Helicobacter pylori* which is changed to a spherical (coccoid) shape both in extended culture and in stomach infections [62] and the uropathogenic *E. coli* cells, which are making long filaments as part of an immune evasion response [63].

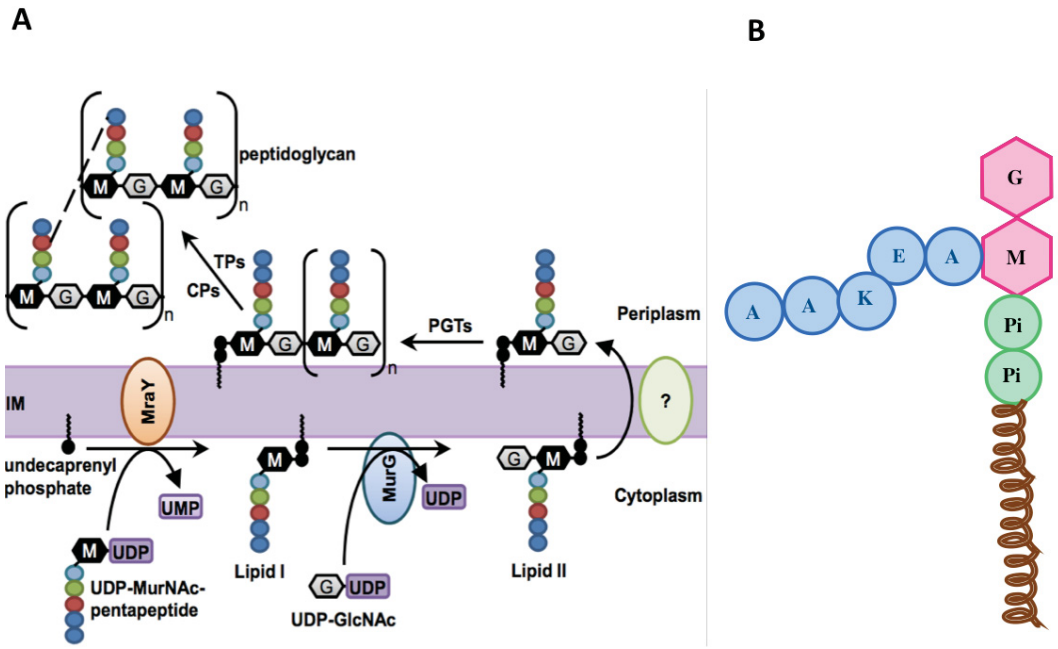

Figure 9. (**A**) Cell-wall synthesis cycle. Assembly of the cell-wall subunit. Lipid I is synthesized by tansfering UDP-MurNAc-pentapeptide to undecaprenyl phosphate by MraY. Next, Lipid II is formed by adding GlcNAc (from UDP-GlcNAc) to Lipid I by MurG. Lipid II is translocated across the membrane by a flippase. Then the glycan chain is assembled by glycosyltransferases (PGTs). The cross-link of the peptides of these chains is occurred by transpeptidases (TPs) [64] **(B)** Detailed structure of Lipid II. [48]

Lipid II consists of large hydrophilic head groups, comprising of the amino sugars, N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) which are linked via a pyrophosphate to a pentapeptide chain with a typical sequence L-alanyl- γ -D-glutamyl-diamino-pimelyl (or L-lysyl)-D-alanyl-D-alanine that is attached to MurNAc as shown in figure 9b. Two pyrophosphate groups are also attached to MurNAc and connected, on the opposing end, to a polyisoprenoid anchor consisting of eight isoprene units.

The exact target of many lantibiotics has been determined recently. It has been shown that Lipid II is the target of nisin, subtilin and epidermin , where these lantibiotics bind to Lipid II via their lanthionine rings A and B. The NMR studies showed that they are binding to the pyrophosphate moiety of Lipid II [80].These lantibiotics are able also, after binding to Lipid II, to form pores in the membrane of the target bacteria. Subtilin, which is from nisin group, permeabilizes lipid membranes and binds the pyrophosphate like nisin. While it is reported that the C-terminal of nisin is responsible for the pore formation, the N-terminal amino acids of subtilin seem to have an important role in its ability to form pores [65]. However, it is important here to mention that the last 3 amino acids in the C-terminal part of subtilin are crucial for the activity but not for the pore formation mode of action. The epidermin shows similar activity of binding lipid II and pore formation.

Mersacidin, a type B lantibiotic, interacts with lipid II but does not form pores in the membrane. Firstly, the accumulation of the peptidoglycan precursor, UDP-MurNAc-pentapeptide, in mersacidin-treated cells, suggested that mersacidin blocks the first step in trans-glycolysation pathway and later it was shown that this is due to the interaction of mersacidin with lipid II itself and not with the trans-glycosolase enzymes [66]. Both disaccharide GlcNAc and the pyrophosphate group, with its conserved motif (TxS/TxEC motif) [52], seems to play a role in binding [67]. The conserved motif of the pyrophosphate group has been found also in some other type AII lantibiotics like plantaricin C, mutacin II and lacticin 481 which all indeed bind to lipid II. Additionally, mersacidin interacts electrostatically with lipid II.This was shown by NMR studies of the mersacidin-lipid II complex where a small hinge region gives mersacidin a flexibility to open and close the ring structure upon this interaction [67,68].

Other peptides of the type B lantibiotics family, like microbisporicin, also inhibit cell wall biosynthesis by forming a complex with lipid II via their N-terminus which show high similarity to the phosphate cage build by type A lantibiotics [52]. Lacticin 3147, a two-component lantibiotic (peptide A1 and peptide A2) produced by *Lactococcus lactis* subspecies lactis DPC3147 is also shown to interact with Lipid II. Here, both peptides are involved in this binding where first peptide A binds which, subsequently leads to the binding of peptide B in order to form pores. This first binding to Lipid II prior to pore formation is similar to the mode of action of nisin [53 69].

The lantibiotic Nisin and its biosynthesis system

Nisin is the most prominent member of the lantibiotic superfamily. Its biosynthesis pathway, genes operon and mode of action are widely studied and serve as a model for lantibiotics. Nisin is produced by many strains of *Lactococcus lactis* and three natural variants have been identified, Nisin A, Nisin Z and Nisin Q. The sequence of NisZ varies at position 27 compared to NisA and at four positions (Val15, Leu21, Asn27 and Val30) in the case of Nisin Q [70 71].

It is synthesized ribosomally as a precursor NisA and then post-translational modified via the NisBC modification machinery. Subsequently, NisT, an ABC transporter, transports the modified, but inactive, nisin out of the cell to be activated via the leader peptide-cleavage of the protease NisP. Active nisin consists of 34 amino acids and contains five lanthionine/methyl-lanthionin rings (Figure 10) with a net positive charge. Its structure is divided into three regions, an N-terminal part, where the rings A and B are installed, a flexible hinge region and a C- terminal part, where the rings C, D and E are located. The presence of these unique rings in nisin, as well as in most lantibiotics, is very important for their antimicrobial activity and stability.

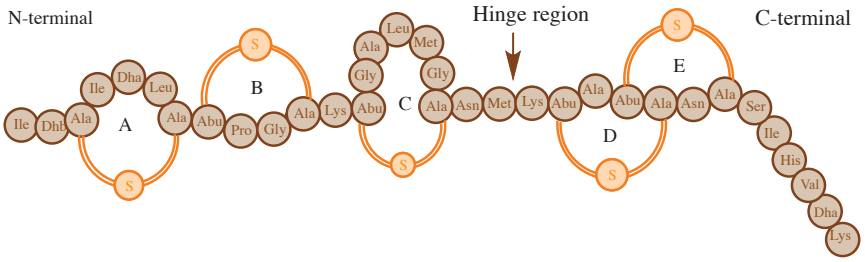

Figure 10. Structure of nisin showing lanthionin rings A, B, C, D, E and the flexible hinge region between rings A-C and rings D-E.

For the past 50 years, nisin was used in industry for a wide range of applications especially in the food industry for cheese production, canned vegetables and diverse pasteurized dairy as well as the production of salad dressing (de Vuyst 1993). Although nisin has been identified as a safe product to be used in foodstuff since 1969 by the Joint Food and Agriculture Organization/World Health Organization Expert Committee of Food Additive, it was only in 1983 that it was added to the European food additive list as number E234 [25]. It can be delivered to the food in different ways, like lantibiotic-producing strains which are used as a starters, or as adjunct strain in fermented food [72] or as a commercial food additive, e.g. nisaplin (Aplin & Barrett Ltd., Applied Microbiology Inc.) which contains 2.5% nisin A provided with other salt and milk solids derived from the fermentation of modified milk medium by nisin producing strains of *L. lactis* [73].

Additionally, nisin is also considered to be a good candidate for pharmaceutical use like treatment against systemic infections, where nisin showed 8-16 times better activity than vancomycin against *S. pneumonia,* which causes Pneumonia in humans, in a mouse model [74]

and treatment of bovine mastitis, where nisin has been evaluated as a contributor in the treatment with other lantibiotics like epidermin [25], and the important affect of nisin to inhibit the growing of *Listeria* in frozen food [75]. Upon combination with other lantibiotics, the activity of nisin is broadened. For example, addition of both nisin and lacticin 3147, showed a potential effect against the treatment of *C. difficile-associated diarrahoea* (CDAD), the most common hospital-acquired *enterocolitis* [76].

Nisin binds Lipid II and inhibits thereby cell wall synthesis [77] (Figure 11A). It is also mentioned that Lipid II serves as a "docking molecule" for nisin, which enables it to form pores in the cytoplasmic membrane of the target bacteria (Figure 11B). In the presence of Lipid II, the activity of nisin in model membrane systems is increased by three orders of magnitude compared to the activity of nisin against susceptible bacteria [78].

In 2004, the solution structure of the Lipid II-nisin complex has been reported to give a solid evidence of the that the N-terminal rings A and B of nisin are binding to the pyrophosphate moiety of Lipid II [79]. After this binding, nisin needs to be inserted into the bilayer of the membrane and thus requires a specific perpendicular orientation with respect to the membrane surface which takes place in the presence of Lipid II [80]. This orientation is stable and leads to the formation of the pore mediated by specific ratio of nisin and Lipid II, where four Lipid II and eight nisin molecules are needed. [81] Additional details on the antimicrobial mechanism of nisin have been provided by Hasper et al in 2006. Here it was shown that Nisin binds Lipid II and transports it away from the septum, the site of cell division [82].

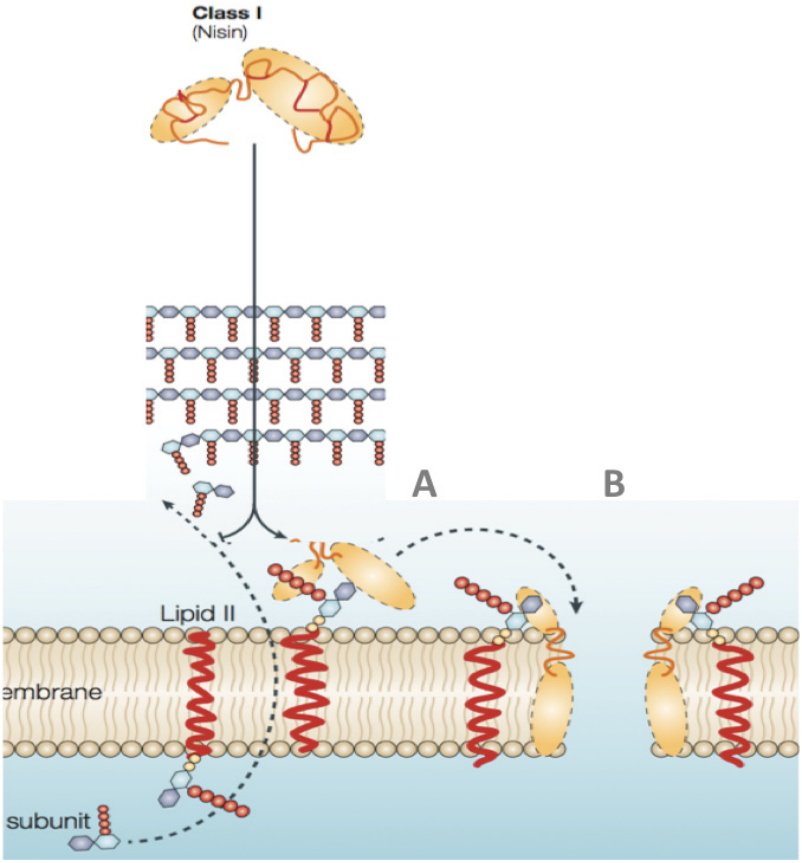

Figure 11. Modes of action of nisin. Binding to lipid II (A), formation of pores (B)[26]

Many experiments were performed using different mutants of nisin in order to determine which parts of nisin are involved in its modes of action. To study the role of the N-terminal part of nisin, where the first two rings A and B are located, variants of nisin with mutations in the conformational of rings A and B were designed [83]. These mutants showed a significant reduced binding ability of nisin to lipid II, and a higher concentration of these nisin mutants were required to achieve pore formation. The NMR study of the nisin-Lipid II complex revealed that both rings A and B are part of the pyrophosphate cage [79]. Other mutants in the hinge region of the peptide resulted in the inability of nisin to permeabilize the membrane [83]. However, some *in vivo* activity for such mutants was observed which could be due to the remaining ability of nisin to bind Lipid II via its first two rings A and B. It has been also proven that ring C is very crucial for the activity and more specifically for the step of pore formation, while removing the ring C resulted in almost non-active nisin [84].

The removal of 5 residues of C-terminal of nisin decreases the activity of nisin 10 fold [84], while removing the last nine C-terminal amino acids caused a huge decrease in activity (100 fold). This deletion caused the absence of the ring E. Replacing valine at position 32 of nisin with a negatively charged glutamate residue drastically reduced the lipid-dependent binding of nisin [85], while the binding was improved by replacing valine-32 with lysine. Serine at position 29 showed a great effect on the function of nisin as a truncated form of nisin (nisin1-28) showed a markedly reduced affinity for the *lactococcal* membrane, a significantly

diminished pore-forming potency in the target membrane, and a 100-fold-lower bactericidal activity against *L. lactis* MG1363 strain[86].

Immunity against Lantibiotics

Lantibiotics are produced by different species of Gram-positive bacteria and have antimicrobial activity against a wide range of Gram-positive bacteria as explained above. They should be able to produce these agents without 'committing suicide'. The producer strains should protect themselves against their own products, and thus they have developed an "immunity system". The immunity system of the lantibiotic producer is expressed from genes located on the same operons that are responsible for the biosynthesis of these lantibiotics (Figure 12). The proteins expressed can be classified as LanI, which is a lipoprotein and LanFEG which forms an ATP binding cassette (ABC transporter.

LanFEG is part of the immunity system in different lantibiotic operons like nisin, subtilin, epidermin (figure 12) and lacticin 3147, streptococcin AFF-22, mutacin II and lacticin 481, respectively. In contrast, in the operons of Pep5, cytolysin, epicidin, lactocin S and epidermin only require a lanI protein is encoded. Interestingly, those systems that contain both, the genes *lanFEG* and *lanI*, express lantibiotics that can both bind to Lipid II and also create pores in the membrane [87]

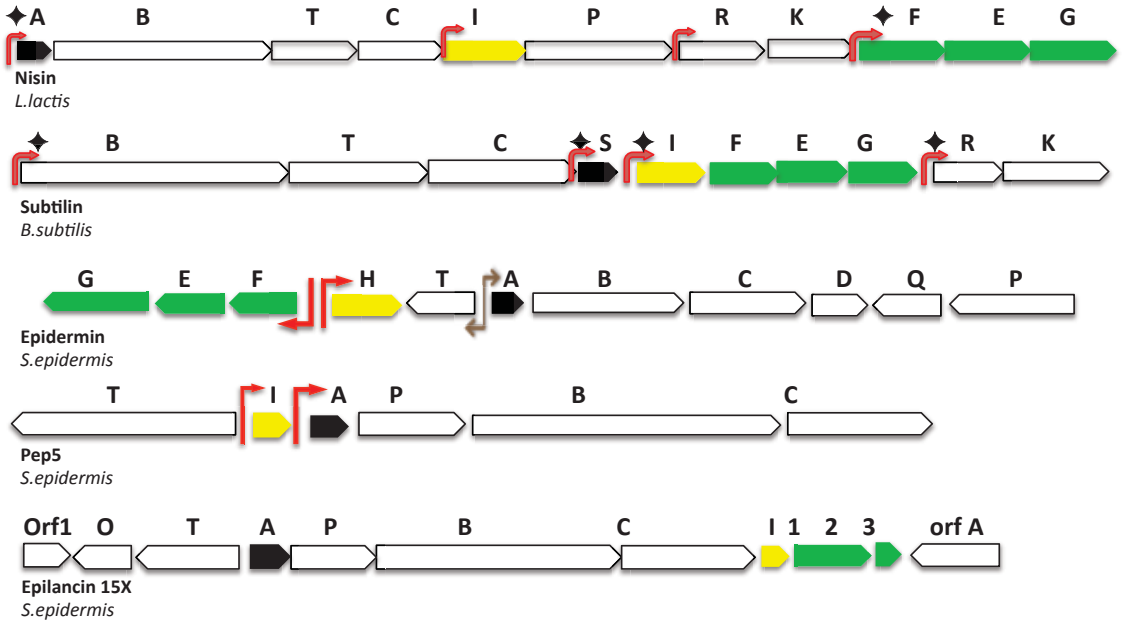

Figure 12. Operons of different lantibiotics. Genes expressing immunity system are labeled in yellow and green.

Both immunity proteins were found in a subtilin producer strain, SpaI a protein, which is similar to NisI. It is proposed that SpaI sequesters subtilin at cytoplasmic membrane, thus preventing pore formation and ultimately an attack of the own cell [88]. Besides, the expression of these *spaIEFG* genes in a subtilin sensitive strain *B. subtilis* MO1099 confirmed their functions. Better immunity than the normal subtilin producer *B. subtilis* ATCC 6633 resulted

in a recovery of approximately of two thirds of the applied subtilin from the supernatant [88], suggesting that SpaIFEG exports subtilin and no modifications or degradation occurred.

In general, all *LanI* genes showed no significant sequence homology with each other or with any other immunity protein [89] with some exceptions like PepI, which showed 74.2% homology to EciI, the immunity gene of epicidin 280 [90]. Generally, an immunity system is very specific for its own lantibiotic and provides no resistance against other lantibiotics. Even very homologous lantibiotics like nisin and subtilin showed no cross-immunity[91]. There are some exceptions. PepI, which provides immunity against epicidin 280. This cross-immunity could be a result of the fact that in this immunity system no ABC transporter is present [92].. Moreover, introducing both nukacin ISK-1 immunity systems, NukEFG and NukH, into *L. lactis* provided full immunity against lacticin 481 [93], which possess only the LctFEG as immunity system.

Immunity of *Lactococcus lactis* against its own nisin

The immunity system of nisin is considered to be a model for lantibiotic immunity systems and comprises of the ABC transporter NisFEG and the lipoprotein NisI.

THE ABC TRANSPORTER NISFEG

ATP binding cassette ABC transporters are a large family of proteins. They are present in all species, from bacteria to human, and transport various substances, including ions, nutrients up to large proteins across cellular membranes [94]. They are generally comprised of a conserved structure of four domains, two nucleotide-binding domains (NBDs), which have a highly conserved sequence, and two transmembrane domains (TMDs), which are not conserved between the different transporters. While NBDs are involved in the binding and hydrolysis of ATP, the (TMDs) transports the substrate through the transmembrane pore [95].

NisFEG is considered to be an ABC transporter as both NisF showed high homologous to the ABC transporters of the HisP family and sequence comparison showed that NisF contains all specifc sequence motifs generally found in cytoplasmic ATP binding proteins. In a functional complex NisF would need to dimerize to be able to hydrolyse ATP. NisE and NisG are hydrophobic proteins residing in the membrane, which together form the integral-membrane segment of the NisFEG ABC transporter [96] [39] . The suggested functional stoichiometry would be the $NisF_2EG$ complex [97] as depicted in Figure 13.

This $NisF_2EG$ complex is expelling nisin, prior to pore formation, into the surrounding media[45]. It has been shown that NisFEG exports the nisin molecules without modifying or degrading it. The knockout of either *nisE* or *nisF* led to cells displaying a higher nisin sensitivity in comparison to cells where all three proteins were present. The deletion of NisG however, did not have a significant effect on the immunity [39]. Interestingly, a reduction of 80 % in immunity was shown when NisFEG was expressed in a NisI-deficient *L. lactis* strain [96].

These results confirmed that NisFEG has a crucial role in protecting *L. lactis* from the antimicrobial activity of nisin.

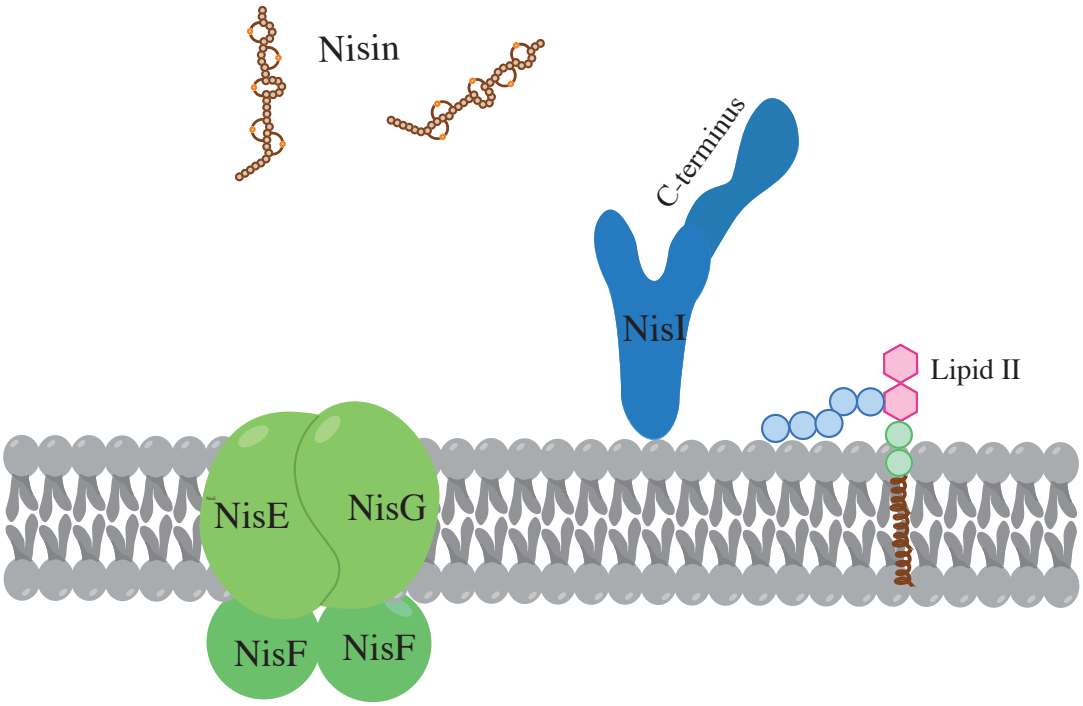

Figure 13. Immunity system against nisin. Both, NisI, a lipoprotein and NisFEG, an ABC transporter, are involved in immunity against nisin.

THE LIPOPROTEIN NISI

NisI is a lipoprotein anchored in the membrane of *L. lactis* after the removal of the N-termianl signal sequence of 19 residues [98]. It consists of 245 amino acids and contributes to the immunity of *L. lactis* against nisin. Studies mentioned a physical interaction between NisI and nisin where NisI intercepts nisin, which causes a reduction in nisin concentration in the surrounding growth media [99]. Also, a knockout of NisI resulted in more sensitive cells even more sensitive than a NisFEG knockout strain [39]. Thus it was believed that NisI might play a bigger role in immunity against nisin that NisFEG [92].

The exact mechanism of NisI mediated immunity is still largely unknown, although Takala and his colleagues, mentioned the importance of C-terminal of NisI in the interaction with nisin Their study identified a 21 amino acid C-terminal deletion of NisI where, the immunity is reduced to 14% compared to the native immunity level of NisI [100]. Interestingly, the replacement of the 21 C-terminal amino acids of the subtilin-specific immunity protein SpaI with the C-terminal 21 amino acid of NisI (SpaI´-NisI´) created a protein, which confers immunity against nisin [100], while this immunity has not been observed with the full length SpaI protein. This suggests that the C-terminus is able to confer immunity by itself.

Although those different lipoproteins NisI, SpaI and PepI confer immunity, which is specific against their cognate lantibiotic, no significant sequence homology was observed. Only recently, the structure of the lipoprotein SpaI was determined [101]. The authors used a truncation of SpaI and demonstrated that the N-terminus is unstructured in solution, which however folds in the presence of lipids resembling the cell membrane in these studies.

To conclude, both NisI and NisFEG are important to provide immunity, as knockout studies for NisFEG alone or NisI alone showed 4-20% of the immunity compared to the full immunity achieved when both proteins are active [96]. However the mechanisms by which both, NisI and NisFEG, participate in the protection against nisin are still unknown.

AIM OF STUDY

The nisin immunity system of the Gram-positive bacteria *Lactococcus lactis* was the main focus of this PhD thesis.

This system consists of two distinct proteins, the lipoprotein NisI and the ABC transporter NisFEG. It has been shown that both, NisI and NisFEG, contribute to the immunity of *L.lactis* against nisin by a knockout approach [40]. Surprisingly, NisI and NisFEG, if expressed individually, exhibited only 4 - 20% of the immunity observed when both proteins are expressed together.

The main question of this PhD thesis was the identification of the molecular mechanism(s) of nisin immunity mediated by NisI and NisFEG and to tackle the open question of how these proteins co-operate to provide maximal protection of *L. lactis* cells against nisin. To achieve this, the first aim of this thesis was to establish a homologous *in vivo* expression system using a nisin-sensitive *L. lactis* strain. Thereby, the direct influence of NisI or NisFEG expression in nisin immunity can be visualized. Furthermore, such an expression plasmid would allow the genetic manipulation of the proteins and would shed light on which parts of the protein are important for function.

After successful establishing such a system, the next goal of this thesis was an investigation of how NisI and NisFEG are able to confer immunity. This was achieved by analyzing growth inhibition as well as pore formation of nisin in the presence of NisI, NisFEG or both protein systems.

The cooperative mode of action of NisI and NisFEG should be visualized by expressing these proteins simultaneously in *L. lactis*. Since the molecular understanding of lantibiotic immunity is still poorly understood, results obtained by this PhD thesis would give important insights into this topic and might be of general importance for understanding immunity of against antibiotics as well.

CHAPTER I

Lantibiotics: How do producers become self-protected?

By

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Lantibiotics: How do producers become self-protected?

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ABSTRACT

Lantibiotics are small peptides produced by Gram-positive bacteria, which are ribosomally synthesized as a prepeptide. Their genes are highly organized in operons containing all the genes required for maturation, transport, immunity and synthesis. The best-characterized lantibiotic is nisin from *Lactococcus lactis*. Nisin is active against other Gram-positive bacteria via various modes of actions. To prevent activity against its producer strain, an autoimmunity system has developed consisting of different proteins, the ABC transporter NisFEG and a membrane anchored protein NisI. Together, they circumvent the ability of nisin to fulfill its action and cause cell death of *L. lactis*. Within this review, the mechanism of regulation, biosynthesis and activity of the immunity machinery will be discussed. Furthermore a short description about the application of these immunity proteins in both medical and industrial fields is highlighted.

1. Introduction

Lantibiotics are produced by Gram-positive bacteria. Well-known examples are nisin from *Lactococcus lactis* and lacticin 481 from *Streptococcus lactis* (Bierbaum and Sahl, 2009). They can function as quorum sensors to activate their own biosynthesis like nisin (Kuipers et al., 1995) or cytolysin from *Enterococcus faecalis* (Coburn et al., 2004). Quorum sensing is used by bacteria to regulate cell-density dependent gene expression (Kleerebezem et al., 1997). Herein, cells sense a specific molecule, which functions as a signal and induces gene expression (Kuipers et al., 1998). For example, nisin production and secretion starts at the early growth stage of *L. lactis* and the produced nisin is absorbed on the cell surface and induces its own biosynthesis (Hilmi et al., 2006). Due to a drop in pH during growth, absorbed nisin is released from the cell surface into the media during the mid-logarithmic growth phase at a pH below 5.5. Only then, nisin activity can be detected in the medium and nisin production reaches a maximum at the early stationary phase (Kleerebezem et al., 1997).

Furthermore, some lantibiotics act as morphogens like SapB from *Streptomyces coelicolor*, which functions as a biological surfactant decreasing the surface tension at the air–water interface. This allows its producer strain to escape the aqueous milieu and grow in air (Kodani et al., 2004).

Lantibiotics are ribosomally synthesized as a prepeptide, post-translationally modified by several enzymes and finally converted into the active form (Hansen, 1993). The terminus "lantibiotic" is derived from "*lan*thionine containing an*tibiotic*" (Schnell et al., 1988). Lanthionines are thioether bridged amino acids and introduce intramolecular ring formation within the peptide (Hansen, 1993). The fact that nisin and Pep5 from *Staphylococcus epidermidis 5*, which contain (methyl)lanthionine rings, were not degraded by proteases, led to the conclusion that (methyl) lanthionine rings protect lantibiotics against proteolytic degradation (Bierbaum et al., 1996; van der Meer et al., 1993).

Three classes of lantibiotics are known according to their biosynthesis and function (Willey and van der Donk, 2007). Class I lantibiotics like nisin, subtilin and epidermin are posttranslationally modified by two distinct enzymes, LanB and LanC, and subsequently this modified prepeptide is exported by a dedicated ABC transporter, LanT. The maturation of class I lantibiotics is terminated after the leader peptide is cleaved off by a protease. Class II lantibiotics like lacticin 481 or mersacidin are posttranslationally modified by a single enzyme, LanM, which catalyzes both modification reactions (Siezen et al., 1996). A single, multifunctional protein performs the export of the lantibiotic and the cleavage of the leader peptide. This membrane protein is also designated LanT. Both classes have significant biological antimicrobial activity, while class III of lantibiotics is lacking this activity, which is the definition of the latter class (Willey and van der Donk, 2007).

Both, class I and class II lantibiotics are active against Gram-positive bacteria and exert various mechanisms of activity. Some of these mechanisms are described below. Nisin inhibits cell-wall

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Table 1
Lantibiotics are listed together with the producers strains, its classification, as well as the immunity proteins present within its strain.

Lantibiotics	Strain	Class I or II	Immunity protein
Nisin	*Lactococcus lactis*	Class I	NisFEG, NisI
Subtilin	*Bacillus subtilis ATCC6633*	Class I	SpaFEG, SpaI
Ericin	*Bacillus subtilis A1/3*	Class I	EriFEG, EriI
Epidermin	*Staphylococcus epidermidis*	Class I	EpiFEG, EpiH
Gallidermin	*Staphylococcus gallinarium*	Class I	GdmFEG, GdmH
Pep5	*Staphylococcus epidermidis*	Class I	PepI
Epicidin	*Staphylococcus epidermidis BN280*	Class I	EciI
Epilancin	*Staphylococcus epidermidis K7*	Class I	ElxI
Lacticin 481	*Lactococcus lactis CNRZ 481*	Class II	LctFEG
Mutacin II	*Streptococcus mutans T8*	Class II	MutFEG
Nukacin ISK-1	*Staphylococcus warneri ISK-1*	Class II	NukFEG, NukH
Lactocin S	*Lactobacillus sakei L45*	Class II	LasJ
Lacticin 3147	*Lactococcus lactis DPC3147*	Class II	Ltn FEG, LtnI
Mersacidin	*Bacillus sp. strain HIL*	Class II	MrsEFG

synthesis by binding to lipid II (Hasper et al., 2006), an essential membrane-anchored cell-wall precursor. Another mechanism is the binding of nisin to lipid II, which induces membrane integration of nisin resulting in the formation of pores composed of nisin and lipid II molecules (Hasper et al., 2004).

There is a highly specific interaction between nisin and lipid II and this is reflected by low micromolar concentrations of nisin, which are sufficient to permeabilize the membrane of the targeted bacteria (van Heusden et al., 2002). The bactericidal activity of lantibiotics is generally measured by quantifying growth inhibition of the target bacteria, either on agar plates or in liquid culture. On agar plates, the zones of growth inhibition of the indicator organism can be visualized and allow a precisely determination of the minimum inhibitory concentration (MIC) (Wiedemann et al., 2001). Alternatively, bacterial growth can be monitored in liquid culture in dependence of the lantibiotic concentration by measuring the optical density. This method allows a simple and quantitative determination of the IC_{50}, the concentration of the antibiotic that inhibits cell growth by 50% (Oman and van der Donk, 2009). However, due to the differences in the purification protocols, a quantitative comparison between different studies is difficult as slight difference in the preparation can result in up to 10-fold difference in the determined IC_{50} (Abts et al., 2011).

Since low amounts of a lantibiotic are already sufficient to harm or even kill the producer strain, it has to prevent such a suicidal mode of action. Thus the producer strain regulates the expression of an immunity system induced via measurement of the extracellular concentration of the lantibiotic. The proteins participating in immunity are generically called LanI and LanFEG. Table 1 summarizes some of the known immunity genes.

We focus on the self-immunity proteins of the strains producing antimicrobial peptides with a special emphasis on the nisin immunity proteins located in the nisin operon of *L. lactis*. In this review, the genetic structure, expression, and mode of action of these immunity proteins will be summarized.

2. Biosynthesis and gene organization

All lantibiotics biosynthesis pathways are arranged in a single "gene cluster" or "operon" within the genome of the organism. These operons are responsible for the synthesis of these lantibiotics and are, in general, induced by their own products or by environmental changes. Although the genes have been given the generic locus symbol "*lan*", each member of this family has its own designated name based on the produced lantibiotic (e.g., *nis* for nisin, *epi* for epidermin). The operon may be found on a transposon (e.g. nisin), on the chromosome of the host (e.g. subtilin), or on a plasmid (e.g. epidermin) (Champak Chatterjee, 2005). Several sequences of the genes involved in lantibiotic maturation, regulation, transport as well as immunity have been revealed. The ability of organisms to produce and secrete novel lantibiotics can be detected based on the sequence homology of candidate proteins found in newly sequenced genomes.

Genes with similar function have been found in almost all lantibiotic gene clusters, sometimes however arranged differently (see Fig. 1). One report indicates that no uniform orientation or order in the position of the genes in different operons can be derived (Siegers and Entian, 1995), while some exceptions exist, for example the *nis* (*L. lactis*) and *spa* (*Bacillus subtilis*) operons, which have a high similarity, or the *epi* and *gdm* operons, which are identically organized.

Operons of the class I as well as class II lantibiotics are depicted in Fig. 1. To highlight their similarity, genes are color-coded according to the function of the resulting protein. Thereby, the similarity in the operon structure as well as their encoded proteins are visualized according to their involvement in biosynthesis and maturation (green), transport (orange), processing (purple), expression and regulation (dark blue), as well as immunity (yellow).

The nisin operon consists of 11 genes expressing the following proteins (Fig. 1): NisA, which is composed of a leader sequence corresponding to amino acids 1–23 at the N-terminus and 34 amino acids that encode the active peptide, NisB, the dehydratase, catalyzes the dehydration of serine and threonine residues in the prepeptide (Karakas Sen et al., 1999) and specifically recognizes parts of the leader sequence (Mavaro et al., 2011). In the next step of maturation, dehydrated prenisin is modified by the cyclase NisC, which catalyzes the condensation of dehydrated residues to C-terminal positioned cysteine residues to form (methyl)lanthionine rings (Koponen et al., 2002). After that, the ABC transporter NisT exports fully modified prenisin (Qiao and Saris, 1996), which is still inactive until the signal sequence is cleaved off by the cell-membrane anchored protease NisP (van der Meer et al., 1993).

For class II lantibiotics, i.e. lacticin 481, (Fig. 1), the functions of the dehydratase and the cyclase are fused to a single gene coding for a protein called LctM (Paul et al., 2007). Furthermore, the LctT transporter has two functions, it exports the modified peptide, similar to class I lantibiotics, and it cleaves the leader sequence (Champak Chatterjee, 2005).

Compared to nisin, other class I lantibiotics gene operons have similar operon structures. The subtilin operon, shown in Fig. 1, shares a high degree of similarity with the nisin operon with a single exception. Here, the protease LanP is missing in the subtilin operon. However, it has been shown that pre-subtilin is cleaved by several extracellular serine proteases, which are not encoded on the subtilin operon (Corvey et al., 2003). Another example is epidermin. This operon contains additional genes like the *epiD* gene encoding an enzyme responsible for specific post-translational modifications on the N-terminus of epidermin during biosynthesis (Majer et al., 2002).

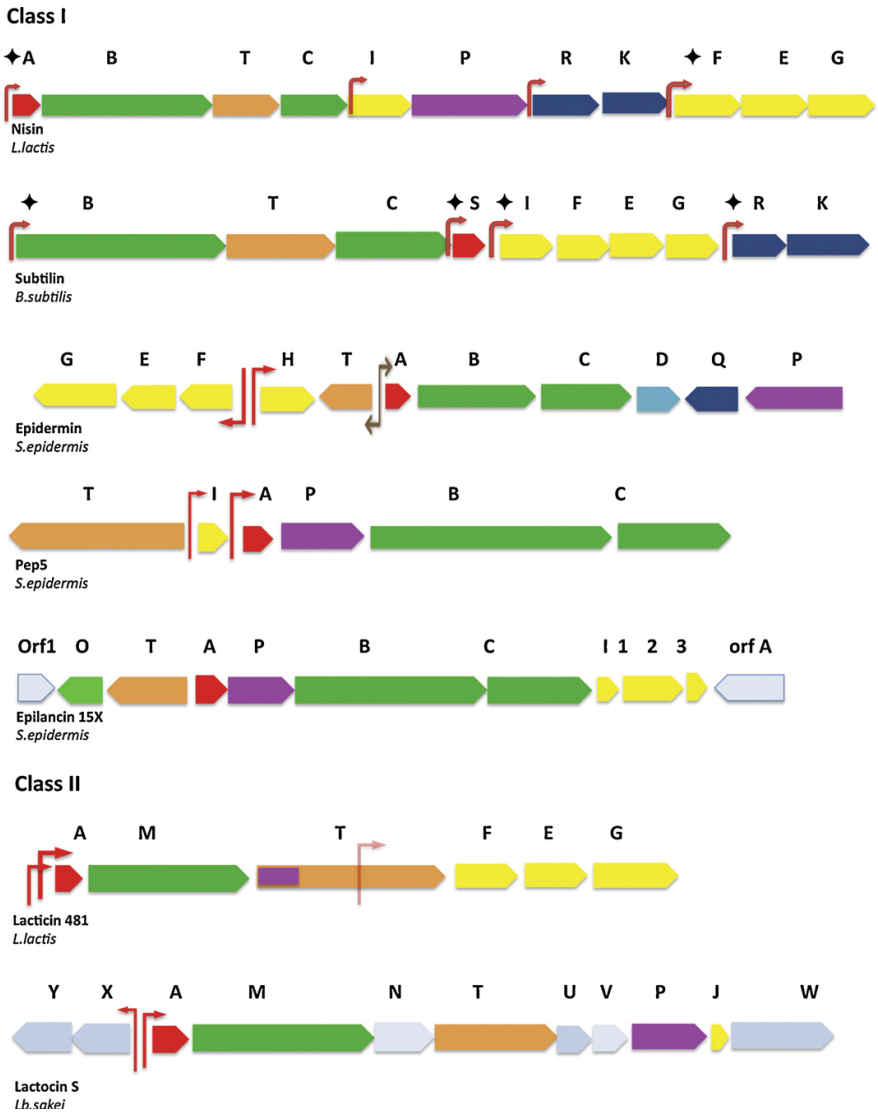

Fig. 1. Representative gene clusters of selected class I and class II lantibiotics. The selected examples are nisin, subtilin, epidermin, Pep5, epilancin, lacticin 481 and lactocin S. Genes with the same function are presented in identical colors. Operons associated with a transcriptional promoter (constitutive promoter) are highlighted by a red arrow, while arrows with * refers to a regulated promoter.

Nisin biosynthesis is auto-regulated by a specific two-component regulatory system, NisR and NisK, which are also found in other lantibiotic systems like the subtilin operon of *B. subtilis* (Cheigh and Pyun, 2005) (described in more details in paragraph 3 of this review).

The nisin operon contains four other genes (shown in yellow in Fig. 1): *nisF*, *nisE* and *nisG*, which, when expressed, assemble an ABC transporter and *nisI* encoding a lipoprotein. These proteins together form an immunity system within *L. lactis* against nisin (Siegers and Entian, 1995). The exact regulation and functions of these proteins will be discussed in more details below.

A comparison of the immunity systems of all lantibiotics reveals that nisin, subtilin, epidermin, lacticin 3147, streptococcin AFF-22, mutacin II and lacticin 481 all harbor a member of the LanFEG family as part of the immunity system. In contrast, Pep5, epicidin and lactocin S only require a LanI protein – PepI, EciI and LacJ – to gain resistance against their own lantibiotic.

Interestingly, lantibiotics that are produced by the strains that contain both proteins, LanFEG and LanI, form pores and bind lipid II (Guder et al., 2002). Since, mersacidin which is produced by a strain that only contains LanFEG, binds to lipid II, but does not form pores, it is assumed that both, LanFEG and LanI, are required to inhibit pore formation (Champak Chatterjee, 2005) within the membrane of the producer strain.

3. Regulation of lantibiotic biosynthesis and its immunity system

The expression of many lantibiotics, like nisin or subtilin, is regulated by a two-component regulatory system, consisting of

a receptor histidine kinase (LanK) and a transcriptional response regulator (LanR) (Chatterjee et al., 2005; Stock et al., 2000). In case of nisin or subtilin, the extracellular lantibiotic acts as a signal, which is recognized by the corresponding LanK, and induces autophosphorylation of a histidine residue within LanK (Bierbaum and Sahl, 2009). The high energetic potential of the phosphoryl group is subsequently transferred to an aspartate residue within the response regulator protein (LanR) (Stock et al., 2000). This initiates the binding of LanR to distinct promoters, which activates the transcription of the lantibiotic gene as well as the genes of the modification and immunity proteins (Chatterjee et al., 2005). In non-lethal concentrations, these lantibiotics serve as a signal and induce their own biosynthesis (Bierbaum and Sahl, 2009; Chatterjee et al., 2005; Willey and van der Donk, 2007). The biosynthesis of some other lantibiotics is regulated by systems unrelated to a two-component regulatory system, for example, a change in pH of the environment induces lacticin 481 production via the transcription regulator RcfB (Madsen et al., 2005). Furthermore, the production of the two-component lantibiotic cytolysin from *E. faecalis* is stimulated by the presence of a potential target cell (Coburn et al., 2004). Cytolysin consists of $CylL_S$ and $CylL_L$, which form a stable complex that has neither toxic nor regulatory functions. In the presence of a target cell, the $CylL_L$ preferentially binds to the target membrane. On the other hand, monomeric $CylL_S$ functions as a signal peptide to induce cytolysin production, which is repressed by CylR2 in the absence of target cells (Coburn et al., 2004).

Similar regulation loops of lantibiotic production and immunity were observed for subtilin from *B. subtilis* (Stein et al., 2002) (Fig. 1). Here, three subtilin inducible promoters regulate the production of the subtilin prepeptide, the modification enzymes as well as the immunity proteins SpaIFEG. In contrast to the nisin system, the two-component system SpaRK of the subtilin-producer strain *B. subtilis* ATCC 6633 is positively controlled by the sigma factor H, SigH. The transcription of *sigH* is negatively controlled by the suppressor AbrB when the *B. subtilis* culture is at the late growth phase (Stein et al., 2002). Nisin, the activator of the *nisA* promoter, results in the transcription of the *nisABTCIP* genes (Kuipers et al., 1995) (see Fig. 1). Another three promoters have been identified within the nisin operon. Two of them depend on the concentration of external nisin, the *nisA* and *nisF* promoters (Qiao and Saris, 1996). Whereas, the *nisR* promoter ensures a constitutive expression of the sensor, NisK and regulator, NisR (de Ruyter et al., 1996). Additionally, the activation of the *nisA/Z* promoter also leads to transcription of *nisRK* (Ra et al., 1996). This guarantees that the signal transduction cascade is always present at sufficient basal levels. The fourth promoter is the *nisI* promoter, which is also a constitutively active promoter ensuring a basal level of immunity against nisin. Without these feed-back loops, the first expression of nisin would be a severe problem for any host. However, upon the activation of the *nisA* promoter, the expression of NisI is also elevated. Thus, an elevated level of resistance is provided. Full immunity, however, is only obtained via the activation of the *nisF* promoter, which initiates the expression of the *nisFEG* genes. The *nisF* promoter is also induced via the NisR/NisK system as disruption of the *nisR/nisK* genes leads to higher susceptibility of *L. lactis* strains against nisin, due to the lack of the full or elevated transcription of the *nisFEG* as well as *nisI* genes (Saris et al., 1996).

However, the *nisF* promoter has a weaker transcription efficiency compared with the *nisA* promoter (de Ruyter et al., 1996). Higher nisin concentrations are required to reach the same transcription levels of *nisF* promoter dependent genes as compared with the *nisA* promoter controlled genes (de Ruyter et al., 1996). As a consequence, genes directed by the *nisF* promoter became transcript at high external nisin concentrations.

4. Immunity against nisin

Lantibiotics, produced by Gram-positive bacteria, possess an antimicrobial activity and are produced to ensure survival of the organism in times of, for example, nutrition shortage. Furthermore it has been shown that small peptides are used for cell–cell communication (Sturme et al., 2002). The producer strain, however, should not be affected by the lantibiotic. Therefore, they developed an immunity mechanism containing a distinct set of genes, which when expressed, lead to a specific resistance against their own produced lantibiotics. These self-immunity proteins are called LanFEG and LanI (Draper et al., 2008).

LanFEG is an ABC transporter, which is energized by binding and/or hydrolysis of ATP and allows transport (export or import) of substances across the membrane of a bacterial cell. The second protein, LanI, is an immunity protein expressed in the cells. Some part is covalently attached to a palmotyl moiety at the N-terminus, and thereby localized to the outer leaflet of the cell membrane, while the remaining part is secreted into the medium.

4.1. The ABC transporter NisFEG

ATP binding cassette (ABC) transporters comprise one of the largest families of membrane proteins present in all kingdoms of life. They transport a large variety of substrates ranging from small ions to large proteins of up to 800 kDa of size (Hinsa et al., 2003). They can be subdivided in two major classes, the export and import ABC transporters (Davidson et al., 2008). Generally ABC transporters consist of four domains, two hydrophobic membrane domains (TMDs) and two hydrophilic cytosolic nucleotide binding domains (NBDs). The NBDs show a high sequence similarity between all ABC transporters despite their widely different transport substrates, while the TMDs display very little sequence homology. In the genome of an organism, NBDs are recognized by certain sequence motifs that are important for ATP binding and hydrolysis. Strikingly, all of the characteristic sequence motifs of an ABC transporter reside within the NBD. These are the Walker A, the Walker B, the H-loop and the two hallmarks of ABC transporters, the C-loop or ABC signature motif (LSGGQ) (Schmitt and Tampe, 2002), and the equally distinctive feature, the D-loop (Higgins and Linton, 2004; van der Does and Tampe, 2004; Zaitseva et al., 2006). Generally, within an operon, the membrane component(s) are located next to the NBD. Although, in some cases both proteins are fused to one polypeptide.

Several high-resolution structures of full length ABC transporters and several NBDs have now been solved (Dawson and Locher, 2006; Hollenstein et al., 2007; Hvorup et al., 2007; Locher et al., 2002; Schmitt et al., 2003; Smith et al., 2002; Zaitseva et al., 2005a). Based on these structures as well as biochemical experiments, we know that ABC transporters function as dimers (Fetsch and Davidson, 2002; Zaitseva et al., 2005a,b). They need an ATPase domain that is involved in the binding and hydrolysis of ATP that, in turn, provides energy for the transport of the substrate via the transmembrane domains. ABC importers contain an extra domain (Berntsson et al., 2010), either fused to the transporter or as an extra lipophilic membrane associated protein which binds substrate with high affinity and releases it into the designated transport for subsequent import into the cell. Since the function of these proteins is to bind substrate, they are called “substrate binding proteins (SBPs)”. These SBPs undergo a substantial domain movement upon binding of the substrate as well as during release of the substrate to the ABC importer, which has been called the “Venus Fly trap” mechanism and has been well studied over the last decades (for a recent review see Berntsson et al., 2010). Generally, there are at least two ABC transporters found in the lantibiotic operon, like LanT, which transport the prepeptide and LanFEG, which is involved

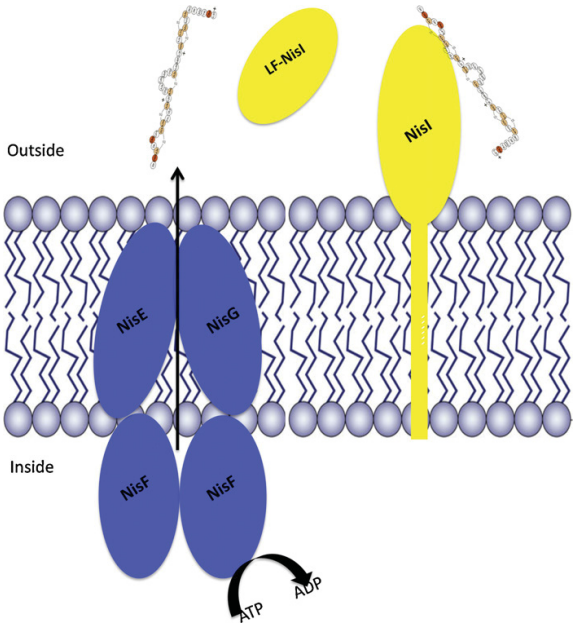

Fig. 2. The nisin immunity system. The nisin immunity system consist of NisFEG (color coded in blue) as well as NisI (color coded in yellow) which can be anchored at the membrane or in a soluble form (LF-NisI). For more details see text.

in the immunity. A schematic view of the immunity transporter is shown in Fig. 2. Here, NisF represents the NBD, which binds and subsequently hydrolyzes ATP. The membrane component consists of two membrane proteins, called NisE and NisG, forming a heterodimeric membrane complex. NisG is a 24 kDa integral membrane protein consisting of 214 amino acids with six predicted transmembrane spanning helices. NisE is a 28-kDa integral membrane protein containing a number of six predicted transmembrane helices as well. Assembled as a complex, these three proteins form a functional ABC transporter. Deletion of one of the subunits abolishes the resistance to the lantibiotic completely (Siegers and Entian, 1995).

The composition of an ABC transporter by three single polypeptide chains is not unusual for ABC transporter, however it is mainly found for ABC importers. Two of the best characterized systems are the maltose uptake system of *Escherichia coli*, $MalFGK_2$ where MalF and MalG are the transmembrane components (Davidson et al., 2008), and the histidine uptake system consisting of an ABC transporter $HisQMP_2$ and a soluble substrate-binding receptor HisJ (Ames et al., 2001). In both cases, the ABC transporter consists of three proteins. Only one example of a bacterial ABC exporter is known where the ABC transporter consists of three individual polypeptides. The $LolD_2CE$, which is involved in sorting of lipoproteins and subsequent detachment from the membrane leading to the water soluble lipoprotein LolA in the periplasmic space of *E. coli* (Narita, 2011).

Next to the above mentioned sequence motifs that are characteristic for ABC transporters, the NBD harbor a highly conserved glutamine in the so-called "Q-loop". This glutamine is involved in rigid-body motions within the NBD upon ATP binding and seems to act as a sensor for ATP. Recently, Okuda et al. suggested that this glutamine of the Q-loop is replaced by a glutamate residue in the family of lantibiotic immunity ABC transporters. In addition to the sensor function, the Q-loop has been shown to be involved in communication between the NBD and transmembrane segments of the TMD (Dawson et al., 2007; Oldham et al., 2008). Mutational studies of the NukFEG transporter, which gives resistance to nukacin ISK-1 revealed that resistance has been lowered or completely abolished upon mutation of this glutamate residue to either Gln or Ala (Okuda et al., 2010). This highlights the important role of the unusual sequence of the Q-loop in the immunity activity of lantibiotic ABC transporters.

Sequence comparison revealed that NisFEG is conserved in all species producing nisin and also shares a significant amount of similarity with strains producing other lantibiotics like for example *B. subtilis*, which produces subtilin.

Hydrophobicity analysis of NisE and NisG demonstrated that both proteins contain six transmembrane helices. However, the conservation of tryptophan residues within these sequences is more important (see Fig. 3). An alignment of the NisE and NisG with homologues from *Streptococcus uberis* (NusE and NusG), *Bacillus cereus G9241* (BsaE and BsaG), *B. subtilis* (SpaE and SpaG) and *Finegoldia magna ATCC 53516* (MutE and MutG) is shown in Fig. 3A and B. The appearance of tryptophan residues is generally rare in proteins. In NisE and NisG 9 (out of a total of 242; 4%) and 5 (out of a total of 214; 2.5%) tryptophan residues are present, respectively. More striking is the fact that these Trp residues are almost exclusively located within the predicted transmembrane helices. In helix II of NisE, two Trp residues are located in the middle, while in helix IV, one Trp is located slightly more towards the cytoplasm. At the end of helix V, a conserved Trp is followed by a – PYTY – sequence motif. This motif is conserved among the immunity genes and suggests that these aromatic or even Trp residues fulfill a specific role during substrate transport. Also, sequence alignment of NisG revealed a conserved Trp residue at the end of helix V. In the middle of helix V as well as after helix VI two other Trp residues are observed within BsaG, SpaG, and MutG. These Trp are not conserved in NusG and NisG. The latter two, however, have two other Trp residues located in the middle of helix III and IV; maybe these residues counterbalance missing Trp residues (see Fig. 3). The conservation of these residues distributed at the cytosolic, middle and exterior site of the transmembrane helices suggest a distinct transport mechanism conserved among all these proteins.

The NisFEG complex is expelling nisin molecules, before or during pore formation into the surrounding media. It is confirmed that NisFEG exports nisin molecules from the membrane, not modifying or degrading it, as most of the nisin could be recovered when applied to *B. subtilis* cells expressing NisFEG (Stein et al., 2003).

The relative contribution of NisFEG to immunity, first studied in 1995 (Siegers and Entian, 1995), was determined by a knockout approach. Disruption of NisF and NisE made cells more sensitive as in the wild type background. However, mutation of NisG did not have a significant effect on immunity (Siegers and Entian, 1995). Nevertheless, maximal immunity of *L. lactis* cells can only be achieved, if the immunity proteins NisI and NisFEG and the modification and processing machinery NisBTCP are expressed. The independent deletion of *nisABTCI* genes and the effect on the immunity and nisin production is well described (Ra et al., 1999). The NisFEG proteins maintain the crucial part of the nisin immunity in *L. lactis*. This is confirmed by gene deletions (e.g. Δ*nisA*, Δ*nisB*, Δ*nisC*, and/or Δ*nisT*), which result in a nisin non-producing strain. If nisin is not produced, the histidine kinase NisK does not phosphorylate NisR and consequently the *nisA* and *nisF* promoters are not activated. Thus only minimal levels of the immunity proteins are present. If these knockout strains are supplemented with minimal concentrations of nisin, the immunity increases due to the expression of NisI and NisFEG (Ra et al., 1999).

4.2. NisI, a second line of defense?

A second protein family involved in lantibiotic immunity is the LanI family. In the case of the nisin operon, NisI is a 245 amino acid

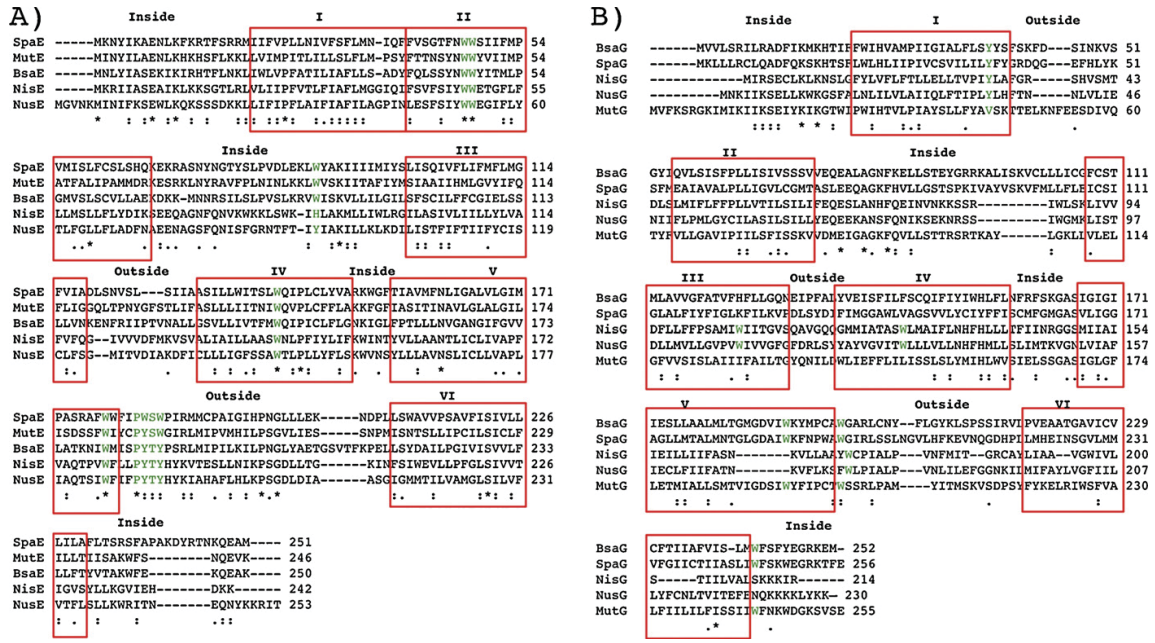

Fig. 3. Sequence alignment of NisE and NisG. (A) Sequence alignment of NisEG homologs, SpaEG from *B. subtilis*, MutEG from *Finegoldia magna ATCC 53516*, BsaEG from *Bacillus cereus G9241* and NusEG from *Streptococcus uberis*. Transmembrane helices predicted by a hydrophobicity plot are highlighted by a red square. Tryptophan residues are highlighted in green. Loops, which are predicted to be cytosolic are named "inside", while extracellular loops are labeled "outside".

lipoprotein, with an N-terminal signal sequence, removed during posttranslational modification resulting in the anchoring of NisI to the extracellular side of the cell wall (Qiao et al., 1995). Some of the expressed NisI escape this lipid modifications. The group of Koponen showed that approximately half of the produced NisI is secreted into the medium (Koponen et al., 2004; Takala et al., 2004).

NisI contributes to an immunity against nisin in *L. lactis*, along with NisFEG and displays a high specificity towards nisin. A knock out of NisI resulted in cells more sensitive than the corresponding NisFEG knock out (Siegers and Entian, 1995). This observation resulted in the hypothesis that NisI plays a more important role in the immunity against nisin (Draper et al., 2008). NisFEG conferred around 20% of immunity when expressed alone comparing to the wild type immunity (Ra et al., 1999). There is not much known about the LanI protein family and the proteins within this family exhibit a rather low sequence similarity, presumably due to the specific lantibiotic characteristics that is bound. NisI contains a hydrophobic N-terminal region containing a lipid-protein sequence found also in other LanI proteins like for example PepI the immunity protein of Pep5.

When expressed in *B. subtilis*, NisI provides significant immunity against nisin. However, simultaneous expression of NisFEG and NisI in *B. subtilis*, resulted in higher levels of immunity. This confirmed the initial hypothesis that the specificity of the nisin immunity system against nisin can be transferred into different bacterial species (Stein et al., 2003).

The exact mechanism of NisI to function as an immunity protein is still unknown, although Takala and his colleagues mentioned the importance of the C-terminus of NisI to interact with nisin. Their study identified a 21aa deletion at the C-terminus of NisI, reduced the immunity to 14% compared to the native level. However, this C-terminal region of NisI is not involved in the co-operation with NisFEG as the truncated NisI showed a cooperative effect of nisin resistance when co-expressed with NisFEG (Takala and Saris, 2006). Moreover, they showed that the replacement of the 21 C-terminal amino acids of the subtilin-specific SpaI with the C-terminal 21 amino acid of NisI (SpaI′-NisI′) confers immunity against nisin (Takala and Saris, 2006).

Similar results were obtained for PepI. Hoffmann et al. showed that the C-terminal part mainly provides immunity, while the N-terminal part is more important for exporting PepI out of the cells (Hoffmann et al., 2004). PepI is the simplest immunity system among lantibiotics, which protects *Staphylococcus epidermidis 5* from its own product Pep5. Similar to NisI, it is located and functions at the membrane-cell wall interface, as shown by fusions of PepI and green fluorescent proteins (Hoffmann et al., 2004).

4.3. Immunity by non-producer strains: NSR – the nisin resistance protein

Nisin producing strains have developed a resistance machinery to oppose their own lantibiotic consisting of the protein NisI and the ABC transporter NisFEG (Stein et al., 2003). Nevertheless not all species belonging to the *L. lactis* subfamily produce and secrete nisin molecules. Remarkably, the non-producing *L. lactis* strains have developed different mechanisms to prevent growth inhibition when encountering a high level of nisin in the media. In non-producing strains, an enzyme is found which protects the cell against nisin. This enzyme is called nisin resistance protein (NSR) (Froseth et al., 1988; Froseth and McKay, 1991; Tang et al., 2001). Froseth et al. first discovered an open reading frame of 957 nucleotides encoding a 319 amino acid protein on a plasmid in *L. lactis* subsp. *lactis biovar diacetylactis* DRC3. The amino acid composition predicted a N-terminal hydrophobic region from amino acid 7–28, which likely is membrane associated. By sequence homology studies several other NSR like proteins where found in three other Gram-positive bacteria families (Froseth and McKay, 1991). Fig. 4 shows an alignment of these proteins.

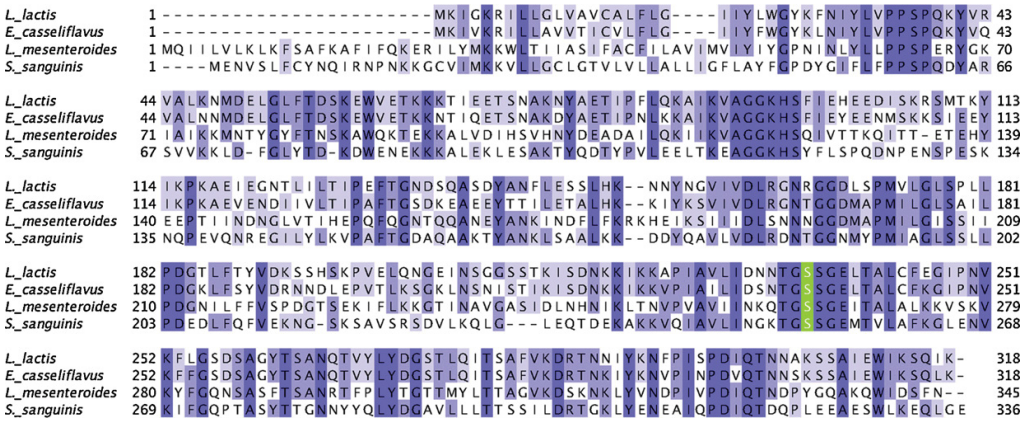

Fig. 4. Sequence alignment of the NSRs. Shown are sequences from *L. lactis* subsp. *lactis* (lane 1) and *Enterococcus casseliflavus* EC10 (lane 2), *Leuconostoc mesenteroides* subsp. *cremoris* ATCC 19254 (lane 3) and *Streptococcus sanguinis* SK1087 (lane 4). The percentage of amino acid identity are given in different blue colors, where light blue represent low and dark blue high identity. The putative catalytically active serine is highlighted in green.

In 2009, Sun et al. expressed the *nsr* gene in *E. coli* and *L. lactis*. The first construct (NSR) corresponds to the full-length protein including the hydrophobic sequence at the N-terminus (Sun et al., 2009). The second construct lacks the first 27 amino acids and was called NSR-SD. Localization studies showed that NSR is mainly located at the cell membrane whereas NSR-SD is mainly expressed as a soluble cytosolic protein. In vitro studies of NSR-SD revealed a proteolytic digestion of nisin between position MeLan28 and Ser29. The obtained nisin fragment, which still contains the five characteristic lanthionine rings, is 100-fold less active against the nisin sensitive strain *L. lactis* MG1363. Cleavage of the last 6 aa shortens nisin, therefore, it is no longer able to span the target membrane and pore formation is thereby inhibited. The remaining antimicrobial activity is due to the binding of nisin to lipid II, thereby inhibiting cell wall synthesis (Hasper et al., 2006).

In vitro activity of the membrane associated NSR could not be observed (Sun et al., 2009). This indicates that although no full resistance can be observed, non-nisin producing strains developed their own immunity system to deal with the presence of nisin in the medium.

5. Immunity system of other lantibiotics

Homologous proteins of the immunity proteins NisI and NisFEG are found in *B. subtilis*, which produces subtilin. SpaI is similar to NisI. It contains an N-terminal hydrophobic region with a lipoprotein signal and it functions as assistance in immunity. It is proposed that SpaI sequesters subtilin at the cytoplasmic membrane, thus preventing subtilin from forming pores and attacking its own cells (Stein et al., 2005). Meanwhile, SpaF showed significant homology to other LanF proteins and the SpaEG proteins have similar sizes and hydrophobicities comparable to other LanEG proteins (Stein et al., 2002). Besides, the expression of the *spaIEFG* genes in the subtilin sensitive *B. subtilis* strain MO1099 confirmed their functions and resulted in even higher levels of immunity than the normal subtilin producer *B. subtilis* ATCC 6633 (Stein et al., 2005). Even in the case of nisin, 90% of nisin was recovered in the media. Both results supported the hypothesis that NisI-FEG and SpaI-FEG are exporting nisin and subtilin, respectively, without modifications or degradations of the lantibiotic (Stein et al., 2003).

In general, all *lanI* genes showed no homology with each other or with any other immunity protein (Twomey et al., 2002). This resulted in sme difficulties to study them. PepI, which showed 74.2% homology to EciI, the immunity gene of epicidin 280, is the huge exception (Heidrich et al., 1998). Although PepI provided immunity to epicidin 280, this is not the case for lantibiotics in general, which are known to be specific to their respective lantibiotic. This cross-immunity could be a result to the fact that the immunity system in both Pep5 and epicidin 280 are simple and no ABC transporter is involved in immunity (Draper et al., 2008). In contrast, nisin and subtilin showed no cross-immunity although they are closely related with 63% of sequence homology (Stein et al., 2005).

6. Application of nisin and its immunity pathway

Lantibiotics are of increasing interest in the biotechnological as well as the pharmaceutical industry, since the specific inhibition of bacterial cell growth can be exploited in many ways. Active nisin for example, has now been used for over 50 years in the food industry, for example within cheese- and milk production, canned vegetables, diverse pasteurized dairy and salad dressing productions (de Vuyst and Vandamme, 1993). Although nisin has been added to the European food additive list in 1983 (number E234), it has been identified as a safe product to be used as an additive in food since 1969 by the "Joint Food and Agriculture Organization/World Health Organization Expert Committee of Food Additive" (Cotter et al., 2005).

Besides the lantibiotic itself, the nisin operon has been exploited for industrial and/or basic research purposes. For example, the two-component system (NisR and NisK) has been successfully used as an expression system in *L. lactis* for over more than a decade. This nisin-controlled gene expression system (NICE) is of great interest due to its properties of being tightly regulated and its high degree of induction (Mierau and Kleerebezem, 2005). With NICE system, many proteins can be expressed in order to study their function and/or to produce them for industrial purposes, like for example metabolic proteins, bacteriocins as well as antigens (Zhou et al., 2006). *L. lactis* is a non-pathogenic and a non-toxic bacteria, and therefore it is considered an excellent host for the production of heterologous proteins for experimental or commercial applications (Nouaille et al., 2003). The expression of a protein of interest is induced by external addition of nisin and the "dose" of gene expression can be controlled in some way by the amount of added nisin (Mierau and Kleerebezem, 2005). Thus, both *L. lactis* as well as the

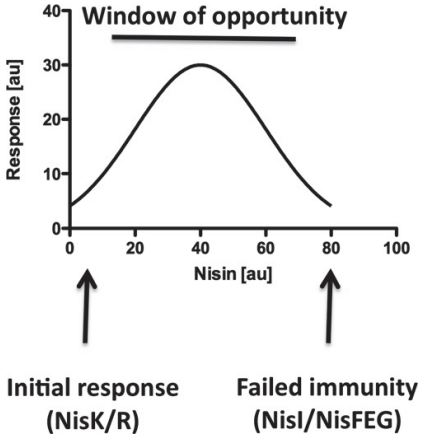

Fig. 5. Schematic summary of "working principle" of the NICE system in biotechnological applications. At the first arrow ("initial response") the concentration of nisin in the media is high enough to induce response of the two-component system and expression of the gene under the control of the corresponding *nis* promoter. Increasing concentrations of nisin (plotted in arbitrary units [au]) increases the response (also plotted in arbitrary units [au]). However, the response reaches a maximum at a certain concentration of nisin and starts to decline, because of the bacteriocidal effect of nisin on the producer strain. This effect is counterbalanced by the immunity gene, but at a certain concentration of nisin (indicated by the right arrow). This scheme represents the basic boundaries of the nisin system highlighting the "window of opportunity" by either increasing the affinity of the two component system or by increasing the tolerance towards nisin would extend the possibilities of biotechnological applications.

nisin derived NICE-expression system result in a versatile device to be used for protein/enzyme production.

There is however, one drawback. Since nisin is active against *L. lactis*, the usage of increasing nisin concentrations, which, in principle, should enhance expression levels, is not possible due to the subsequent "killing" activity of the inducer. In Fig. 5, a theoretical dose–response curve is shown. Here, the expression of any gene of interest under the control of a nisin promoter is shown in arbitrary units. At initial concentrations of nisin, where the NisK/NisR response is low, only low amounts of the protein of interest is produced. At a certain concentration of nisin, a critical level is reached and *L. lactis* cells are attacked and eventually lysed by the added inducer (nisin). The usage of the nisin immunity genes will shift this dose–response curve and will create a maximum at higher concentrations of external nisin. Accordingly, an implementation of the immunity genes in the NICE expression system, will allow usage of higher amounts of inducer (nisin) and consequently increase the amount of the expressed protein.

A recent study mentioned that the *nisI* gene could act as an extra factor for the NICE system, as the expression of a recombinant protein (GFP) was roughly 2-fold higher when the *nisI* gene was inserted into the NICE expression vector (Oddone et al., 2009). Before this report, the *nisI* gene was used as a food-grade selectable marker by Takala and Saris in 2002 when they constructed a vector, pLEB590, for lactic acid bacteria (LAB) showing a high and easy selection of the transformants on nisin plates (Takala and Saris, 2002). This *nisI*-mediated vector enabled a selection for many LAB bacteria other than *L. lactis*, like *lactocacilli* bacteria. Lately, in 2011, both *nisI* and *nsr* (nisin-resistance gene) genes were used as a selection markers with 5 μg nisin/ml for *nsr* gene (Li et al., 2011). Here, as noticed, the knowledge about the immunity system can help, after understanding its mechanism, to gain a better controlled expression system eventually leading to higher levels of the interested protein.

Some of lantibiotic producing bacteria are human pathogens. For example, *Streptococcus agalactiae* causes pneumonia and meningitis in neonates and the elderly. *Streptococcus suis*, which is an important pathogen of pigs and causes severe infections including meningitis, septicaemia, endocarditis, or deafness when transferred to human. Another example is *Streptococcus pyogenes*, which is the cause of many human diseases, ranging from mild superficial skin infections to life-threatening diseases. Infections typically begin in the throat or skin, and, in general, a lot of medical treatments are using antibiotics (one or even a cocktail of several different ones) and due to this high usage of antibiotics, the occurrence of resistant strains is a severe problem in the treatment of the patients.

As the genome sequences of these organisms revealed the presence of only lantibiotics immunity genes, a detailed understanding of the LanFEG and LanI protein families could gain insight into their resistance mechanism. Since a specific inhibitor against one of the immunity proteins would inhibit the self-protection of these strains specifically and thereby these human pathogenic strains would become susceptible to their own lantibiotic. This would mean that the pathogenic bacteria would kill itself by the expression of lantibiotics.

The same holds true for pathogenic strains containing a nisin resistance protein, which inactivates lantibiotics by C-terminal cleavage. If a specific inhibitor would exist, these strains could, in principle, be treated by a cocktail of LanI inhibitors and for example nisin.

The major advantage of a medical usage of lantibiotics is that lantibiotics do not harm human cells and therefore will not cause any side effects. Thus, they seem to be an excellent candidates for medical purposes. Although, this is currently only a vision, the potential is truly present and might be used by further studies of the immunity genes encoded in the operons of lantibiotic producing Gram-positive bacteria.

7. Concluding remarks

The nisin operon contains eleven proteins, which all have different functions. Interestingly, all eleven proteins act as a symphonic orchestra and only together an efficient and effective production of nisin is possible. The auto-immunity of producer strains against their own lantibiotics, highlights the enormous power of the immunity system, since in comparison the target cells lacking an immunity response are already killed by nM amounts.

Next to the biosynthesis and modification apparatus, the two-component system as well as the immunity system attracts more and more interest of both, basic and industrial researchers.

The immunity against lantibiotics has been observed in producer strains but up to date not in target cells. The LanFEG and LanI superfamilies of proteins have been studied in the past, but due to their potential role in possible medical and industrial applications, they will be move more and more in the research focus of many disciplines within the next years.

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CHAPTER II

NSR from *Streptococcus agalactiae* confers resistance against nisin and is encoded by a conserved *nsr* operon

By

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Short Communication

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NSR from *Streptococcus agalactiae* confers resistance against nisin and is encoded by a conserved *nsr* operon

Abstract: Nisin is a lantibiotic produced by *Lactococcus lactis* (*L. lactis*), which is active against many Gram-positive bacteria. However, in various pathogenic and non-pathogenic bacteria, the presence of a nisin resistance protein (NSR) confers resistance against nisin. Here, we show that NSR from *Streptococcus agalactiae* (*Sa*NSR) confers 20-fold resistance when expressed in *L. lactis*. We also show that *Sa*NSR is encoded by an operon structure comprising of a lipoprotein and an ATP-binding cassette transporter as well as a two-component system that is putatively involved in expression and regulation. This organization of the operon is conserved in several (non) pathogenic strains that do not produce nisin themselves.

Keywords: ABC transporter; immunity; lantibiotic; lipoprotein; nisin resistance protein; regulation.

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Lantibiotics are ribosomally synthesized peptides that are characterized by the extensive post-translational modifications they undergo and are approximately 19–38 amino acids in length (Sahl and Bierbaum, 1998). The dehydration of serine and threonine residues in the prepeptide forms dehydroalanine and dehydrobutyrine amino acids (Chatterjee et al., 2005a), which then covalently link to the free cysteine thiols to form (methyl) lanthionine rings (Ingram, 1969). These lanthionine rings are important for the activity because they stabilize the peptide and protect it from proteolytic degradation (Bierbaum et al., 1996). Lantibiotics have a broad antimicrobial spectrum against various Gram-positive bacteria and thus are interesting candidates for pharmaceutical applications (Willey and van der Donk, 2007).

One of the best-characterized lantibiotic is nisin, a type I lantibiotic. It is an amphiphilic, cationic peptide of 34 amino acids. It is produced by *Lactococcus lactis* and was first discovered in 1928 (Rogers, 1928). Nisin contains one lanthionine ring and four methyl-lanthionine rings (Chatterjee et al., 2005b) and has bactericidal activity against many Gram-positive bacteria including *Streptococcus pneumonia*. It also prevents the outgrowth of many *Clostridium* and *Bacillus* spp. (Harris et al., 1992). The bactericidal efficiency of nisin is due to its capability to inhibit the cell wall biosynthesis (Reisinger et al., 1980) and its pore-forming ability (Wiedemann et al., 2001; van Heusden et al., 2002), where it uses the cell wall precursor lipid II as a docking molecule (Hasper et al., 2006). This activity can be quantitatively measured by growth inhibition of the target bacteria either on agar plates [minimum inhibitory concentration (MIC)] (Wiedemann et al., 2001) or in liquid medium [the concentration of lantibiotic that inhibits cell growth by 50% (IC_{50})] (Oman and van der Donk, 2009). Because nisin is bactericidal in nature (low nanomolar concentrations of nisin are enough to permeabilize the target membrane), there is a mechanism in nisin-producing strains conferring immunity against their own harmful lantibiotic and thus preventing a suicidal effect. A specific lipoprotein, NisI, and an ATP-binding cassette (ABC) transporter, NisFEG, together fulfill this role (Siegers and Entian, 1995). Interestingly, both these proteins act cooperatively, and only 20% of the total immunity is conferred in the absence of either of the two (Ra et al., 1999).

However, there are some Gram-positive human pathogenic bacteria such as *Staphylococcus aureus* (Carlson and Bauer, 1957) and *Streptococcus agalactiae* (Hirsch, 1950) that display substantial resistance against nisin (Harris et al., 1992). Interestingly, these strains themselves do not produce nisin.

In non-nisin-producer *L. lactis* subsp. *diacetylactis* DRC3, the nisin resistance determinant was found to be associated with the nisin resistance gene, *nsr* (Froseth and McKay, 1991). Sequencing of this *nsr* gene revealed an open

reading frame of 318 codons. The encoded 35-kDa protein nisin resistance protein (NSR) was recently characterized and was found to be membrane-associated, which is likely due to its hydrophobic N-terminus (Froseth and McKay, 1991). It has been shown that NSR from *L. lactis* TS1640 degrades nisin proteolytically by cleaving the peptide bond between MeLan28 and Ser29, thus resulting in the reduced bactericidal efficiency of nisin (Sun et al., 2009). The cleaved nisin, still having the five characteristic lanthionine rings, was found to have a reduced affinity for the cell membrane and showed 100-fold less activity (Sun et al., 2009). NSR belongs to the S41 family of peptidases, also known as the C-terminal processing peptidase (CTP). CTPs are characterized by an active site consisting of a catalytic dyad made up of serine and lysine and an N-terminal signal peptide (Keiler and Sauer, 1995; Rawlings et al., 2012). Based on homology modeling, it was later identified that NSR contains a tail-specific protease domain at its C-terminus (Silber et al., 1992) that is responsible for the C-terminal specific cleavage of its substrate.

By sequence homology studies, we identified an *nsr* gene in *S. agalactiae* ATCC 13813 (Gene Accession No.: HMPREF9171_1170). The gene product, here referred to as *Sa*NSR, shows to confer resistance when expressed in a nisin-sensitive *L. lactis* strain (Figure 1). A shift in IC_{50} toward higher nisin concentrations indicates that *Sa*NSR confers resistance. Comparing the IC_{50} values of nisin incubated with the sensitive strain harboring the empty plasmid (IC_{50}=3.8±0.4 nM) and the *Sa*NSR-expressing strain (IC_{50} of 69±1.2 nM) highlighted the fact that NSR from *S. agalactiae* confers a 20-fold resistance. As previously shown, NSR proteins act as serine proteases and the catalytic serine is conserved among the NSR homologues. When mutating this serine to alanine (gene product annotated as *Sa*NSR-S236A), the resistance against nisin drops to merely 8±1.1 nM. This slight increase when compared with the sensitive strain containing the empty plasmid is likely due to the fact that although catalytically deficient, the expressed *Sa*NSR-S236A protein still binds to the nisin molecules, resulting in a slightly higher number of nisin molecules, which are needed to inhibit the cell growth.

Additionally, through genomic data and comparative sequence analysis and using the *nsr* gene from *S. agalactiae* ATCC 13813 as the query sequence, we found that this gene is localized in a specific operon within the genome, termed here as *nsr* operon encoding six different proteins. Similar to the immunity system present in the producer strains (Alkhatib et al., 2012), the *nsr* operon consists of a lipoprotein, NSR, and an ABC transporter, termed here as NsrFP (NsrF is named after LanF found in the producer strains and P stands for the permease). Furthermore, a two-component system is also present, consisting of the response regulator and the histidine kinase (designated as NsrR and NsrK, respectively). Likely, this nisin resistance mechanism (schematically shown in Figure 2) is similar to the process of immunity in nisin-producing strains because of the high similarity between the genes present in both the systems.

This *nsr* operon is found to be present in some nonpathogenic species *Corynebacterium casei* and *Corynebacterium ammoniagenes*; in various pathogenic strains of *Leuconostoc mesenteroides*, *Leuconostoc carnosum*, *Enterococcus faecium*, *Staphylococcus epidermis*, *Streptococcus ictaluri*, *Streptococcus sanguinis*; and in different strains of *Streptococcus dysgalactiae* and *S. agalactiae*.

Further analysis showed variation in the orientation and the order of the genes in the *nsr* operon, which can be categorized in four different groups (Figure 3). The first group (I) comprises the nonpathogenic strains *C. casei* and *C. ammoniagenes*, which contain *nsrR*, *nsrK*, *nsrFP*, and *nsr* in their operon. However, here, the translational direction of *nsrFP* and *nsr* genes is opposite of *nsrR* and *nsrK* genes. The second group (IIa and IIb) comprises the

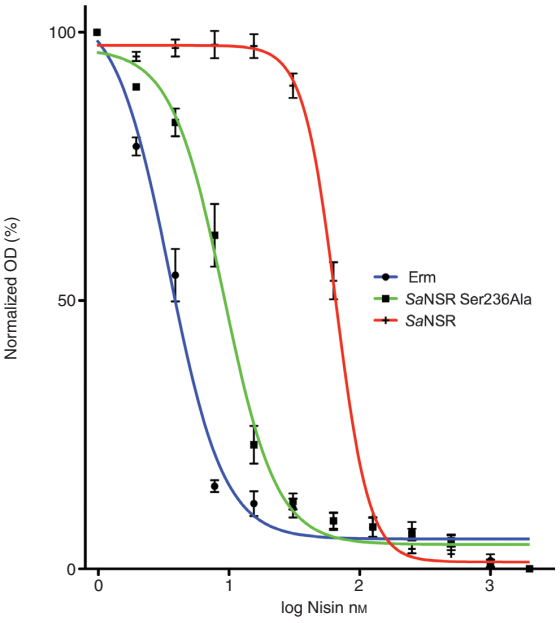

Figure 1 IC_{50} determination of nisin incubated with strains expressing *Sa*NSR and *Sa*NSR-S236A. Shown are the inhibition curves for strains expressing *Sa*NSR (red), *Sa*NSR-S236A (green), and the nisin-sensitive *L. lactis* strain NZ9000 (blue). The IC_{50} measurements were performed according to the protocol mentioned by Abts et al. (2011).

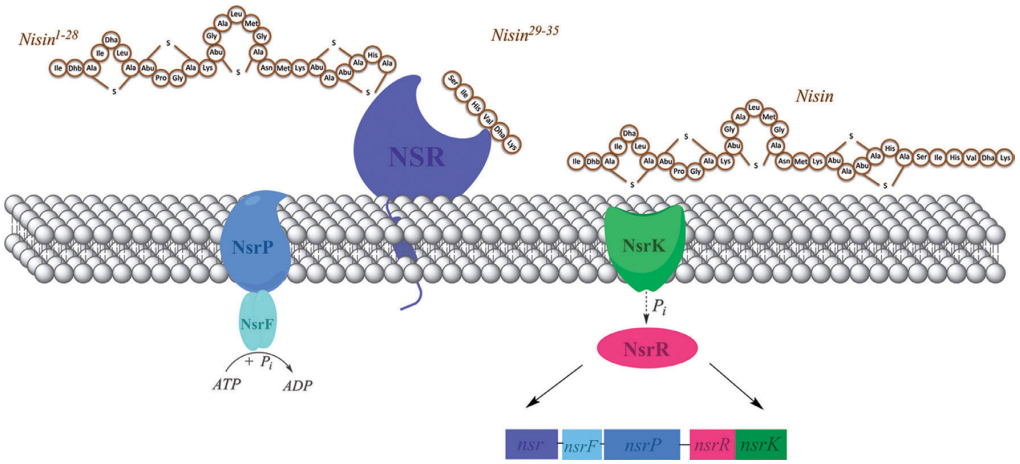

Figure 2 Mechanism of nisin resistance. The nisin resistance system consists of membrane-associated NSR, histidine kinase NsrK, transcriptional regulator NsrR and an ABC-transporter NsrFP. NSR cleaves the nisin molecule into nisin$^{1-28}$ and nisin$^{29-35}$ fragments, which show reduced activity.

pathogenic *Streptococcus* species where the gene order is different, encoding *nsr* and *nsrFP* genes first and then the two-component system genes, *nsrR* and *nsrK*. *S. ictaluri* also belongs to this group, however, here the *nsrP* gene is much smaller than that observed in the other operons (see below) and is therefore classified separately into group IIb. One interesting observation is that the *nsr* operon found in *S. sanguinis* encodes two additional *nsrFP* ABC transporters, suggesting that it might be resistant against different lantibiotic peptides, where every ABC transporter might be responsible for resistance against a specific antimicrobial peptide (group III, Figure 3), which is also reflected by its large operon size. However, the members of group IV do not have the complete operon and in *L. carnosum*, *E. casseliflavus*, and *E. faecium*, only the *nsr* gene is present, whereas the putative genes for regulation and transportation could not be identified. In contrast, the operons of *S. epidermis* and *L. mesenteroides* have *nsr* and *nsrFP*, whereas *nsrR* and *nsrK* genes were not identified. (Note: The genomes of the species of group IV are not fully sequenced or completely assembled, and only DNA contigs could be found, which might be the reason for the missing genes.) Recently, a similar operon structure has also been found, which is associated with nisin resistance in *Streptococcus mutans* UA159 (Kawada-Matsuo et al., 2013). Despite the variation in the operon structures, the same set of genes putatively involved in resistance, regulation, and transport remain present in these groups.

The proteins NsrF and NsrP together encode a functional ABC transporter (Figure 2). ABC transporters comprise one of the largest families of membrane proteins that are present in all kingdoms of life and are subdivided into two major classes, the exporters and the importers (Davidson et al., 2008). Generally, an ABC transporter consists of two hydrophobic transmembrane domains (TMDs) and two hydrophilic cytosolic nucleotide-binding domains (NBDs). Within the *nsr* operon, the protein encoded by *nsrF*, designated NsrF, represents the NBD, and NsrP encoded by *nsrP* is the TMD, which forms the transport pore within the membrane. Sequence analysis of NsrF protein reveals that it contains all the ABC transporter sequence motifs. These are the Walker A, the Walker B, the H-loop, and the two hallmarks of ABC transporters, the C-loop (or ABC signature motif, LSGGQ) (Schmitt and Tampé, 2002) and the D-loop (Higgins and Linton, 2004; Van Der Does and Tampe, 2004; Zaitseva et al., 2006). These sequence motifs are important for ATP binding and hydrolysis and are needed to energize the transport of the substrate *via* the TMDs.

NsrFP belongs to the ISVH family of ABC transporters that comprises a macrolide-specific ABC-type efflux carrier (MacAB), which confers resistance to macrolides, and proteins involved in cell division (FtsE), and lipoproteins released from the cytoplasmic membrane (LolCDE) (Holland et al., 2003). Furthermore, the ABC transporter present in the genus *Enterococcus*, which has been proven to confer resistance against the antimicrobial peptide bacitracin, also belongs to this ABC transporter family (Matos et al., 2009).

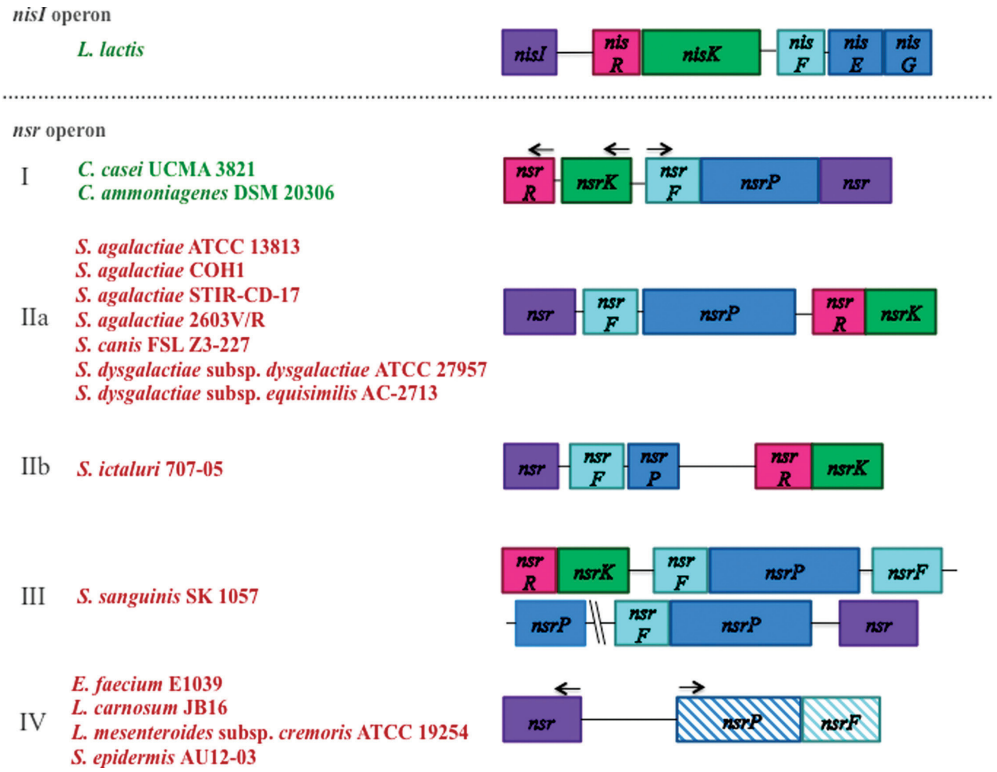

Figure 3 Structure of the nisin resistance operon. The operon structure of *nisI* in producer strain and *nsr* in different nonproducer strains has been shown. Genes performing similar functions are color-coded identically. Nonpathogenic strains are indicated in green, whereas the pathogenic strains are represented in red. Homologues of NSR from *S. agalactiae* ATCC 13813 (GI: 319745028) were manually retrieved from NCBI using a BLAST search (Altschul et al., 1990). Sequences having an E-value <1e-10 and percentage identical amino acids >30 were retained; partial sequences were excluded. Using UniProt (Magrane, 2011) and NCBI (Sayers et al., 2010), the genome of the selected microorganisms were searched for the *nsr* gene, and subsequently, other genes were also identified. *The genome and gene accession numbers are provided in Table 2 of the Supplementary Information.

Generally, the TMDs of ABC transporters display a low sequence similarity, reflecting the large variety of substrates they transport, ranging from small ions to large proteins of up to 900 kDa. A noteworthy feature of the *nsrP* gene is its size (around 2000 bp, encoding 630 amino acids), which is relatively large for a single TMD of an ABC transporter. Analysis of the membrane helical content of NsrP showed that it contains 10–12 transmembrane helices (Bernsel et al., 2009) (Figure 4), indicating that it might be harboring both the TMDs needed for a functional ABC transporter, which would result in the stoichiometry of a monomer of NsrP and a dimeric NsrF. One exception here is the NsrP of *S. ictaluri*, which might be forming a homodimer in the membrane, as it is predicted to contain only six helices (Bernsel et al., 2009) (group IIb, Figure 3).

Interestingly, in the NsrP proteins, a large extracellular loop of 200–250 amino acids (18–24 kDa) is present between helices 7 and 8, representing an extra domain (highlighted in red, Figure 4). The *nsrP* gene of *S. ictaluri* is smaller; consequently, the extracellular loop present in its NsrP (between helices 5 and 6) is also smaller than that present in other NsrP proteins.

Analysis of the loop sequence of NsrFP using BLAST searches (Altschul et al., 1990) did not reveal any sequence similarities to other proteins, except the NsrP-like ABC transporters that are involved in lantibiotic resistance. Furthermore, sequence comparison and structure prediction programs such as PDB-BLAST (Altschul et al., 1990) and Phyre II (Söding, 2005), yielded no significant structural homology with the known crystal structures, suggesting that this extracellular loop is an exclusive characteristic of the lantibiotic resistance-associated family of ABC transporters. Extra domains fused to ABC transporters are not unusual and perform some specific functions. In

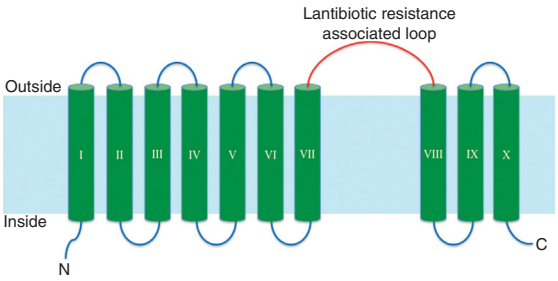

Figure 4 Predicted secondary structure of NsrP. NsrP is composed of 10 transmembrane helices (Bernsel et al., 2009) shown in green. Between helices 7 and 8, an extra loop domain (red) thought to be associated with lantibiotic resistance is present.

the glycine betaine ABC transporter, OpuA from *L. lactis*, an extra domain [identified as substrate-binding protein (SBP)] is fused to the membrane permease. Located at the C-terminus, the main purpose of this SBP is to bind to the substrate in the extracellular space and deliver it to the transporter (van der Heide and Poolman, 2002). A peptidase domain localized at the N-terminal is present in the ABC exporter NukT that transports Nukacin ISK-1 (a class II lantibiotic produced by *Staphylococcus warneri* ISK-1), which cleaves off the signal sequence prior to the transport of the lantibiotic into the external environment (Nishie et al., 2011). Similar to the presence of an extra domain between the helices in NsrP, an extra domain is also localized between transmembrane helices 2 and 3 in $MalFGK_2$ (the maltose uptake ABC transporter system of *Escherichia coli*). The membrane protein MalF contains a large extracellular loop (called P2-loop), which folds into an Ig-like domain, extending 30 Å away from the membrane surface and interacts with MalE (SBP) (Oldham et al., 2007) for regulatory purposes.

Because NsrFP is an ABC transporter, it should either export or import the substrate. Using the lantibiotic immunity system as a model, we hypothesize the function of NsrFP. The immunity ABC transporter SpaFEG in the subtilin-producing systems is known to act as an exporter, transporting the subtilin molecules into the external environment (Stein et al., 2005). Similarly, in the nisin-producing strains, NisFEG has also been shown to function as an exporter, expelling the nisin molecules out of the membrane into the extracellular space (Stein et al., 2003). Studies involving the expression of NisIFEG in *B. subtilis* showed that all the nisin molecules could be recovered from the media and the strain became resistant (Stein et al., 2003). Thus, LanFEG builds a so-called second line of defense in the lantibiotic-producing strains.

It is known that the lipoprotein NSR captures the nisin molecules and cleaves off the last six C-terminal residues, thereby lowering its activity (Sun et al., 2009). However, at higher nisin concentrations, it is likely that NSR is not cleaving all the nisin molecules. We propose that NsrFP would have a similar activity as the NisFEG, rescuing the microorganism from the nisin molecules that escape the first line of defense provided by NSR.

In nisin-producing *L. lactis* strain, the expression of NisI and NisFEG is regulated by the two-component system, NisR and NisK. The presence of *nsrR* and *nsrK* genes, together encoding a two-component regulatory system in the operon, suggests that the expression of NSR and NsrFP is also tightly regulated. In the producer strains, the genes for biosynthesis and immunity are regulated *via* signal transduction involving a two-component regulatory system composed of a receptor NsrK, *nisK*, and a transcriptional NsrR, *nisR* (Kuipers et al., 1995). The transcription of the immunity genes is controlled by *nisR* and *nisK*, based on the concentration of extracellular nisin (Kleerebezem, 2004). Thus, nisin acts as a pheromone and regulates its own biosynthesis and expression (Kuipers et al., 1995). Recently, the involvement of the two-component system NsrRS of *S. mutans* UA159 was confirmed in nisin resistance, which also regulates the expression of other genes found in the operon (Kawada-Matsuo et al., 2013). Thus, we postulate a similar function for NsrR and NsrK, which might also be induced by the amount of external nisin.

In conclusion, the *nsr* gene found in the genome of *S. agalactiae* confers resistance to nisin when expressed in *L. lactis*. *Sa*NSR is encoded by an operon consisting of *nsr*, *nsrR*, *nsrK*, and *nsrFP* genes, which are found in other nisin-nonproducing strains as well. The observed operon structure resembles the one found in nisin-producing strains. However, the exact underlying mechanism of the correlated functioning of these genes is still unknown, and some extensive studies on the encoded proteins are required for proper understanding. An in-depth knowledge about this resistance would open new avenues for the treatment of bacterial infections using lantibiotics.

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Chapter III

Lantibiotic immunity; Inhibition of nisin mediated pore formation by NisI.

By

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Lantibiotic immunity:

Inhibition of nisin mediated pore formation by NisI

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Abstract

Nisin, a 3.4 kDa antimicrobial peptide, is the most prominent member of the lantibiotic family, produced by some *Lactococcus lactis* strains. Nisin can inhibit cell growth and penetrates the target Gram-positive bacterial membrane by binding to Lipid II, an essential cell wall synthesis precursor. The assembled nisin-Lipid II complex forms pores in the target membrane. To gain immunity against its own-produced nisin, *Lactococcus lactis* is expressing two immunity protein systems, NisI and NisFEG. Here, we show that the NisI expressing strain displays an IC_{50} of 73 $\pm$ 10 nM, an 8-10-fold increase when compared to the non expressing sensitive strain. The cells expressing full-length NisI stop growing when the nisin concentration is raised above 70 nM rather than being killed. NisI is inhibiting nisin mediated pore formation, even at nisin concentrations up to 1 μM. This effect is induced by the C-terminus of NisI that protects Lipid II. Its deletion showed pore formation again.

The expression of NisI in combination with externally added nisin mediates an elongation of the chain length of the *Lactococcus lactis* cocci. While the sensitive strain cell-chains consist of mainly two cells, the NisI expressing cells display a length of up to 20 cells.

Both results shed light on the immunity of lantibiotic producer strains, and their surviving at high levels of their own lantibiotic in the habitat.

Introduction

Since the 1920s the heterogeneous group of bacteriocins became an interesting research topic for different applications e.g. as food preservatives or as antibiotic alternatives [1]. Bacteriocins are small, heat stable ribosomally synthesized peptides showing antimicrobial activity [2,3]. They are mostly produced by Gram-positive bacteria and act mainly against other Gram–positive species. Therefore they are candidates with high potential for the treatment of bacterial infections in humans with multiple resistances against antibiotics like the pathogenic VRE or MRSA strains [4,5].

Within the group of bacteriocins, there is a large family called lantibiotics [6]. They contain characteristic thioether bridges, called lanthionine rings, which are post-translationally introduced. These lanthionine rings provide a high level of protection against peptide-digesting enzymes, and more importantly ensure high antimicrobial activity against mainly Gram–positive bacteria, reflected by the low nanomolar amount needed to fulfill their activity [7,8].

Lantibiotics are produced and secreted in a nonactive form. Afterwards they are activated by cleavage of the specific N-terminal leader peptide. These active lantibiotics are able to lyse mainly Gram-positive bacteria and also some Gram-negative bacteria strains are affected [7,9].

Within lantibiotic producer strains, the structural genes for the biosynthesis, modification, and transport across the cellular membrane as well as the regulation are localized on a single gene cluster [7,10,11]. Additionally, genes encoding a lantibiotic specific immunity system are present, preventing that lantibiotics harm their own producer strain. Although, lantibiotics are grouped in different classes based on their sizes and activities [11,12], the lantibiotic specific immune system seems to be conserved all along. Two functional proteins are mediating this immunity. LanI, a

72 membrane associated protein, and LanFEG, an ABC transporter localized in the cellular
73 membrane [13]. In most lantibiotics encoding operons , both immunity proteins are
74 present [10].
75 Nisin is the best-known and most extensively studied lantibiotic and is in slight variants
76 produced by *Lactococcus lactis* (*L. lactis*) strains [14]. Due to the high bactericidal
77 activity in combination with the low toxicity in humans, nisin is used since decades as a
78 natural preservative in the food industry [8]. Active nisin consists of 34 amino acids and
79 contains five lanthionine rings. Here, the first three rings are separated from the two
80 intertwined rings four and five by a flexible hinge region [15]. The first two rings are
81 able to bind Lipid II and thereby inhibiting cell wall synthesis [16], the hinge region and
82 the last two rings, which are intertwined, are able to flip into the membrane and create
83 pores [9,17,18].
84 The mode of action of nisin has been thoroughly studied since its discovery. It was
85 suggested that nisin kills bacteria by inhibiting cell-wall synthesis via binding to Lipid II,
86 as observed for many other lantibiotics. However, the immediate release of small
87 cytoplasmic compounds such as amino acids, ATP or pre-accumulated rubidium from
88 cells [19], highlighted that nisin acts by the distinct permeabilization of the plasma
89 membrane. The nisin–membrane interaction was extensively studied, with a focus on
90 the interaction between the cationic nisin peptide and the abundant anionic lipids of the
91 plasma membrane of Gram-positive bacteria [19-21]. More recently, it has been shown
92 that nisin uses Lipid II as a 'docking molecule' to form pores in a targeted manner with
93 high efficiency [22,23]. Here, in presence of Lipid II, the activity of nisin in model
94 membrane systems is increased by three orders of magnitude compared to the activity
95 of nisin against susceptible bacteria. Therefore, nisin can permeabilize membranes by
96 two different mechanisms: I) through a low-affinity permeation mechanism that is only

observed in model systems; II) by a much higher nisin-Lipid II-dependent targeted pore-formation mechanism. In the first case, which requires micromolar concentrations of nisin and the presence of anionic lipids in the target membrane, nisin binds to the anionic lipids, and is subsequently inserted in the membrane at the position of the phospholipid head groups [24-27]. The accumulation of nisin in the outer lipid leaflet of the target membrane drives aggregation of nisin monomers, which is followed by the formation of short-lived pore-like structures.

The second mode of action of nisin, which is dependent on the presence of Lipid II in the membrane (as present in Gram-positive bacteria), can be described as follows: specific recognition and binding of Lipid II, which is followed by pore assembly and formation. Interestingly, the pores formed by nisin in the presence of Lipid II are much more stable than pores formed in the absence lacking this lipid [28,29]. Nisin has a specific transmembrane orientation in the presence of Lipid II, indicating that the formed pores are stable [17]. This also indicates that the role of Lipid II in the nisin-mediated pore-formation is not only binding. It has been shown that Lipid II is a constituent of the formed pore, which consists of four Lipid II molecules and eight nisin molecules [18].

In 2006 Hasper *et al.,* proposed an additional, third mode of action for nisin. Here, nisin binds to the pyrophosphate moiety of Lipid II, which is displaced afterwards from its location in Gram-positive bacteria. Since Lipid II is essential for the cell wall synthesis, and is therefore localized in the septum, this binding leads to growth inhibition. This sequestering effect is a distinct mode of bactericidal activity [30].

The nisin producer *L. lactis* strains are protecting themselfs from this high activity of nisin by expressing two protein systems; the lipoprotein NisI and the ABC transporter NisFEG. When both are expressed, a high level of immunity against nisin, up to ~ 750 nM nisin (1000 IU/ml), is provided [31]. Interestingly, both immunity proteins act

122 cooperatively and each of them displays only 10-30% of the full level of immunity when
123 expressed alone [32,33].
124 NisI is a 245 amino acids lipoprotein, with a N-terminal signal sequence, removed
125 during posttranslational modification resulting in the anchoring of NisI to the
126 extracellular side of the cytoplasmic membrane [34]. Koponen *et al.* showed that a
127 significant percentage of expressed NisI is secreted but not anchored in the membrane
128 thereby NisI is released into the extracellular media [35]. The presence of this "lipid-free"
129 NisI may have a biological function, complexing external nisin before it can interact with
130 the cell surface, thereby acting as an additional mechanism of self-protection [35].
131 The importance of NisI for the nisin immunity in *L. lactis* cells was observed via deletion
132 of the *nisI* gene. A *nisI* knockout resulted, which were more sensitive to nisin than the
133 corresponding *nisFEG* knockout [36]. This observation lead to the hypothesis that NisI
134 plays a more effective role in the immunity against nisin, although the differences are
135 small [37].
136 The exact molecular mechanism of NisI to provide immunity is still unknown. Takala
137 and his colleagues showed the functional importance of the C-terminus of NisI, likely
138 interacting with nisin [38]. Their study identified that a deletion of 21 amino acids at the
139 C-terminus of NisI, reduced the NisI mediated immunity compared to the level observed
140 with full-length NisI. Interestingly, this C-terminal region of NisI is not involved in the
141 co-operation with NisFEG, as the truncated NisI still showed a cooperative effect of nisin
142 resistance when co-expressed with NisFEG [38]. Moreover, the replacement of the 21 C-
143 terminal amino acids of the subtilin-specific immunity protein SpaI with the C-terminal
144 21 amino acids of NisI (SpaI´-NisI´) created a protein, which confers immunity against
145 nisin [38], while this has not been observed with the full length SpaI protein. Similar
146 function of the C-terminus was observed for the PepI, an immunity protein against the

147 lantibiotic Pep5. Here, it was shown that the C-terminal part mainly provides immunity,
148 while the N-terminal part of PepI is more important for its membrane localization [39].
149 Although those different lipoproteins NisI, SpaI and PepI share the fact that they confer
150 immunity, which is specific against their cognate lantibiotic, no significant homology in
151 their primary sequence was observed. These results highlighted that the C-terminus of
152 the NisI protein contains an important function for the immunity although the
153 underlying mechanism is not known.
154
155 In this paper, we focus on the contribution of the lipoprotein NisI to the immunity of *L.*
156 *lactis* against nisin. We revealed by using a fluorescence-based method that NisI inhibits
157 pore formation even at concentrations up to 1 µM nisin. Furthermore, the simultaneous
158 presence of nisin and NisI induced a reversible long chain formation of the *L. lactis* cells.
159 Both mechanisms allow the survival of the L. lactis cells at high nisin concentration
160 albeit only for a certain period of time.
161
162 **Material and Methods**
163
164 ***Cloning of the shuttle vector pNZ-SV:***
165 To allow more efficient DNA-manipulation and cloning, the *L. lactis/E. coli* shuttle vector
166 pNZ-SV was created in the first step by standard genetic manipulations as described by
167 Sambrook *et al.* [40]. The *L. lactis* plasmid pNZnisA-E3 [41] was linearized by PCR using
168 the primer pair pNZE3-BglIIfor (GATGCATCGATAGATCTAGTCTTATAAC) and pNZ-
169 BamHIrev (CTAGATCTATCGATGGATCCCTTAACTTAC). With the primers pET24aBglIIfor
170 (CTTGCGGTATTCGAGATCTTGCACG) and pET24BamHIrev
171 (CTAAATACATTCAAATATGGATCCGCTC) applying pET24a as template the coding

region of Kan, which confers resistance to Kanamycin in *E. coli*, and the pBR322 origin were amplified. The PCR-products were hydrolysed with BamHI and BglII and ligated. In a second step, the *nisA*-gene was replaced by the multiple cloning site (MCS) of pET24a using the In-Fusion HD-Cloning Kit according to manufactures protocol (Clontech). The vector pNZ-SV-nisA was linearized by PCR with the primer pair pNZ-SV-for (GCTTTCTTTGAACCAAAATTAG) and pNZ-SV-rev (GGTGAGTGCCTCCTTATAAT). The MCS of pET24a was amplified by PCR applying the primers MCS-pET24-Inf-for (AAGGAGGCACTCACCGAATTCGAGCTCCGTCGACAAG) and MCS-pET24-Inf-rev (TGGTTCAAAGAAAGCTGTTAGCAGCCGGATCTCAGTG), both primers with a 15 bp homology to the vector for the in-Fusion reaction. Restriction analyses and sequencing verified the correct sequence in the resulting plasmid pNZ-SV.

Cloning of pNZ-SV-nisI and pNZ-SV-nisIΔ22:

The *nisI* gene was amplified from the genome of *L. lactis* NZ9700 by PCR and inserted into the pNZ-SV by In-Fusion® HD Cloning. The used primers were pNZ-nisI-for (AAGGAGGCACTCACCATGAGAAGATATTTAATACTTATTGTGGCTTAATAG) and pNZ-nisI-rev (TGGTTCAAAGAAAGCCTAGTTTCCTACCTTCGTTGCAAGCTTAAAAT). The ends of the *nisI*-PCR product contained a 15 bp homology overhang to the pnZ-SV vector. After linearization of the vector pNZ-SV by PCR (primers: pNZ-for (GCTTTCTTTGAACCAAAATTAGAAAAC) and pNZ-rev (GGTGAGTGCCTCCTTATAATTTATTT)) the In-Fusion reaction was carried out according to the manufacturers' recommended conditions. Site-Directed mutagenesis was used to delete the last 22 amino acids of the C-terminal NisI protein by using two primers: the pNZ*nisIΔ22aa*–for (CCATTCTATTAGAGGAAAATAGCTTACTGAAGCATTTG) and the complement primer as a pNZ *nisIΔ22aa*–rev. This *nisI* variant is called *nisIΔ22* and

was verified by sequencing. After the successful cloning of pNZ-SV-*nisI* and pNZ-SV-*nisIΔ22*, the plasmids were transformed into *L. lactis* NZ9000 by electroporation at 1 kV, 25 µF, 5.0 ms, and the corresponding strain were termed NZ9000NisI and NZ9000NisIΔ22. An empty vector pNZ-SV was also transformed into the NZ9000 strain and was used as a control (that exclude any possible effect of the plasmid), and this strain is called NZ9000Erm. Transformation was performed as previously described [42].

Expression of NisI and NisIΔ22 in L. lactis NZ9000:

The NZ9000NisI or NZ9000NisIΔ22 strain was grown in GM17 media supplemented with 5µg/ml erythromycin to an OD_{600} of 0.8. By the addition of nisin (at a final concentration of 1 ng/ml), the expression was induced and the culture was further grown overnight. These cells were used for the assays described below.

To analyse the expression, the cells were harvested at OD_{600} of 2.0 by 5000 ×g for 30 min. The pellet was suspended in 1 ml of a 50 mM HEPES pH8.0, 150 mM NaCl, 10% (w/v) glycerol, and 700 kU/ ml lysozyme and was incubated 30 min at 37°C followed by 5 minutes at 50°C allowing lysozyme to lyse the cell wall. Afterwards the buffer with lysozyme was removed by another centrifugation step and the pellet was resuspended in SDS-loading dye ((0.2M Tris-HCl, pH 6.8, 10% (w/v) SDS, 40% (v/v) glycerol, 0.02% (w/v) bromophenol) and β-mercaptoethanol) and analysed via SDS-PAGE analysis. Western blot analysis was carried out using a polyclonal antibody against NisI (Eurogentec).

Purification of nisin:

Nisin was purified as described in [43].

222 Briefly, commercial available nisin powder (sigma) was dissolved in 50 mM lactic acid
223 pH 3. The nisin solution was purified by using 5ml HiTrap SP HP cation exchange
224 column (GE Healthcare) pre-equilibrated with the same buffer. Nisin was eluted with
225 400 mM NaCl and monitored online at a wavelength of 215 nm, since nisin lacks
226 aromatic amino acids in its sequence. In the last step, nisin was precipitated by TCA and
227 dried out after washing it with cold acetone [43]. The concentration of nisin was
228 measured by using RP-HPLC [44].
229
230 ***Determination the activity of nisin by IC_{50}:***
231 Cells from the different expressing strains were grown overnight in GM17
232 supplemented with with 5 µg/ml erythromycin in presence of 1 ng/ml nisin. The diluted
233 cells (final OD_{600} was 0.1) were incubated with a serial dilution of nisin in a 96 well plate.
234 The total volume in each well was 200 µl, consisting of 50 µl nisin and 150 µl GM17
235 containing the corresponding *L. lactis* strain. The highest concentration of nisin used
236 was adapted to the corresponding maximum immunity displayed by each strain.
237 The plate was incubated at 30°C. After 5 hours, the optical density was measured at 620
238 nm via 96 plate reader BMG. The normalized optical density was plotted against the
239 logarithm of the nisin concentration in order to calculate the IC_{50} of nisin and the data
240 was evaluated using the following equation (1):

241 $$y = OD_{min} + \frac{OD_{max} - OD_{min}}{1 + 10^{(\log(IC50) - x) * p}} \quad (1)$$

242 The OD_{max} value describes the normalized optical density value where no nisin was
243 added, while the OD_{min} value corresponds to the normalized optical density of the cells
244 grown in the highest nisin concentrations. Y presents the resulted normalized optical
245 density value and X represents the logarithmic of the nisin concentration added. The IC_{50}

value is the concentration of nisin where the growth of the *L. lactis* strain is inhibited by 50% as described in more detail earlier [43].

SYTOx green nucleic acids binding assay:

SYTOx green nucleic acids binding dye possesses a high binding affinity towards nucleic acids. It enters cells only when they contain a pore in the plasma membrane and never crosses the intact membranes of living cells [45].

The cells of NZ9000Emr, NZ9000NisI, NZ9000NisIΔ22 were grown overnight in GM17 supplemented with 5 μg/ml erythromycin in presence of 1 ng/ml nisin. The next day, the overnight culture was diluted to an OD_{600} of 0.1 in fresh media supplemented with 5 μg/ml erythromycin. The cultures were grown until the OD_{600} reaches 0.5, the SYTOx green dye was added at a final concentration of 5 μM and incubated for 5 minutes according to the manual of the manufacturer (Invitrogen). The fluorescence signal, which was measured at an excitation and emission wavelength of 504nm and 523nm, respectively, was monitored for 400 seconds to obtain a stable baseline. At 400 seconds nisin was added and the fluorescence was monitored for 15 minutes.

Regrowth experiment of the different* L. lactis *strains:

Cells of NZ9000Erm, NZ9000NisI and NZ9000NisIΔ22aa were grown overnight in GM17 media. A main culture was inoculated with overnight culture to an OD_{600} of 0.1. After a 30 min preincubation at 30 °C the GM17 broth was supplemented with 0 (control) or 10-fold IC_{50} concentration (nM) of nisin and cells were incubated for 1, 2, 3, 4 and 5 h. Cells (out of 3 ml GM17 medium) were harvested and the cell pellet was washed three times with fresh GM17 medium. The recovered cells were used to inoculate fresh GM17 medium to OD_{600} of 0.1 and incubated at 30 °C in 96-well plate (vol. 200 μl) for a

maximum of 15 hours. To count the number of living cells 100 µl (after a 1:100 or 1:10.000 dilution) of every sample was plated on GM17 agar plates supplemented with 5 µg/ml erythromycin and incubated at 30 °C for two days and the grown colonies were counted.

Morphology study

To study the effect of nisin on cell morphology, the overnight culture was diluted to an OD_{600} of 0.1. The cells were incubated with 0, 1, 10, 30 nM of nisin for 3 hours and then they were harvested at 13.000 rpm for 15 min. Harvested cells were washed with PBS buffer (50 mM phosphate buffer pH 7.2, 150 mM NaCl) and fixed with a 1:1 mixture of absolute ethanol and PBS buffer. Afterwards 10 µl of the fixed cells were applied to poly-L-lysine cover slides. Followed by the addition of 5 µl of mounting medium, the sample was dried before use. For long-term storage, nail polish was used to seal the cover slips. Those samples were monitored using a Nikon Eclipse Ti inverted microscope with a CFI60 100x/1.35 oil objective. The phase contrast pictures were obtained after a raster scan of 8x8 pictures with 5 areas per sample. The Nikon Nis-Elements imaging software was used to control the microscope and the imaging software ImageJ Version 1.47 was used for analysis.

Results

Activity of NisI and NisIΔ22 in *L. lactis*

The *nisI* gene was cloned in a pNZ-SV vector, which was complemented with an origin of replication for *E. coli* allowing rapid cloning and mutagenesis in all standard *E. coli* laboratory strains. After successful cloning the plasmids were transformed into *L. lactis*

296 for homologous expression of the NisI or NisIΔ22 protein. We used *L. lactis* NZ9000,
297 which is a derivative of the plasmid-cured *L. lactis* MG1363 and contains the *nisRK* genes
298 inserted in the chromosomal pepN locus [46]. This strain is commonly used as the host
299 for the nisin-controlled gene expression system (NICE) [47]. However, since this
300 NZ9000 strain lacks the nisin immunity genes, *nisI* and *nisFEG*, it is highly sensitive to
301 nisin [48]. The transformation of an empty plasmid pNZ, a plasmid harbouring wild type
302 NisI and a C-terminal truncation of NisI into *L. lactis* NZ9000 resulted in strains termed;
303 NZ9000Erm, NZ9000NisI and NZ9000NisIΔ22, accordingly. The expression of NisI and
304 NisIΔ22 was monitored by Western blot analysis using a polyclonal NisI antibody (see
305 Figure 1A). Here, a slight double band is visible for full-length NisI. The upper band
306 resembles NisI, which is not processed and still contained the secretion signal. It
307 remained inside the cell and therefore did not contribute to the nisin immunity activity
308 of NisI observed below. From this Western blot it can be judged that both NisI (lower
309 band) and NisIΔ22 were expressed in similar quantities.
310 To quantitatively assess the growth inhibitory activity of nisin, a liquid culture assay
311 was performed using the NZ9000Erm, NZ9000NisI and NZ9000NisIΔ22 strains. The
312 optical density of the corresponding *L. lactis* strain cultures after 5 hours of growth was
313 plotted against the logarithm of the different added nisin concentrations. Thus, the
314 activity of nisin can be measured and quantified by calculating the amount of nisin
315 required to inhibit cell growth by 50% (IC_{50}) using equation 1. In case of the control
316 strain, NZ9000Erm, nisin exhibits a high activity (IC_{50}= 9.1 $\pm$ 0.7 nM (Figure 1B black
317 curve)). The expression of NisI as observed for the NZ9000NisI strain reduced the effect
318 of the nisin activity almost 8-10 fold as reflected by the IC_{50} value of 73.0 $\pm$ 10.2 nM
319 (Figure 1B blue curve). The strain expressing NZ9000NisIΔ22 displayed an intermediate

320 IC_{50} value of 25.3 ± 1.7 nM (Figure 1B red curve). This highlights that NisI is capable of
321 conferring immunity and that the C-terminus of NisI adopts an important role in this.
322
323 **Pore formation by nisin**
324 As it is mentioned before, nisin conducts several modes of action. The binding of nisin to
325 Lipid II, a cell wall precursor, which leads to the inhibition of cell growth, is the
326 predominant one. Upon binding, nisin is also able to form pores in the membrane, which
327 leads to membrane disruption and subsequently rapid cell death. The latter one can be
328 visualized by a SYTOx green nucleic acid dye. In this assay when pores are formed, the
329 dye enters the cells of *L. lactis* and binds to the DNA resulting in a rapid increase in the
330 fluorescence signal, which can be monitored in real time [45,49]. The chosen conditions
331 guarantee that the *L. lactis* cells were in their exponential growth phase (OD_{600}= 0.5) to
332 ensure that the cells were in a good shape. Different concentrations of nisin were used
333 reflecting concentrations slightly below or above the IC_{50} values of each strain
334 determined above (10, 30 and 1000 nM nisin, Figure 2). The measurement in which no
335 nisin was added (buffer control) was used as a control, where no effect on the
336 fluorescence signal was observed.
337 When incubating the NZ9000Erm, NZ9000NisI and NZ9000NisIΔ22 strains with the
338 SYTOx green dye, a stable baseline is reached. After 400 seconds nisin was added
339 (indicated with an arrow in Figure 2) and the fluorescence signal was monitored
340 continuously.
341 By the addition of 30 nM nisin to the NZ9000Erm strain an increase of the fluorescence
342 could be measured starting at 800 seconds reaching its maximum after 2000 seconds,
343 which indicates pore formation in the cytoplasmic membrane (Figure 2A). In contrast,
344 no increase of the fluorescence signal was observed for the NZ9000NisI and the

NZ9000NisIΔ22 strains at the same nisin concentration indicating that the SYTOx dye did not enter the cells and thus no pore formation occurred (Figure 2A). This is in line with the IC_{50} measurement data since a nisin concentration of 10 nM is slightly above the IC_{50} of the NZ9000Erm strain, but below the IC_{50} of the NZ9000NisI and NZ9000NisIΔ22 strains (Table 1).

When adding 30 nM of nisin the curve of the NZ9000Erm strain increases more rapidly (already shortly after addition) and also the slope was steeper (Figure 2B). The maximum of the fluorescence signal was also higher when compared to the curve obtained upon addition of 10 nM nisin indicating that more cells were lysed. For the NZ9000NisIΔ22 strain an increased fluorescence signal was also observed. Here, the signal increases rapidly at roughly 700 seconds. At this nisin concentration, the NZ9000NisI strain however, shows only a minimal increase of only 10% of the value observed for the NZ9000Erm strain (Figure 2B). When the nisin concentration was further increased to 1000 nM, which is 100-fold above the IC_{50} for the NZ9000Erm strain, which was 50-fold above the IC_{50} for NZ9000NisIΔ22 strain and even 15-fold above IC_{50} for the NZ9000NisI strain, the curves became even more pronounced. The fluorescence signal observed for the NZ9000Erm strain increased immediately after the addition of nisin (Figure 2C). Similarly, the NZ9000NisIΔ22 strain displayed an increase of the fluorescence signal directly after nisin treatment, with a similar slope. However, a different maximum of the fluorescence signal was reached. Interestingly, only a small increase of the fluorescence signal was observed for the NZ9000NisI strain (Figure 2C). Also the curve shape demonstrated that this is a gradual effect rather than a sharp and sudden effect suggesting that this increase cannot be assigned to rapid cell lysis due to a nisin induced pore formation.

From these results, we can conclude that the presence of NisI inhibits the pore formation activity of nisin. This inhibition seems to be a stable effect since even at concentrations 15-fold above the determined IC_{50} values no pore formations could be observed. Furthermore, this inhibition is mediated by the C-terminus since its deletion displayed pore formation at concentration higher than the IC_{50} value determined for the NZ9000NisIΔ22 strain.

These results prompted us to re-evaluate our IC_{50} values in more detail. For the NZ9000NisI strain, cell growth was inhibited by 50% when a nisin concentration of 70-80 nM was added. When adding higher concentration of nisin; no growth was observed by measuring the optical density (Figure 1B). Here, it is important to mention that the IC_{50} is measured after 5 hours of growth. As visualized by the SYTOx green assay no pore formation was observed although a slight increase of the fluorescence was observed, suggesting that the NZ9000NisI strain was not suffering from nisin induced pore formation, but rather that the strain stopped growing.

Recovery experiment: Regrowth of NZ9000NisI

The recovery experiment was performed to determine the ability of *L. lactis* NZ9000NisI cells to re-grow after being exposed to a high concentration of nisin.

The NZ9000Erm, NZ9000NisI and NZ9000NisIΔ22 strains were incubated with a nisin concentration 10-fold higher than their corresponding IC_{50} values, being 100 nM, 600 nM and 300 nM for the NZ9000Erm, NZ9000NisI and NZ9000NisIΔ22, respectively. As a control the same strains were used without adding nisin. After incubation of 1, 2, 3, 4 and 5 hours, the cells were harvested by a centrifugation step, extensively washed and re-suspended in fresh media lacking nisin to an final OD_{600} of 0.1. The growth was monitored for 15 hours by measuring the OD_{600} online (Figure 3).

394 The number of cells re-growing after reducing the nisin concentration was used as a
395 parameter to determine whether the strains were protected against nisin.
396 For both NZ9000Erm and NZ9000NisIΔ22 strains, no growth was observed indicating
397 that they were killed by nisin (Figure 3A and 3C), while the control of these strains
398 displayed an exponential growth. In contrast, the NZ9000NisI strain started exponential
399 growth, although after a delay time of 5-8 hours (Figure 3B).
400 This shows that some NZ9000NisI cells could survive in an environment containing a
401 high nisin concentration and can start growing again when transferred into fresh GM-17
402 medium. Furthermore, there was a considerable effect of the incubation time with high
403 nisin concentrations. When NZ9000NisI cells were incubated for 1-3 hours they started
404 growing after 3 hours whereas the potential of the cells to regrow was reduced when
405 the cells were incubated for longer times (4 and 5 hours). This was also visible by the
406 final OD_{600} of the cells incubated with high nisin concentration, being 0.7, 0.64 and 0.6
407 for cells incubated for 1, 2 and 3 hours, respectively. The cells incubated for longer time
408 did not grow and showed a final OD_{600} of 0.4 and 0.3 for 4 and 5 hours of incubation
409 (Figure 3B). The cells incubated only for a shorter time period, reached a similar OD_{600}
410 as the cells that were not incubated with nisin, indicating that they were fully recovered.
411 The long lag-phase in the growth curve indicated that not all NZ9000NisI cells survived
412 the treatment with a nisin concentration corresponding to 10x the IC_{50}, value,
413 suggesting that the OD_{600} 0.1 included besides living cells also cells, which did not
414 survive. Therefore, we plated a fraction of the cells onto agar plates and counted the
415 appeared colonies. For the NZ9000Erm strain were no nisin was added prior $4x10^{07}$
416 (100%) cells were growing, which decreased to 0.1% after treatment with 10-fold the
417 IC_{50} concentration of nisin for only 1 hour (Figure 3D). The NZ9000NisI strain showed
418 >12-14 times more cells surviving the nisin treatment. Here, after 1 hour 1.4% of the

cells when compared to the untreated cells where growing. Interestingly, the number of cells surviving correlates with the incubation time with nisin (Figure 3D). The longer nisin was present the fewer colonies appeared on the agar plate. One has to take into account that the nisin concentration used in this assay varies, since it was adjusted to 10-fold the IC_{50} value. Although the NZ9000NisI strain was treated with 600 nM nisin and the NZ9000Erm strain with 100 nM, much more cells of the NZ9000NisI strain survived.

The number of colonies that appeared when using the NZ9000NisIΔ22 strain were comparable to the number observed with the NZ9000Erm strain again highlighting the importance of the C-terminus in the immunity mediated by NisI.

This assay showed that a significant number of NZ9000NisI cells were capable to survive nisin concentrations 10-fold above the IC_{50} value for a certain time period. In clear contrast, with the NZ9000Erm and NZ9000NisIΔ22 strain which were not able to survive such a high nisin concentration even when incubated for only 1 hour.

Phenotype of NisI and NisIΔ22 expressing strains

Different expressing *L. lactis* cells were monitored using increasing amounts of concentrations of nisin, e.g. 0, 1, 10 and 30 nM. Here, the growth was measured after 3 hours and the cells were transferred onto a cover slide and monitored using a Nikon Eclipse Ti microscope. The growth phase of each strain was adjusted to the exponential deviation phase. The chain length was observed and the number of cells forming one chain were counted and grouped according to the number of present cells (Figure 4). In the control experiments (no nisin added), the typical double cocci morphology of *L. lactis* was observed for all the strains, NZ9000Erm, NZ9000NisI and NZ9000NisIΔ22, respectively (Figure 4).

For the NZ9000Erm strain, which is highly susceptible to nisin, the majority of chains consisted of double cocci (Figure 4). Even at a nisin concentration of 30 nM , this strain did not change its phenotype.

A similar observation was found for the NZ9000NisI strain, as it displayed almost exclusively chains consisting of double cocci (>90% of the population) when no nisin was added. The phenotype changed drastically upon the addition of 1 nM nisin. Longer chains were formed in almost 50% observed cells in this population. When the nisin concentration was set to 30 nM, a further increase was observed and almost 80% of the cells were localized in long chains. Here, the chain length varies between 3-5 cells (50%) and more than six cells (30%) (Figure 4B). It is worth mentioning that even longer chains were visible (up to 30 cells in one chain), but a quantitative analysis was not possible since they were in almost all cases lying on top of each other.

For the NZ9000NisIΔ22 strain, more than 90% of the cells were double cocci in the absence of nisin. The addition of nisin (below the IC_{50}) resulted in an increase of the chain length. At a nisin concentration of 10 nM, more than 70% of the cells were localized in chains consisting of 3-10 cells (Figure 4B).

Interestingly, at 30 nM nisin, these longer chains were not observed as frequently. Hence, the majority (>70%) of the cells formed double cocci (Figure 4B). This can be explained by the fact that the addition of nisin at a concentration of 30 nM, is above the IC_{50} value. At this concentration, the NZ9000NisIΔ22 strain is suffering from nisin mediated pore formation as observed by the SYTOx green assay (see above).

This data suggests that the presence of low nisin concentrations, in combination with the expression of NisI resulted in long chain formation of *L. lactis* cells. A phenotype, which was observed with the NZ9000NisI as well as the NZ9000NisIΔ22 strain, suggesting that this phenotype is mediated NisI but not via its C-terminus.

Discussion

Several *L. lactis* strains produce the lantibiotic nisin, a 3.4 kDa antimicrobial peptide harbouring five lanthionine rings in its fully active conformation, which are installed by posttranslational modifications [50]. These rings are crucial for the high level of antimicrobial activity as well as its stability [51]. To confer immunity, the nisin producer *L. lactis* strain co-expresses the membrane associated protein systems NisI and NisFEG, which are not localized next to each other on the encoding gene cluster [52] but both seems to have distinct promoter sites for regulation albeit by the same regulator NisR. Since this regulation is induced by the external addition of nisin, the amount of the immunity proteins in the membrane correlates with the external nisin concentration present in the habitat [31]. The specific NisI promoter is however leaky, which thereby ensures a low but always present immunity [53], which can be up-regulated.

We characterized the function of the homologously expressed nisin specific immunity protein NisI and showed that it can act independently. When over-expressed in *L. lactis,* NisI confers immunity with an IC_{50} value for nisin of around 73 nM, which is almost 8-10% of the IC_{50} value observed for the producer strain where both NisI and NisFEG are expressed in our laboratory (data not shown). The last 22 amino acids are important for the function of NisI, since a deletion of these residues decreased the IC_{50} value to almost one third (22 nM). These results are in-line with the results of Takala *et al.*, which reported a decrease to 22% for the same truncated version [38]. The slight variation could be due to the different experimental setup since in comparison to our study they determined MIC values. Furthermore, in our study the purification protocol for nisin was performed differently [43].

The ability of nisin to form pores in the cytoplasmic membrane of Gram-positive bacteria is believed to represent the mode of action guaranteeing the high activity of

494 nisin upon addition of nM amounts [9]. Intriguing is the fact that NisI seems to inhibit
495 exactly this nisin mediated pore formation as shown with the SYTOx green assay. This
496 assay has been used in the past to visualize pore formation, for example for salivaricin 9
497 from *Streptococcus salivarius,* which is also a pore forming lantibiotic [54]. In principle
498 the capturing of nisin by NisI before it reaches the membrane would be sufficient to
499 prevent this pore formation. However, when the nisin concentration rises at a certain
500 point all NisI proteins in the membrane would be occupied. By further increase of the
501 nisin concentration above this threshold, some nisin molecules would be able to reach
502 the membrane and induce pore formation. This is not observed in our assay, rather we
503 showed that NisI can inhibit pore formation even up to 1000 nM nisin (15-fold of the
504 IC_{50}) and this implies that the immunity mediated by NisI is not solely due the formation
505 of a NisI-nisin complex at the membrane. This consequently raises the question, whether
506 there is an additional function of NisI ensuring the survival of the host cells at high
507 concentration of nisin ?
508
509 Depletion of the last 22 amino acids of NisI (NZ9000NisIΔ22), leads to pore formation
510 already at lower concentration of nisin indicating that the C-terminus is responsible for
511 the inhibition of the pore formation. The last 22 amino acid has been shown to be
512 sufficient to confer some resistance to nisin when fused to another protein. Here, SpaI-
513 NisI hybrids were created, where the last 22 amino acids of NisI are attached to the SpaI
514 protein normally conferring immunity against subtilin in *B. subtilis*. These hybrids were
515 able to confer resistance to nisin whereas the full-length SpaI protein is not. This
516 elegantly showed that the C-terminus of NisI is responsible for as well as enough to
517 confer resistance against nisin [38]. The NZ9000NisIΔ22 strain however, is still showing
518 some resistance although substantially lower. This was observed in the here presented

519 IC_{50} values, as well as the SYTOx green assays. This is likely arising from a nisin binding
520 event to the rest of the NisI protein. Due to this interaction event, a higher amount of
521 nisin is needed to fulfil the activity of nisin e.g. pore formation as reflected by the higher
522 IC_{50} value of the NZ9000NisIΔ22 strain compared to the NZ9000Erm strain.
523
524 Interestingly, in our IC_{50} measurements no growth was observed at concentrations
525 above 70 nM nisin for the NZ9000NisI strain. Here, we observe the formation of chains
526 consisting of high numbers of *L. lactis* cells (Figure 4). Our data showed that this
527 clustering of cells, which can reach up to 10-20 cells, is directly correlating with the
528 concentration of externally added nisin. Increasing the nisin concentration, leads to
529 longer chains. Only the normal double cocci chains were observed, when no nisin is
530 added although NisI is present. This shows that when NisI is expressed upon the
531 addition of nisin induces a morphology change of the *L. lactis* cells, which is more
532 pronounced at higher nisin concentrations. Since this is also observed in the
533 NZ9000NisIΔ22 strain, this activity lies, in contrast to the pore formation inhibition,
534 elsewhere in the NisI protein and is not mediated by the C-terminus.
535 This chaining event could be the first step towards biofilm formation, especially when
536 considering the potential of the NZ9000NisI strain to survive at high nisin concentration.
537 It is worth to mention that cells with a long chain morphology sediment faster than cells
538 with short chains [55]. This sedimentation can be observed when the NZ9000NisI strain
539 was incubated with a high nisin concentration (data not shown). This chaining event
540 wass reversible. When the NZ9000NisI strain is incubated with high amounts of nisin a
541 significant percentage of the cells survive and started growing again when nisin is
542 removed. This suggests this phenotype depends on the external nisin concentration.
543 Whether this chaining phenotype is an effect of the NisI overexpression or really

544 displays an immunity mechanism can not be distinguished by our experiments at the
545 moment and is currently under investigation.
546
547 One intriguing question still remains: how does the C-terminal part of NisI inhibit pore
548 formation?
549 In the recently reported SpaI structure, a rather flexible N-terminus has been found
550 which folds upon lipid binding [56]. It is tempting to speculate that such a flexible
551 termini is also present in NisI albeit here it is localized at the C-terminus. Maybe the C-
552 terminus is directly binding to Lipid II and thereby inhibiting the binding of nisin to its
553 docking partner, resulting in the inhibition of pore formation.
554 A similar inhibition of the nisin-Lipid II binding has been indirectly observed when
555 vancomycin was added prior to nisin. Here, vancomycin was provided first to nisin-
556 sensitive cells. Since Lipid II was occupied with vancomycin, which does not have any
557 pore formation activity, the nisin molecules, which have been added afterwards, were
558 not able to bind Lipid II anymore. Subsequently no nisin mediated pore formation was
559 observed [23]. This shows that when Lipid II is occupied by another compound, nisin
560 cannot form pores. So maybe the C-terminus of NisI is binding to lipid II, thereby
561 ensuring that no nisin-Lipid II complex can be formed. Thereby, *L. lactis* becomes
562 immune against nisin even at concentrations above the determined IC_{50} value. This
563 immunity mechanism of NisI is intriguing protects *L. lactis* itself from nisin without
564 degrading or damaging it. Not only the inhibition of pore formation is ensured, but also
565 the alternative mechanism of Lipid II displacement by nisin [30] would be circumvented.
566 Moreover, when the concentration of nisin decreases this NisI - Lipid II interaction
567 appears to be dissociating and at lowered nisin concentrations the *L. lactis* cells continue
568 to grow.

Conclusions

Bacterial strains that produce antimicrobial peptides like lantibiotics must protect their own membrane against the activity of their own peptides. *L. lactis* protects itself against nisin by the expression of two protein systems; NisI and NisFEG. The latter has been shown to expel nisin from the membrane into the extra cellular media [32].

Here, we show that NisI comprises a role in inhibiting nisin mediated pore formation via its C-terminus even at very high nisin concentrations. Additionally, the NisI expressing strains form long chain cluster of *L. lactis* cells, which are reversible and counteracts high concentrations of nisin. In the habitat of *L. lactis,* it is likely that such high concentrations of nisin are only reached for a short-period of time, since the nisin molecules will diffuse away into the media. During this short time, NisI is however able to confer immunity.

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758
759
760

761 **Table**

762

763 **Table 1: IC_{50} values determined for the different strains.**

764

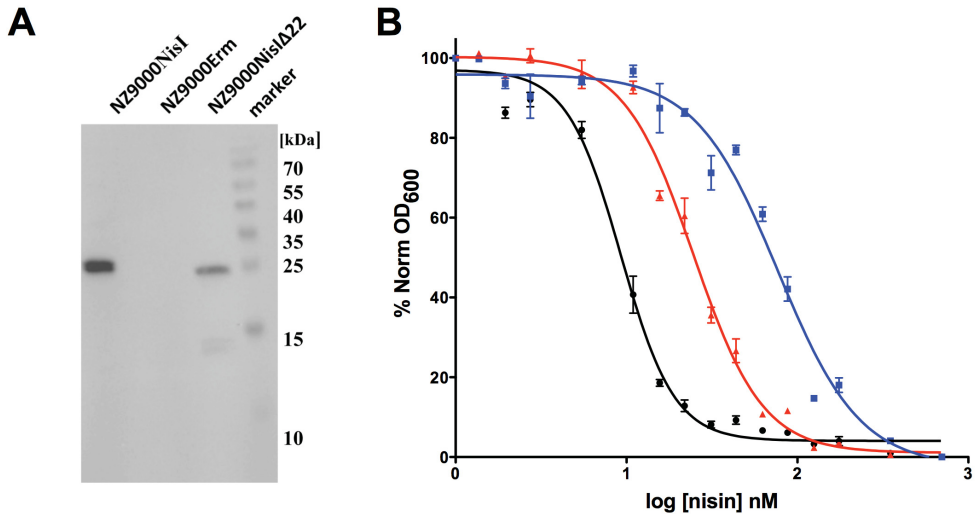
	NZ9000Erm	NZ9000NisI	NZ900NisIΔ22aa
Nisin	9.1 ± 0.7 nM	73.0± 10.2 nM	25.3 ± 1.7 nM

765

766

767 **Figure legends**

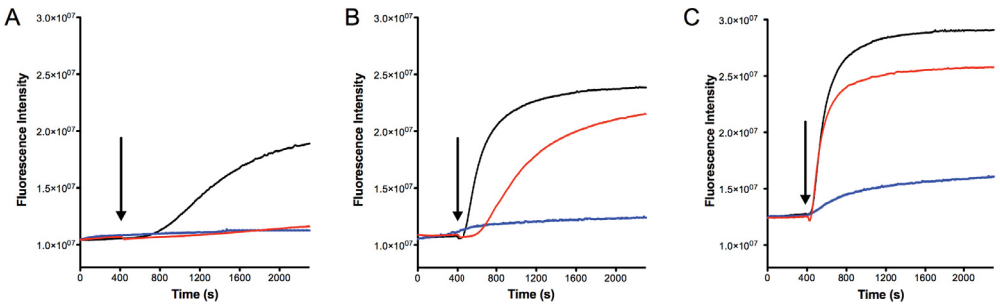
768



769

770 Figure 1: **(A) Western blot analysis using a polyclonal NisI antibody.** Shown are the
771 *L. lactis* strains: NZ9000Erm, NZ9000NisI and NZ9000NisIΔ22 strain. **(B) IC_{50}**
772 **determination of different strains**. Growth inhibition experiments were performed
773 with nisin using different strains. Black line: NZ9000Erm strain; blue line: NZ9000NisI
774 strain; red line: NZ9000NisIΔ22 strain. Data was fitted and evaluated according to
775 equation (1). Each experiment was performed at least in triplicates.

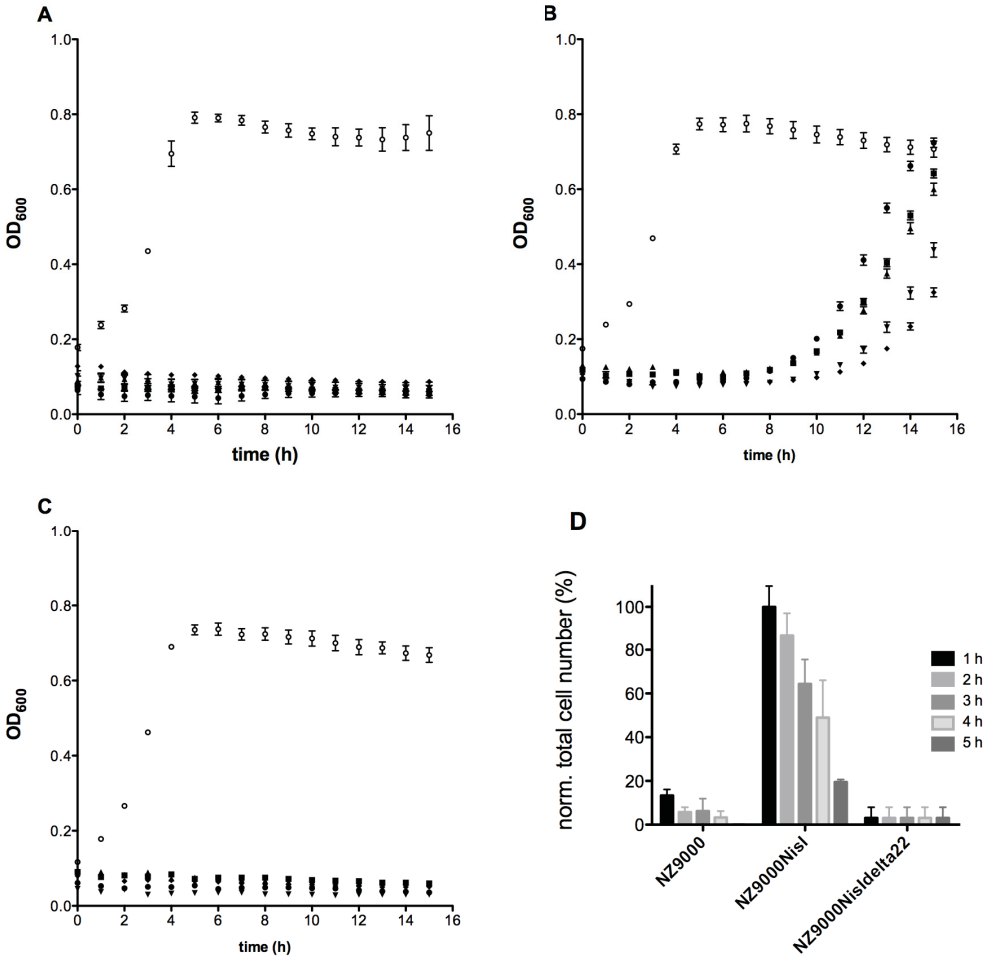
776



777

778 Figure 2: **SYTOx green assay to visualize pore formation mediated by nisin**. The
779 NZ9000Erm strain (black line), NZ9000NisI strain (blue line) and the NZ9000NisIΔ22
780 strain (red line) were grown and incubated with the SYTOX green dye. The fluorescence
781 signal was monitored online using a fluorolog (Horiba III). After 400 seconds a stable
782 baseline was reached and nisin was added (A) 10 nM (B) 30 nM and (C) 1000 nM. The
783 addition of nisin is indicated with an arrow. The rapid increase of the fluorescence signal
784 indicated pore formation.

785



786

787 Figure 3: **Growth recovery assay**. The different strains were incubated for 1 (●), 2 (■),
788 3(▲), 4(▼) and 5(◆) hours at an OD_{600} of 0.1 with nisin at a concentration which
789 represents 10-fold the IC_{50} determined, being 100 nM, 300 nM and 600 nM for the
790 NZ9000Erm (A), NZ9000NisIΔ22 (B) and the NZ9000NisI (C) strain respectively. The
791 cells were separated from the growth media by centrifugation and extensively washed
792 with media to remove the remaining nisin. Afterwards the cell were transferred into
793 fresh medium at a final OD_{600} of 0.1 and the growth was monitored by measuring the
794 optical density at 600 nm. As a control (❍) the corresponding strains without the
795 addition of nisin during pre-incubation were used. Each experiment was performed 4

796 times. Within the different experiments the interval of recovery comprised between 5
797 and 8 hours. Furthermore the end point OD_{600} (after 15 hours growth) was in a range of
798 65-100 % recovery ability (compared with the end point OD_{600} of the control). To
799 control the number of cells survived the incubation with a high nisin concentration, the
800 resuspended cells were striked on GM17 agar plates.The number of colonies on these
801 plates resemble the total number of living cell in the cell suspension with on OD_{600} of 0.1.
802 A normalisation of the total cell number between the strains NZ9000Erm, NZ9000NisI
803 and NZ9000NisIΔ22 shows the relative distribution depending on the living cells (D).
804 The NZ9000 NisI after 1 h incubation time is set as 100 % (total cell number: 261.866
805 ± 32.809) and reflects 1.4 % of surviving cell compared to the control (total cell number:
806 24.800.000 ± 1.844.776). Longer incubation times leads to survibal rate of 20 % for
807 NZ9000NisI. For the NZ9000Erm and NZ9000NisIΔ22 strains even fewer cells survived,
808 only 0.1 % when compared to the control. The error bars indicating the standard
809 deviation of three independent experiments.
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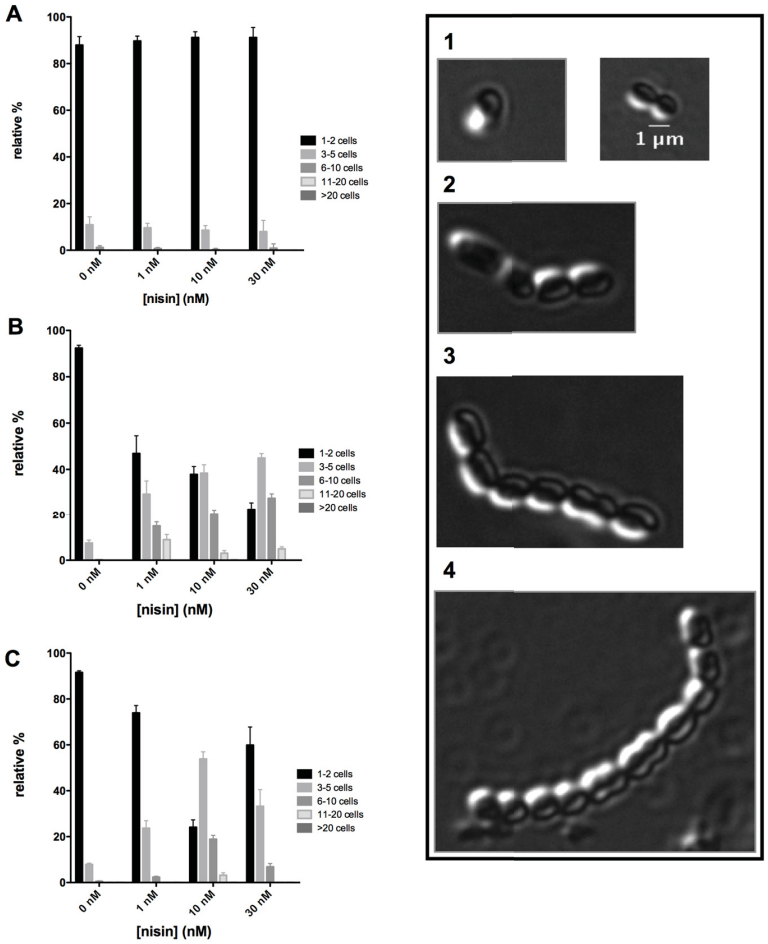

812

813 **Figure 4: Phenotype visualisation of *L. lactis* cells using the NZ9000Erm,**
814 **NZ9000NisI and NZ9000NisIΔ22 strain**. The different strains were grown until
815 exponential phase (OD_{600} = 0.5). During growth different concentration of nisin were
816 added (0, 1, 10 and 30 nM). The cells were transferred and fixed onto a coverslide and
817 the cells were visualised using a Nikon eclipse Ti microscope. The chains were counted
818 and categorized in different classes. Class 1 consists of 1-2 cocci (black bar), class 2
819 consists of 3-5 cells (grey bar), class 3 consists of 6-10 cells (dark grey bar), class 4
820 consists of 11-20 cells (light grey) and class 5 consisting of >20 cells. For each sample
821 the number of counted cells per area was > 50. In total after scanning five different areas
822 at least a quantity of >500 cell chains were observed.

CHAPTER IV

The C-terminus of nisin is important for the ABC transporter NisFEG to confer immunity in *Lactococcus lactis*

By

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Running head: NisFEG recognizes the C-terminal part of nisin

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Abstract

The lantibiotic nisin is a small 3.4 kDa antimicrobial peptide which acts against Gram-positive bacteria in the nM range. Nisin is produced and secreted by several *Lactococcus lactis* strains ensuring advantages against other bacteria in their habitat. Nisin contains five specific lanthionine rings of which the first two are important for Lipid II binding and the last two are crucial for the pore formation in the membrane. To ensure immunity against nisin, the producing strain is expressing an ABC transporter called NisFEG, which expels nisin from the membrane resulting in 6-8-fold more nisin that is needed to kill the cells. This immunity is mediated by the hydrolysis of ATP as shown by an ATP deficient mutant. The C-terminus of nisin is important for the interaction between nisin and NisFEG since deletion of the last six amino acids as well as of the last ring lowered the activity of NisFEG.

Introduction

Lantibiotics are small ribosomally synthesized peptides produced by numerous of Gram-positive bacteria. After posttranslational modifications, lantibiotics are activated, upon the cleavage of the specific secretion signal called leader peptide. These active lantibiotics are able to lyse mainly Gram-positive bacteria as well as a limited number of Gram-negative bacteria and act via different but distinct mechanisms (Wiedemann *et al.*, 2001, Chatterjee *et al.*, 2005b) where they are targeting the membrane of the bacteria. This means that the membranes of lantibiotic producer strains could be also affected. To circumvent this suicidal effect, lantibiotic producer strains express an immunity protein system (Alkhatib *et al.*, 2012). The genes encoding for the immunity system seem to be present in all found gene clusters which encode for lantibiotics (Willey & van der Donk,

2007) . They are called LanI, a membrane anchored lipoprotein, and LanFEG, which is an ATP binding cassette (ABC) transporter localized in the cellular membrane. In most operons both proteins are present although some exceptions are known (Alkhatib et al., 2012).

Nisin is the best-known and the most extensively studied lantibiotic which is produced by some strains of *Lactococcus lactis* (*L. lactis*). Nisin is produced as an inactive precursor containing an N-terminal leader peptide that is cleaved after secretion by the subtilisin-like serine protease NisP (van der Meer *et al.*, 1993). Due to the high bactericidal activity in combination with the low toxicity in humans, nisin is used since decades as a natural preservative in the food industry (Delves-Broughton *et al.*, 1996). Active nisin is a 3.4 kDa peptide, consisting of 34 amino acids and contains five lanthionine-based rings (ring A-E)(Figure 1). Here, the first three rings (A-C) are N-terminally located and are separated from the intertwined rings D and E (located at the C-terminus) by a flexible hinge region. The mode of action of nisin has been thoroughly studied, and it was suggested that nisin attacks bacteria by inhibiting their cell-wall synthesis via binding to Lipid II.

Here, it has been shown that ring A and B of nisin are responsible for binding to Lipid II (Hsu *et al.*, 2004), which is used as a 'docking molecule' for nisin to protrude into the membrane by the hinge region as well as ring D and E and subsequently form a pore. (Wiedemann et al., 2001, van Heusden *et al.*, 2002, Hasper *et al.*, 2004, Brotz *et al.*, 1998, Breukink *et al.*, 1999). The presence of Lipid II is needed for the high activity of nisin, since in model membrane systems an increase of the pore formation activity by three orders of magnitude was observed when Lipid II was present. Furthermore, it has been shown that Lipid II is a constituent of the formed pore, which, when fully assembled, consists of four Lipid II and eight nisin molecules (Hasper et al., 2004).

The nisin producing *L. lactis* strain expresses two protein systems. The lipoprotein NisI and the ATP binding cassette (ABC) transporter NisFEG, which when both are expressed result in immunity against a high level of nisin (Ra *et al.*, 1996). Both immunity systems seem to act cooperatively, since each of them displays only 20-30% of the full immunity when expressed alone (Stein *et al.*, 2003, Ra *et al.*, 1999). Recently, the function of NisI has been described. Here, NisI has been shown to inhibit nisin mediated pore formation at concentration up to 1 µM nisin (AlKhatib *et al.*, 2014). This activity is mediated by the C-terminus of NisI, which is likely involved in the binding or shielding of Lipid II. Takala and co workers also described the importance of the C-terminus and showed that a 21aa deletion at the C-terminus of NisI, reduced the NisI mediated immunity 6-fold compared to the level observed with full-length NisI (Takala & Saris, 2006).

ABC transporters, like NisFEG, comprise one of the largest families of membrane proteins, found in all kingdoms of life. They transport a large variety of substrates ranging from small ions to large proteins of up to 800 kDa of size (Hinsa *et al.*, 2003) and can be subdivided in two major classes, export and import ABC transporters (Davidson *et al.*, 2008). Generally, ABC transporters consist of four domains, two hydrophobic transmembrane domains (TMD) and two hydrophilic cytosolic nucleotide-binding domains (NBD). The NBDs show high sequence similarity and contain characteristic sequence motifs; the Walker A, the Walker B, the H-loop, the two hallmarks of ABC transporters, the C-loop or ABC signature motif (LSGGQ) (Schmitt & Tampe, 2002), and the equally distinctive feature, the D-loop (Zaitseva *et al.*, 2006, Higgins & Linton, 2004, van der Does & Tampe, 2004). The NBDs are involved in the binding and hydrolysis of ATP that, provide energy for the transport of the substrate through the transmembrane pore, which is formed by the TMDs. In NisFEG, NisF represents the NBD, and the membrane component consists of the proteins NisE and NisG, forming a hetero-dimeric

membrane complex, as observed for other ABC transporters (Fetsch & Davidson, 2002, Zaitseva *et al.*, 2005b, Zaitseva *et al.*, 2005a). NisG is a 24 kDa integral membrane protein consisting of 214 amino acids with six predicted transmembrane spanning helices and NisE is a 28-kDa integral membrane protein containing a number of six predicted transmembrane helices as well (Alkhatib et al., 2012). Assembled as a complex, these three proteins form the functional immunity ABC transporter with a proposed stoichiometry of $NisF_2EG$. Deletion of either NisE or NisG abolished the immunity against the lantibiotic nisin completely, highlighting their equal importance for function (Siegers & Entian, 1995). NisFEG has been shown to remove nisin from the membrane prior to pore formation (Stein et al., 2003). Here, the authors showed that nisin can solely be found in the supernatant of the growth media, when NisFEG is expressed, whereas some of the nisin molecules are membrane bound in cells lacking NisFEG. This leads to the conclusion that NisFEG might exhibit a nisin expelling function (Stein et al., 2003).

Other lantibiotic operons also contain genes expressing for an ABC transporter conferring immunity against the produced peptide. For example *smbFT* from *Streptococcus mutans* displays immunity against the two component antimicrobial peptide Smb. Both of the protein-encoding genes are located within the *smb* locus. Also the expressed ABC transporter seems to be rather specific since other tested peptides were not recognized by this transporter (Biswas & Biswas, 2013). Another example is the ABC transporter NukFEG from *Staphylococcus warneri* ISK-1 that is bale to provide immunity against nukacin-ISK-1 (Okuda *et al.*, 2010).

Here, we used a homologous expression system, which allows the expression of NisFEG in *L. lactis* by the *nisA* promotor and measured the immunity arising against nisin. The $NisF_{H181A}EG$ mutant revealed that ATP hydrolysis is needed to perform its activity. The

C-terminal part of nisin is responsible for the pore forming activity, we mutated nisin in such a way that (I) the last ring E and the last two rings D-E are missing, and (II) created truncation mutants where the most C-terminal 6 and the last 14 amino acids are missing. Hereby, we could show that NisFEG is indeed conferring immunity against nisin up to 60 nM, and that the last ring as well as the most C-terminal located 6 amino acids of nisin are needed for NisFEG to be able to fulfil its complete function.

Material and Methods

Cloning of pIL-SV

The *L. lactis*/*E. coli* shuttle vector pIL-SV was cloned using the In-Fusion HD cloning kit (Clontech) according to the manufacturers' protocol. For this the vector backbone of pIL3BTC (Rink *et al.*, 2005) was amplified by PCR with the primers pIL-SV-for and pIL-SV-rev. The PCR, using the primers pUC19Inf-pIL-for and pUC19Inf-pIL-rev and the vector pUC19 as template, amplified a fragment with the coding region of the gene, which confers resistance to ampicillin in *E. coli*, and the pUC origin. In a third PCR the Promotor P_{nisA} was amplified applying the primers pIL-SV-P-for and pIL-SV-P-rev and pIL3BTC as template. The 15 bp overlap extension in the primers allowed the fusion of the three PCR fragments to the vector pIL-SV. Primers used in this study are give n in Table 1

Cloning of pIL-SV-nisFEG and pIL-SV-nisF$_{H181A}$EG

To construct the pIL-SV-nisFEG plasmid, the genes were amplified using isolated genomic DNA from *L. lactis* NZ9700 with the primers nisFEGNotI-for and nisFEGSacI-rev. The PCR-fragment and the vector pIL-SV were both hydrolyzed with *Not*I and *Sac*I and

then ligated. The alanine substitution of the H181 residue by site-directed mutagenesis was performed by PCR using Pfu DNA polymerase, the template pIL-SV-nisFEG and a pair of oligonucleotides (NisF-H181A-for and NisF-H181-rev).

Both plasmids pIL-SV-nisFEG and pIL-SV-nisFH181AEG were verified by sequencing and then transformed into *L. lactis* NZ9000 by electroporation at 1 kV, 25 µF, 5.0 ms, and the corresponding strain were termed NZ9000NisFEG and NZ9000NisF$_{H181A}$EG. The used plasmids and bacterial strains are listed in Table 1 and 2.

Cloning of the prenisin variants

The mutants of nisin were introduced by site directed mutagenesis using the pNZnisA vector, which contains an origin for *E. coli*, and therefor can be propagated in standard *E. coli* lab strains. The resulting plasmids were verified by sequencing. The used primers are listed in Table 1 and the different variants are shown in Figure 1. Four different variants have been constructed genetically, where the last or the last two cysteines where replaced by alanines which when expressed results in active nisin containing only ring A-D or A-C, respectively. The resulting nisin variants are termed CCCCA and CCCAA, respectively (Figure 1). Furthermore, two truncation variants were used; one where the last six amino acids are missing termed Nisin$_{1-28}$ (a kind gift of G. Moll Groningen), and one was newly cloned where the last 14 amino acids are missing termed Nisin$_{1-22}$.

Expression of NisFEG and NisF$_{H181A}$EG in *L. lactis* NZ9000

The NZ9000NisFEG *and* NZ9000NisF$_{H181A}$EG *strains* were grown in GM17 media supplemented with 5 µg/ml chloramphenicol to an OD$_{600}$ of 0.8. By the addition of nisin (final concentration of 1 ng/ml), the expression was induced and the culture was further

grown overnight. After 15 hours, the cells were diluted to an OD_{600} of 0.1 in fresh media supplemented with 5 µg/ml chloramphenicol and the inducer nisin.

Purification of nisin

Nisin was basically purified as described in (Abts *et al.*, 2011).

In short, commercial available nisin powder (Sigma) was dissolved in 50 mM lactic acid pH 3. The nisin solution was purified by using 5ml HiTrap SP HP cation exchange column (GE Healthcare) pre-equilibrated with the same buffer. Nisin was eluted with 400 mM NaCl and the elution fraction online monitored at a wavelength of 215 nm. Nisin containing fractions were precipitated by TCA and dried after washing it with cold acetone (Abts et al., 2011). Upon usage nisin was dissolved in 50 mM lactic acid (pH 3) and the concentration of nisin was measured by using RP-HPLC (Abts *et al.*, 2013).

Expression and purification of prenisin variants

The expression of the nisin variants was performed as described in (Mavaro *et al.*, 2011). The *L. lactis* NZ9000 strain harbouring both pNZnisA containing the variants and pIL3BTC plasmids were used to express, modify and secret the prenisin variants in minimal medium (MM). The expression was induced by the addition of nisin to a final concentration of 5 ng/ml at OD_{600} of 0.4-0.5. The cells were harvested after overnight expression by centrifugation at 8000 x g for 30 min at 4°C.

The supernatant was diluted 1:1 with 50 mM lactic acid, pH 3, and then applied on SP Sepharose column. Here, the buffer was changed to 50mM HEPES buffer pH 7, and the column was washed with 8 column volumes with the same buffer. The prenisin variants were eluted with 50mM HEPES-NaOH pH 7.0, 1M NaCl and 10% (v/v) glycerol. The eluted prenisins were filtered through an Amicon Ultracentrifugal filter (30 kDa cut-off)

to remove high-molecular weight proteins. The flow-through was concentrated afterwards using a 3 kDa cut-off Amicon Ultracentrifugal filter. The concentration of the different prenisins were determined by RP-HPLC (Abts et al., 2013).

Expression and purification of NisP

The expression and purification of the serine protease NisP was previously described in (Abts et al., 2013). In brief, the *L. lactis* strain NZ9000 harbouring the plasmid pNG-$NisP_8$His was used in GM17 supplemented with 5 µg/ml chloramphenicol and grown overnight at 30 °C. Cells were harvested, and transferred into minimal medium with a starting OD_{600} of 0.1 and 0.1 ng/ml of nisin was added to induce the expression of NisP. After harvesting the cells, the pH of the supernatant was adjusted to pH 8.0 by the addition of 3 M Tris-HCl (pH 10) and then applied to an IMAC HP column (GE Healthcare) preloaded with Co^{2+} and pre-equilibrated with low IMAC buffer (50 mM HEPES-NaOH pH 8.0, 150 mM NaCl). After washing with low IMAC buffer the NisP protein was eluted in one step with 50 mM HEPES-NaOH, pH 8.0, 150 mM NaCl, and 300 mM imidazole. The elution fractions were concentrated by ultra- centrifugation (10 kDa MWCO) and the buffer was exchanged using a PD10 column (GE Healthcare) to 50 mM HEPES-NaOH, pH 7.0, 150 mM NaCl, and 10 % glycerol. The resulting NisP protein was aliquoted and stored at −80 °C until further usage.

The different prenisin variants were incubated with NisP overnight at 30°C with a molar ratio of 1000:1. The activated nisin variants were used in all the assays described later. Prenisin and active forms of nisin variants were analysed by RP-HPLC with a LiChrospher WP 300 RP-18 end-capped column using an acetonitrile/water solvent system as described (Abts et al., 2013) and further analysed by Tricine SDS-PAGE.

Determination the activity of nisin and its variants by IC_{50}

The NZ9000, NZ9000NisFEG and NZ9000NisF_{H181A}EG strains were grown overnight in GM17 media supplemented with 5 µg/ml chloramphenicol in the presence of 1 ng/ml nisin. The diluted cells (final OD_{600} was 0.1) were incubated with a serial dilution of nisin or its variants in a 96 well plate. The total volume in each well was 200 µl, consisting of 50 µl nisin and 150 µl GM17 containing the corresponding *L. lactis* strain. The highest concentration of nisin used was adapted to the corresponding maximum immunity displayed by each strain.

The plate was incubated at 30°C. After 5 hours, the optical density was measured at 620 nm via 96-plate reader BMG. The normalized optical density was plotted against the logarithm of the nisin concentration in order to calculate the IC_{50} of nisin and the data was evaluated using the following equation (1):

$$y = OD_{min} + \frac{OD_{max} - OD_{min}}{1 + 10^{(\log(IC50) - x) * p}} \quad (1)$$

The IC_{50} value is the concentration of nisin were the growth of the *L. lactis* strain is inhibited by 50% as described in more detail earlier (Abts et al., 2011).

SYTOx green nucleic acids binding

SYTOx green nucleic acids binding dye possesses a high binding affinity towards nucleic acids. It enters cells only when they contain a pore in the plasma membrane and never crosses the intact membranes of living cells (Roth *et al.*, 1997).

The cells of NZ9000, NZ9000NisFEG and NZ9000NisF_{H181A}EG were grown overnight in GM17 supplemented with 5 µg/ml chloramphenicol in presence of 1 ng/ml nisin. The next day, the overnight culture was diluted to an OD_{600} of 0.1 in fresh media supplemented with 5 µg/ml chloramphenicol. The cultures were grown until an OD_{600} of

0.5 was reached. At this point the SYTOx green dye was added at a final concentration of 5 μM and incubated for 5 minutes according to the manual of the manufacturer (Invitrogen). The fluorescence signal, measured at an excitation and emission wavelength of 504 nm and 523 nm, respectively, was afterwards monitored for 400 seconds to obtain a stable baseline. At 200 seconds nisin was added and the fluorescence was monitored for 15 minutes.

Results

Activity of NisFEG against nisin

Active nisin was purified as previously described (Abts et al., 2011). The plasmid pILNisFEG or pILNisF$_{H181A}$EG was transformed into the *L. lactis* NZ9000 strain (termed NZ9000NisFEG and NZ9000NisF$_{H181A}$EG). The expression of NisFEG was induced by the addition of externally added nisin (de Ruyter *et al.*, 1996). To address the activity of nisin against the NZ9000Erm, NZ9000NisFEG and NZ9000NisF$_{H181A}$EG strains, the IC$_{50}$ value of nisin was determined for the different strains, which reflects the growth inhibition of the corresponding strain by 50%. The IC$_{50}$ value was determined according to equation (1). The H181 in NisF was mutated into alanine, which was identified based on sequence comparison to be the conserved H-loop and this mutation should lead to an ATP hydrolysis deficient mutant.

Nisin is highly active against the NZ9000Erm strain, which lacks the immunity system, as observed by the IC$_{50}$ value of 9 ± 0.7 nM (Figure 2 and Table 3). The NZ9000NisFEG strain exhibited a 7-8 fold higher IC$_{50}$ value of 63 ± 5 nM indicating that the expressed proteins confer immunity against nisin (Figure 2 and Table 3). This highlights that the ABC transporter NisFEG was able to confer immunity against nisin when expressed in *L. lactis*. The NZ9000NisF$_{H181A}$EG strain displayed a lower IC$_{50}$ value of 13 ± 1.2 nM which

is within experimental error the same as the NZ9000Erm strain. When using the *L. lactis* NZ9700 strain the IC_{50} value was 990 ± 54 nM. This strain is a nisin producing *L. lactis* strain, which contains the full nisin operon, and thereby also the immunity system NisI and NisFEG, integrated into its genome (Rauch & De Vos, 1992). So the IC_{50} value determined for this strain comprises the full immunity system. NisFEG when expressed alone is able to confer 6-8% of the immunity observed when using the strain containing the full immunity system.

In summary, this shows that the ABC transporter NisFEG is able to confer a 6-8 fold immunity against nisin and that the $NisF_{H181A}EG$ is not showing any immunity although expressed, indicating that ATP hydrolysis is crucial for the function of the ABC transporter NisFEG.

Pore formation of nisin in the membrane of NZ9000NisFEG cells

Nisin is able to form pores in the membrane of Gram-positive bacteria. This is mediated by the initial binding to Lipid II and subsequently reorientation of the C-terminal part of nisin into the membrane. This leads to membrane leakage and rapid cell death. We visualized this pore formation using a SYTOX green nucleic acid dye (Roth et al., 1997). In our assay the dye enters the cells of *L. lactis* when pores are formed in the membrane and it binds to the DNA. This resulted in a rapid increase of the fluorescence signal, which can be monitored in real time. We monitored the pore forming action of nisin against the NZ9000Erm, NZ9000NisFEG and $NZ9000NisF_{H181A}EG$ strains using three different nisin concentrations, 10, 30 and 100 nM, respectively (Figure 3). As a control we added only the buffer, without nisin, which resulted in no increase of the fluorescence signal as observed by the black lines in Figure 3A and 3C.

In line with the IC_{50} value for the sensitive strain (NZ9000Erm), pore formation was

observed by the addition of 10 nM nisin as indicated by the increase of the fluorescence signal (Figure 3A green curve). This increase was however not observed instantly, rather the effect started with a ≈ 200 seconds delay. When adding a higher amount of nisin (e.g. 100 nM) the increase of the fluorescence signal appeared instantly and reaches a stable plateau already after a couple of seconds (Figure 3A orange curve). This suggested that at a concentration of 100 nM the nisin molecules are acting faster than when 10 nM is used.

The NZ9000NisFEG strain was not affected at a nisin concentration of 10 nM (Figure 3B green curve) as observed by the lack of an increase of the fluorescence signal. When adding 30 nM nisin to the NZ9000NisFEG strain, which corresponds to 50% of the determined IC_{50} value, also no significant increase was observed. Here, however a small linear increase was visible, indicating that some of the cells were lysing (Figure 3B blue line). When using a nisin concentration above the IC_{50} value, e.g. 100 nM, the fluorescence signal was increasing rapidly. The NZ9000NisF_{H181A}EG strain showed almost no effect upon the addition of 10 nM of nisin. This concentration was below the IC_{50} value determined for this strain (see above). In the NZ9000NisF_{H181A}EG strain an increase in the fluorescence signal is, however observed at 30 nM (Figure 3C blue curve). Indicating that this strain is not able to counteract this concentration of nisin. Here, similar to the observed curve for the NZ9000Erm strain with 10 nM nisin, the increase of the fluorescence signal occurs after a delay of 200 seconds. Upon the addition of 100 nM nisin also the NZ9000NisF_{H181A}EG strain displayed a rapid almost instant increase of the fluorescence signal (Figure 3C orange curve).

Altogether this shows that NisFEG confers immunity against nisin when expressed in *L. lactis*, up to 60 nM by preventing pore formation. At nisin concentrations above the IC_{50} value, NisFEG cannot protect the cells against nisin anymore, as a rapid increase of the

fluorescence signal was observed. This indicates that nisin is able to form pores, at these elevated concentration.

Substrate specificity of NisFEG

Nisin contains five lanthionine rings, which are crucial for its antimicrobial function. Whereas, the first two rings are important for docking onto Lipid II, the last two are important for pore formation (Wiedemann et al., 2001, Hsu et al., 2004). We constructed variants of nisin where genetically the last or the last two cysteines were replaced by alanines which when expressed results in active nisin containing only ring A-D or A-C, respectively (primers are listed in Table 1). An overview of the different variants is schematically shown in Figure 1. After successful introduction of the mutations, the resulting plasmids were transformed into *L. lactis* together with the pIL3BTC plasmid encoding for the modification and secretion machinery. This dual plasmid system has been previously used to introduce successfully mutations in the leader sequence (Abts et al., 2013, Plat *et al.*, 2011). The resulting nisin variants are termed CCCCA and CCCAA, respectively (Figure 1). Furthermore, we constructed two truncation variants; one where the last six amino acids are missing termed $Nisin_{1-28}$. Important to note is that all lanthionine rings, A-E are still installed (Figure 1). The $Nisin_{1-22}$ variant contained a stop codon at position 22 and its expressed prenisin variant contained the lanthionine rings A-C, but lacks the rest of the C-terminus (Figure 1). The expression and purification was performed as described in Material and Methods.

After secretion of the prenisin variants, the supernatant was applied onto a cation exchange chromatography (cIEX) and eluted using high salt buffer (see experimental procedures). To remove higher molecular weight species the samples were spawn through a 30-kDa concentrator where the prenisins ended up in the flow trough. Since

these variants are inactive with the leader sequence attached we used the natural protease NisP to cleave off the leader sequence by incubation of the prenisin variants with purified NisP in a molar ratio of 1000:1. We analysed the cleavage reaction via RP-HPLC (Figure 4A). The prenisin variants eluted at a retention time of 17.2 minutes (Figure 4A red line). After cleavage the peak diminished and two new peaks appeared. The first peak eluted at 13.9 minutes and the second peak appeared at a retention time of 22.2 minutes (Figure 4A green line). As a control active nisin purified from the commercial powder was injected onto the RP-HPLC and elutes at a retention time of 22.5 minutes (Figure 4A blue line). The peaks were collected and analysed by mass spectrometric analysis to verify the identity of the peptides eluting and revealed that the cleavage of the prenisin variants reached nearly 100% efficiency. The analysis of the first peak revealed that this contained a peptide, which corresponds to the leader sequence, whereas the second peak contained the corresponding nisin variant. The peaks containing these activated nisin variants were used to determine their antimicrobial potency.

The mutants were >90% pure as judged by SDS-PAGE analysis (Figure 4B). The exact concentration for each variant as well as wildtype nisin was determined via RP-HPLC analysis, by the integration of the corresponding peaks as described before (Abts et al., 2013).

The nisin CCCCA and CCCAA variants, displayed IC_{50} values of 74 ± 1.7 nM and 182 ± 8 nM, against the sensitive NZ9000Erm strain, respectively (Figure 5A and Table 3). This resembles an 8-9-fold and 20-fold reduction when compared to wild-type nisin using the same strain and is in line with previous studies highlighting that the C-terminal part of nisin is crucial for its high activity in the low nM range (Rink *et al.*, 2007).

Similarly, the truncated mutations also showed a significantly reduced activity. The

Nisin$_{1-28}$ variant lacking the last six amino acid showed an IC_{50} of 177 ± 15nM (20-fold) whereas the Nisin$_{1-22}$ variant displayed an IC_{50} of 224 ± 15 nM (25-fold). This shows that not only the last two rings are important for the activity of nisin, but also that the last six amino acids play a crucial role, since their deletion lowered the activity 12-fold (Figure 5A and Table 3).

The NZ9000NisFEG displayed a 6-7 fold immunity when comparing the IC_{50} value of nisin against the NZ9000Erm and NZ9000NisFEG strains (see above). When incubating the NZ9000NisFEG strain with the nisin variants, a 6-7 fold should be observed when the mutation is not interfering with the activity of NisFEG. The fold should decrease when the ring or the truncated nisin variants influences the activity of NisFEG. For example, when no increase in the IC_{50} value is observed, NisFEG was not able protect the cell against this nisin variant anymore. In contrast, when a 6-7 fold reduction of the IC_{50} value of the nisin variants is observed, NisFEG was still able to function for a 100 %.

When incubating the NZ9000NisFEG with the CCCCA variant an IC_{50} value of 237 ± 32 nM (Figure 5B), a 3.2-fold increase of the IC_{50} value is observed when compared to the NZ9000Erm strain using the same nisin variant (Table 3). The CCCAA variant displayed an IC_{50} of 624 ± 87 nM (Figure 5B) a 3.4-fold increase compared to the activity measured of CCCAA variant against the NZ9000Erm strain. The truncation variants displayed an activity of 678± 78 nM and 578 ± 63 nM for Nisin$_{1-28}$ and Nisin$_{1-22}$, respectively. This represents a 3.8 and 2.5 fold immunity mediated by NisFEG (Figure 5B and Table 3).

Discussion

Lantibiotics are small posttranslationally peptides which display a high antimicrobial activity against numerous Gram-positive bacteria. The best-characterized lantibiotic is nisin which is produced by several *L. lactis* strains (Piper *et al.*, 2011). This, 3.4 kDa

antimicrobial peptide comprises five lanthionine rings (ring A-E) in its fully active conformation, specifically introduced by two enzymes (Koponen *et al.*, 2002). These lanthionine rings are crucial for the high level of antimicrobial activity as well as protection against proteolytic degradation (Chatterjee *et al.*, 2005a). To confer immunity against nisin, the producer *L. lactis* strain co-expresses the membrane associated protein systems NisI and NisFEG (Kuipers *et al.*, 1993). The expression of these genes is regulated by a two-component system, consisting of NisR and NisK, which senses the external nisin concentration present in the habitat (Ra et al., 1996).

The proteins of the immunity system, NisI and NisFEG, act cooperatively, since full immunity is only observed when both proteins are present simultaneously (Kuipers et al., 1993, Stein et al., 2003). This was shown in *L. lactis* itself by knockout studies (Ra et al., 1996) as well as by heterologous expression in *Bacillus subtilis* (Stein et al., 2003). This strain gained immunity against nisin even at high concentrations whereas the single expression of one of the genes reduced this nisin resistance drastically (Stein et al., 2003).

In this study, we showed that NisFEG, when homologously expressed in *L. lactis* is conferring a 6-8 fold immunity, as reflected by the increase of the IC_{50} value. Furthermore, we observed that NisFEG protecting the membrane from pore formation up to this concentration. Here, however at nisin concentrations above this IC_{50} value, nisin is able to kill the cells and pore formation can be observed again. These findings are in-line with studies on *B. subtilis* where the quantities of nisin in the culture supernatant were compared between strains, which lack and strains that express NisFEG. Here, the strains expressing NisFEG were found to expel nisin from the membrane into the medium (Stein et al., 2003). The epidermin immunity ABC transporter homologue EpiFEG from *S. epidermidis* appears to have such an expelling

function to confer immunity as well (Otto *et al.*, 1998). NisFEG can only provide a certain level of immunity via this mechanism and the velocity of the ABC transporter dictates its maximum. The nisin molecules added above this concentration will penetrate the membrane and form pores leading to cellular leakage and consequently cell death.

ABC transporters contain specific sequences, which are essential for the binding and / or hydrolysis of ATP. These motifs, Walker A and B, the C-loop and D-loop form the hall marks of this protein super family and are localized in the nucleotide binding domain. In our case they are localized in NisF. By sequence alignment we identified the H181 to be the H-loop in NisF. Mutations of the H-loop have been found to abolish ATP-hydrolysis completely in other ABC transporter systems. For example in the nucleotide binding domain of the Type 1 secretion system of *E. coli* the introduction of the H662A mutation yielded protein which is still able to bind ATP with similar affinities, but the protein is not able to hydrolyse it anymore and therefore it is not functional (Zaitseva et al., 2005a). We expressed the H181A mutant and observed that NZ9000NisF_{H181A}EG was not able to display any significantly resistance to nisin anymore when compared to the NZ9000Erm strain. This highlights that NisFEG is depending on ATP hydrolysis to fulfil its function.

Nisin itself has several modes of action: binding to Lipid II which results in growth inhibition and displacement of Lipid II which also inhibits the growth. Both these mechanism rely on the binding of nisin to Lipid II, which is mediated by the first two lanthionine rings. The high activity of nisin, however relies on the pore formation mechanism initiated by the binding to Lipid II with ring A and ring B. These pores require a specific transmembrane orientation of nisin involving insertion of the C-terminal part (van Heusden et al., 2002) which is possible due to a flexible hinge region (nisin residues 20–22) which is present between ring A-C and ring D-E. Since NisFEG is

expelling nisin from the membrane (Stein et al., 2003) it seems plausible that NisFEG is recognizing the C-terminal part of nisin. We created four different variants of nisin to investigate this, two of them lack ring E or both ring D and E, termed CCCCA and CCCAA respectively. Two other variants were truncations of nisin, where the last six amino acids or the last 12 amino acids were missing, termed $Nisin_{1-28}$ and $Nisin_{1-22}$ respectively. In the $Nisin_{1-28}$ truncation all rings are still present whereas in the $Nisin_{1-22}$ only ring A-C and the linker region are left.

The CCCCA and CCCAA nisin variants still display some antimicrobial activity however lower as the wild-type nisin. Against the NZ9000Erm strain the CCCCA mutant displayed a 8-fold and for the CCCAA mutant an almost 20-fold reduction was observed. This highlights that the rings are crucial for the activity of nisin. Also the truncated mutants displayed a lower IC_{50} against the NZ9000Erm strain. Here, the activity dropped for $Nisin_{1-28}$ by a factor of 20 and for $Nisin_{1-22}$ a 25-fold reduction was observed. In line with previous studies showed that deletion the C-terminal residues leads to a reduction of the activity between 16-100 fold reduction (Sun *et al.*, 2009, Chan *et al.*, 1996). This shows that, not just the rings are important, but also the C-terminal residues located behind the rings of nisin play an important role for the activity.

NisFEG is able to achieve an 8-9-fold immunity against nisin when expressed in *L. lactis*. When performing the same experiments with the nisin variants, the fold of immunity mediated by NisFEG differs. The deletion of ring E resulted in an immunity level drop to 3.6-fold, a similar reduction of the immunity was observed with the $Nisin_{1-28}$ truncation variant, where all five rings are still present. In other words, the activity of NisFEG was lowered by a factor of 2. This highlights that the most C-terminal six amino acids and the ring E are equally important for the activity of NisFEG. Interestingly, the CCCAA as well as the $Nisin_{1-22}$ truncation variants resulted in almost similar level of the NisFEG induced

immunity being, 3.8 and 2.5 fold, when compared to the IC_{50} value found for the nisin sensitive strain, respectively. Since there was no further reduction of the fold of immunity, ring D as well as the amino acids 23-27 in nisin are not crucial for the activity of the NisFEG ABC transporter.

Our nisin mutants showed that the most C-terminal part of nisin is of special need to be expelled by NisFEG. When these amino acids are missing as in the $Nisin_{1-28}$ variant, the ABC transporter is still able to confer some immunity, however at a lower level. This suggests that, besides this C-terminus, also other parts are needed for the immune activity of NisFEG. Presumably, also ring A and B play a role as they bind Lipid II, which is needed prior to pore formation.

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Table 1: Primers used in this study. Shown are the primers used to create the pIL-SV-NisFEG and pIL-SV-NisF_{H181A}EG expression plasmid, as well as the nisin mutants: CCCCA, CCCAA and the truncations $Nisin_{1-28}$ and $Nisin_{1-22}$

Name	**Sequence (5'-3')**
pIL-SV-for	CAGCTTTCTTGTACAAAGTGGTGATGG
pIL-SV-rev	GGAGCTGTAATATAAAAACCTTCTTC
pUC19Inf-pIL-for	TTATATTACAGCTCCTCTTCCGCTTCCTCGCTCAC
pUC19Inf-pILrev	TATTGATCTTGGAGCGAAAGGGCCTCGTGATACGC
pIL-SV-P-for	GCTCCAAGATCAATAGAAACATTAAC
pIL-SV-P-rev	TGTACAAGAAAGCTGGCGGCCGCCTATTTGAGTGC
nisFEGNotI-for	CAAATAGGCGGCCGCATGCAGGTAAAAATTCAAAATCTTT CTAAAACATATAAAG
nisFEGSacI-rev	GAATTCGAGCTCCACAAGAAAAAATACTTTATCTAATCTT TTTTTTAG
NisF-H181A-for	GACAATCTTGATTTCTAGTGCTCAGTTGCACGAAATAAGT AAAG
NisF-H181A-rev	CTTTACTTATTTCGTGCAACTGAGCACTAGAAATCAAGAT TGTC
CCCCA_for	CAGCAACTTGTCATGCTAGTATTCACGTAA G
CCCCA_rev	GCTTACCTGAATACTAGCATGACAAGTTGCTG
CCCAA_for	GGAGCTCTGATGGGTTGTAACATGAAA AC

CCCAA_rev	GTTTTCATGTTACAACCCATCAGAGCTCC
$Nisin_{1-22}$_for	GCTTACGTGAATTTAACAATGACAAGTTGC
$Nisin_{1-22}$_rev	GCAACTTGTCATTGTTAAATTCACGTAAGC

Table 2. Strains used in this study

Strain	Plasmid	Characteristics	Reference
NZ9000	-	nisRK+ and an empty plasmid	(de Ruyter et al., 1996)
NZ9000NisFEG	pILSV-NisFEG	nisFEG	This work
NZ9000NisF$_{H181A}$EG	pILSV-NisF$_{H181A}$EG		This work
NZ9700	-	nisABTCIPRKEFG (Wilde type nisin producer)	(Kuipers et al., 1993)

Table 3: IC_{50} values of nisin and its variants against the NZ9000Erm and NZ9000NisFEG strains. Besides the IC_{50} values also the fold of immunity against the nisin variants mediated by NisFEG is shown.

	NZ9000Erm	NZ9000NisFEG	Fold of immunity
Nisin	9 ± 0.7 nM	59 ± 3.7 nM	6.5
CCCCA	74 ± 1.7 nM	237±32 nM	3.6
CCCAA	182 ± 8 nM	624 ± 87nM	3.4
Nisin$_{1-28}$	177 ± 15nM	678± 78 nM	3.8
Nisin$_{1-22}$	224 ± 15 nM	578 ± 63nM	2.5

Figure 1: **Nisin variants used in this study.** Schematically shown are the wildtype nisin peptide structure as well as the variants CCCCA, CCCAA $Nisin_{1-28}$ and $Nisin_{1-22}$. Highlighted in yellow are the dehydrated residues and the lantionine rings are highlighted with a red line. The lanthionine rings are numbered A-E.

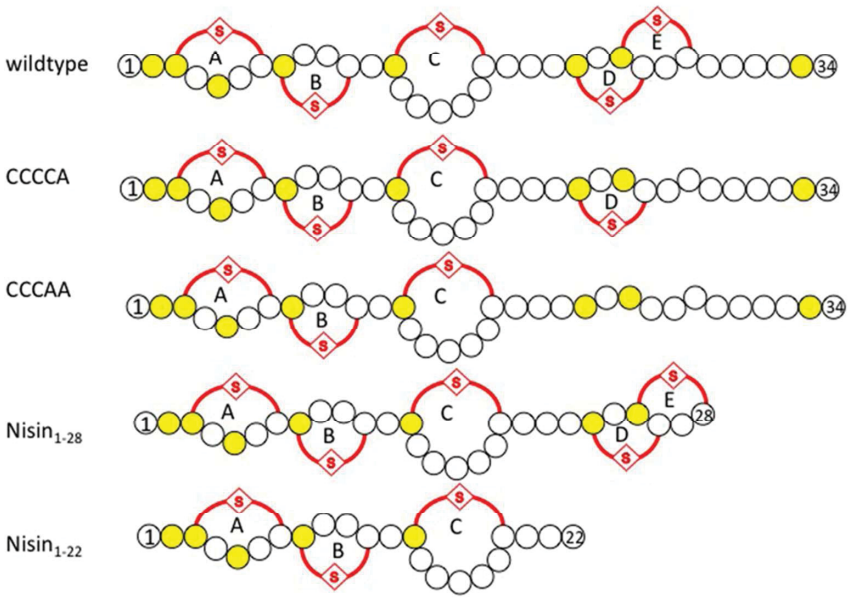
Figure 2: **IC_{50} determination of nisin against different strains**. Growth inhibition experiments were performed with nisin using the NZ9000Erm (λ), NZ9000NisFEG (υ), $NZ9000F_{H181A}EG$ (open square) and NZ9700 strain (τ). Data was fitted and evaluated according to equation (1). Each experiment was performed at least in triplicates.

Figure 3: **SYTOx green assay to visualize pore formation mediated by nisin**. (A) NZ9000Erm strain (B) NZ9000NisI strain (C) NZ9000NisIΔ22 strain (red line) were grown and incubated with the SYTOX green dye. The fluorescence signal was monitored online using a fluorolog (Horiba III). After a stable baseline was reached nisin was added and pore formation was monitored by measuring the increase of the fluorescence signal. Black curve: buffer control green curve 10 nM nisin; blue curve 30 nM nisin; orange curve100 nM nisin. The rapid increase of the fluorescence indicated pore formation.

Figure 4: **Cleavage reaction of prenisin variants analysed by RP-HPLC**: (A) RP-HPLC profile following the cleavage reaction of NisP with the modified CCCCA precursor peptide. The modified precursor CCCCA peptide (**red**) was digested with NisP and analyzed by RP-HPLC. The digested sample (**green**) showed two peaks. One at an elution time of 13.5 min corresponding to the leader peptide, which was confirmed by mass spectrometric analysis. The second peak eluted at 22 min which run similar as the active nisin (**blue**) which was used as control. (B) SDS-PAGE analysis of the activated

nisin variants. Exemplary shown is the activation of the CCCCA variant. Left lane: prenisin form of the CCCCA variants. Right lane: Activated nisin variant after NisP treatment and HPLC analysis.

Figure 5: **IC_{50} determination of the CCCCA, CCCAA, $Nisin_{1-28}$ and $Nisin_{1-22}$ against the (A) NZ9000Erm and (B) NZ9000NisFEG strains**. Growth inhibition experiments were performed with the nisin variant using the NZ9000Erm and NZ9000NisFEG. Data was fitted and evaluated according to equation (1). Each experiment was performed at least in triplicates.



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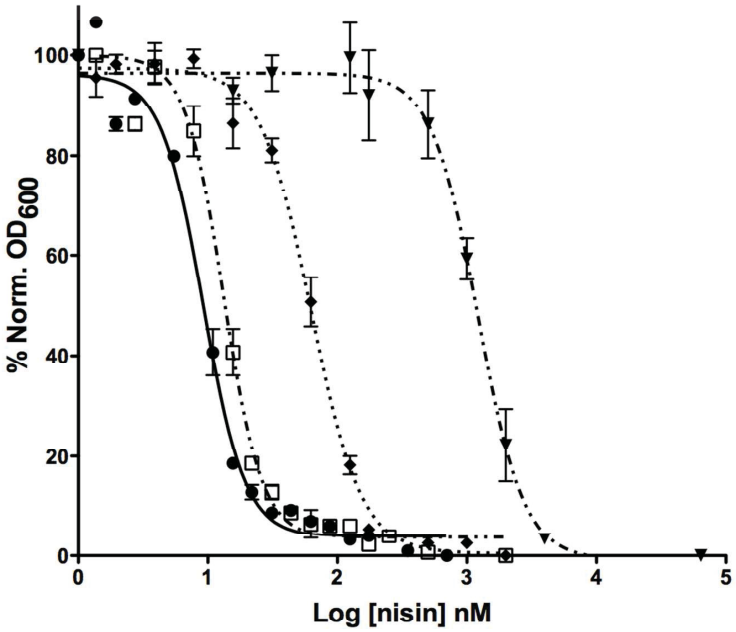

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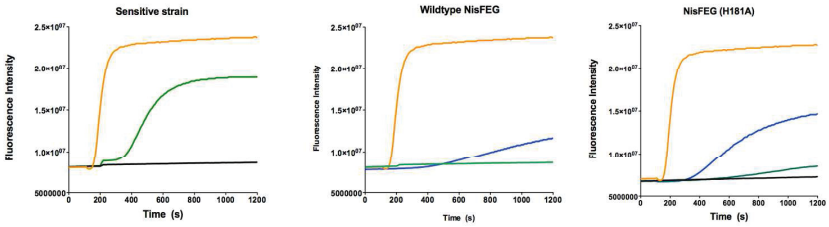

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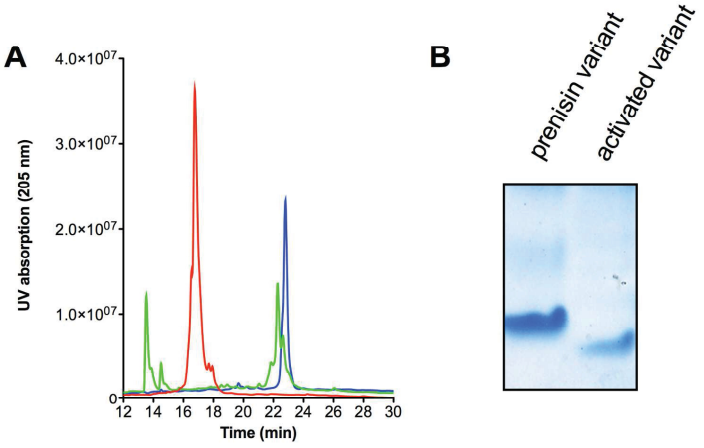

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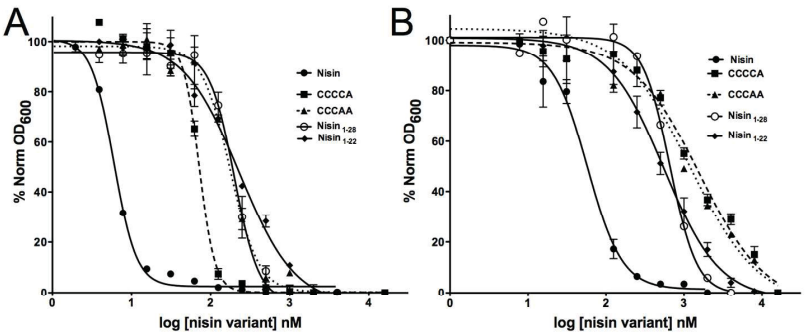

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CHAPTER V

Cooperative mechanism of the lantibiotic immunity proteins NisI and NisFEG

In preparation

Introduction

Lantibiotics are small, ribosomally synthesized peptides which are produced by numerous Gram-positive bacteria. After post-translational modification and secretion the specific secretion signal called leader peptide is cleaved off to activate the mature lantibiotic. These active lantibiotics are able to lyse mainly Gram-positive bacteria as well as few Gram-negative bacteria. They act via different but distinct mechanisms (Wiedemann *et al.*, 2001, Chatterjee *et al.*, 2005b) and target the bacterial membrane of mainly Gram-positive bacteria. This suggests that the membranes of lantibiotic-producer strains can be also harmed. In order to circumvent this suicidal effect, the producer strain expresses an immunity system (Alkhatib *et al.*, 2012). The proteins involved in this immunity are encoded by genes present on the operon near the genes responsible for maturation and secretion. The immunity proteins seem to be present in all the lantibiotic encoding gene clusters (Willey & van der Donk, 2007) . They are LanI, a membrane anchored lipoprotein; and LanFEG, an ATP binding cassette (ABC) transporter, which is localized in the cellular membrane. Although in most of the operons, both these proteins are present, some exceptions are are observed where only one of the two is present (Alkhatib et al., 2012).

Nisin is the best-known and the most extensively studied lantibiotic which is produced by some strains of *Lactococcus lactis* (*L. lactis*). Nisin is produced as an inactive precursor containing an N-terminal leader peptide, which is cleaved directly after secretion by the subtilin-like serine protease, NisP (van der Meer *et al.*, 1993).

Due to its high bactericidal activity in combination with its low toxicity in humans, nisin has been used as a natural preservative in the food industry since decades (Delves-Broughton *et al.*, 1996). Active nisin is a 3.4 kDa peptide, consisting of 34 amino acids and contains five lanthionine-based rings (ring A-E)(Figure 2). The first three rings (namely A-C) are N-terminally located and are separated from the intertwined rings D and E (located at the C-terminus) by a flexible hinge region. The mode of action of nisin has been thoroughly studied, and it is suggested that nisin attacks bacteria by inhibiting their cell-wall synthesis *via* binding to Lipid II.

It has also been shown that the rings A and B of nisin are responsible for binding to Lipid II (Hsu *et al.*, 2004), which is used as a 'docking molecule' for nisin to protrude into the membrane using the hinge region and the rings D and E, which subsequently form a pore. (Wiedemann et al., 2001, van Heusden *et al.*, 2002, Hasper *et al.*, 2004, Brotz *et al.*,

1998, Breukink *et al.*, 1999). The presence of Lipid II is required for the high activity of nisin. This was confirmed by artificial membrane systems, where a three-fold increase in pore forming activity of nisin was observed when Lipid II was present. Furthermore, it has been shown that Lipid II is a constituent of the formed pore, which, when fully assembled, consists of four Lipid II and eight nisin molecules (Hasper et al., 2004).

The nisin producing *L. lactis* strain expresses two protein systems consisting of the lipoprotein NisI and the ATP binding cassette (ABC) transporter NisFEG, which when expressed together, provide immunity against high levels of nisin (Ra *et al.*, 1996). Both immunity systems seem to act cooperatively, since each of them displays only 20-30% of the full immunity when expressed alone (Stein *et al.*, 2003, Ra *et al.*, 1999). This cooperative mode of action is intriguing but till date is quite poorly understood.

In the experiments described in Chapter III, it was observed that NisI mediates an 8-9 fold immunity against nisin (IC_{50} value for nisin of 70 nM for the NZ9000NisI strain compared to 9 nM for the NZ9000Erm strain). Furthermore, the NisI protein is able to inhibit pore formation even at high concentrations of nisin (15- fold above IC_{50} value). In contrast, NisFEG provides a slightly lower immunity against the wild-type nisin (60 nM) and at concentrations above this value, nisin is able to bind to Lipid II and is again able to form pores in the membrane.

In order to further investigate this cooperative mechanism, we created a plasmid termed pILNisIFEG, where both NisI and NisFEG can be simultaneously expressed under the control of externally added nisin and the resulted strain was termed as NZ9000NisIFEG. Although the producer *L. lactis* strain NZ9700 is available, our system has a further advantage that no nisin is produced by this strain during its growth. As a consequence, the effects observed are solely based on the amount of externally added nisin. Additionally, to identify the mechanism of this cooperative mode of action between the NisI and NisFEG proteins, we have used this NZ9000NisIFEG strain together with several variants of nisin.

Material and Methods

Cloning and Expression of NisI, NisIΔ22aa and NisIFEG protein in L. lactis NZ9000:

The NZ9000NisI and NZ9000NisIΔ22 strains were cloned as explained in Chapter III. The NZ9000NisIFEG was cloned with the same procedure as explained in Chapter III, where both NisI and NisFEG were inserted into one vector (pNZSV*nisA*) .
The cloned strains were grown in GM17 media, supplemented with 5µg/ml erythromycin or chloramphenicol, respectively, to an OD_{600} of 0.8. The expression was induced using externally added nisin (at a final concentration of 1 ng/ml), and the culture was allowed to grow overnight. These cells were then used for the assays described below.

Cloning of the prenisin variants

The variations/mutations in prenisin were introduced by site-directed mutagenesis using the pNZnisA vector, which contains an origin for *E. coli*, and therefore, can be propagated in standard *E. coli* lab strains. The resulting plasmids were verified by sequence analysis. The different variants are shown in Figure 2. Four different variants have been constructed genetically, where the last or the last two cysteines where replaced with alanines, resulting in the expression of active nisin containing only rings A-D (termed CCCCA) or A-C (named CCCAA), respectively (Figure 2). Furthermore, two truncated variants, $Nisin_{1-28}$ and $Nisin_{1-22}$, were also constructed, which lacked the last six and last fourteen amino acids, respectively.

Overexpression and purification of active nisin, precursor peptide and its variants

The cloning, expression and purification of mature nisin and the precursor peptide derivatives were performed as earlier published (Abts *et al.*, 2011, Mavaro *et al.*, 2011), using slightly modified elution buffer (50 mM HEPES-NaOH pH 7.0, 1 M NaCl and 10% glycerol) for the cation-exchange chromatography (cIEX) of the different precursor peptide derivatives.

Cleavage off the leader peptide by NisP

NisP was expressed and purified as described (Abts et al 2013). The leader peptide of prenisin (amino acids -23 to -1) is removed using the protease NisP which digests the modified precursor peptide, as mentioned below. The purified, modified precursor peptide variants were incubated with the purified NisP in a molar ratio of 1000:1 at 30 °C overnight. The reaction mixture was then applied to a RP-HPLC column and the eluted nisin variants were fractionated and then lyophilized. The nisin variants were dissolved in Lactic acid buffer pH 3 prior to the usage for the IC_{50} measurements.

Determination of the concentration of nisin and its variants.

The nisin peptides were analysed by RP-HPLC using a LiChrospher WP 300 RP-18 end-capped column with an acetonitrile / water solvent system consisting of solvent A (10% acetonitrile / 90% water / 0.1% TFA) and solvent B (90% acetonitrile / 10% water / 0.1% TFA). After injecting the sample, the elution was performed by a linear gradient spanning over 35 minutes to 60% solvent B at a flow rate of 1 mL min^{-1}.
The specific peptide concentrations were determined by absorption at 205 nm. For the calibration, known amounts of nisin (Sigma) and human insulin (Sigma) were injected and the absorption integrals were determined using EZChrom Elite V.3.3.1. software and plotted against the known masses.

Determination of the nisin activity by IC_{50}:

Cells from NisI or NisIFEG expressing strains were grown overnight in GM17 in the presence of 1 ng/ml nisin. The diluted cells, final OD_{600} of 0.1 were then incubated with a serial dilution of nisin in a 96-well plate. Thus, each well had a total volume of 200 µl, which consisted of 50 µl nisin and 150 µl cells of the corresponding *L. lactis* strain. The highest concentration of nisin used varied for each variant and for each strain.
The plate was incubated at 30°C for 5 hours, and the OD_{600} was then measured at 620 nm using a 96-well plate reader BMG. In order to calculate the IC_{50} of nisin, the normalized OD_{600} was plotted against the log of nisin concentration and the data was evaluated using equation (1):

$$y = OD_{min} + \frac{OD_{max} - OD_{min}}{1 + 10^{(\log(IC50) - x) * p}} \quad (1)$$

The IC_{50} value is then calculated as the value of the used nisin concentration where the growth inhibition is 50%.

Results

Activity of the NZ9000NisIFEG against nisin

To investigate the activity of NisI and NisFEG simultaneously, we used a plasmid system which allows the propagation of the plasmids in *E. coli* and then transformed them into the *L. lactis* NZ9000 strain. The expression of the NisI and NisFEG proteins was induced with externally added nisin.

Nisin is highly active against the NZ9000Erm strain, which lacks the immunity system, as observed by a low IC_{50} value of 9 ± 0.3 nM (Figure 1). Contrarily, the NZ9000NisI and NZ9000NisFEG strains, both exhibit a higher IC_{50} value, 73 ± 10 nM and 63 ± 5 nM respectively, indicating that the expressed proteins confer immunity against nisin (Figure 1). (also described in Chapter III and IV). These values highlight the fact that the NisI protein and the NisFEG ABC transporter are also individually able to provide some immunity against nisin when expressed alone in *L. lactis*. However, in the nisin producer NZ9700 strain, both these proteins are simultaneously expressed and thus, results in a very high IC_{50} value of 1.2 ± 0.1 µM (Figure 1). However, this strain produces active nisin, and the IC_{50} reflects both the externally added as well as newly synthesized nisin. Therefore, to solely rely on the externally added nisin, we constructed a plasmid named pnz*NisIFEG*, where both the *nisI* and *nisFEG* genes are located on the same plasmid having their own promoter region.

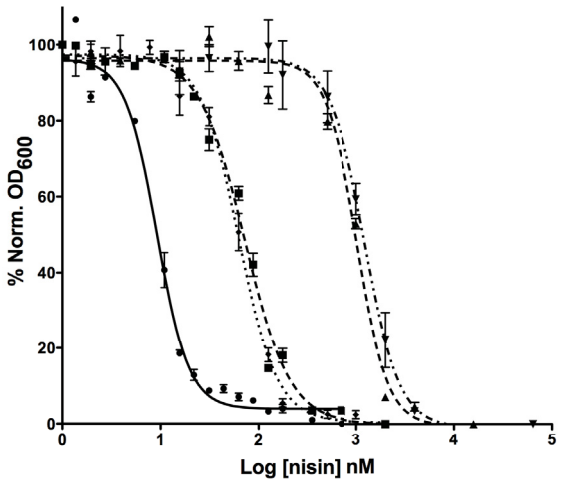

Figure 1. IC_{50} determination of different strains. Growth inhibition experiments were performed (at least in triplicates)with nisin using different strains. (●): NZ9000Erm strain; (◆): NZ9000NisI strain; (■): NZ9000NisFEG strain; (▲): NZ9000NisIFEG strain (▼): NZ9700 strain. Data was fitted and evaluated according to equation (1)..

This resulting plasmid was then transformed in the *L. lactis* strain NZ9000 (termed NZ9000NisIFEG), which allowed the simultaneous expression of both NisI and NisFEG proteins. . This NZ9000NisIFEG strain displayed an IC_{50} value similar to the producer strain, *i.e.,* 990 ± 10 nM (Figure 1), which confirms that both these proteins are expressed in the NZ9000NisIFEG strain and at levels, which resemble the producer strain , supported by the same magnitude of the IC_{50} value.

Effect of the nisin variants on the NZ9000NisIFEG strain

Furthermore, to determine the influence of the different nisin variants on the activity of NisI, the IC_{50} values of the NZ9000NisI strain were determined of each variant. The wild-type nisin displays an IC_{50} value of 73 ± 10 nM against the NZ9000NisI strain, which is almost 8-9 fold reduction of the nisin activity (see above). In case the nisin variant has no effect on the function of NisI, a similar fold of reduction is expected.

Four different nisin variants were created (for details see Chapter IV) and after successful introduction of these mutations, the resulting plasmids were transformed into *L. lactis* together with the pIL3BTC plasmid encoding for the modification and secretion machinery. This dual plasmid system has been previously used to successfully

introduce mutations in the leader sequence of nisin (Abts *et al.*, 2013, Plat *et al.*, 2011). After cleaving off the leader sequence, these nisin variants were activated and their activity was tested against the nisin sensitive NZ9000Erm strain. The first two variants of nisin miss the last or the last two cysteines resulting in an active nisin lacking the ring E or rings D-E, respectively (see chapter IV). The activated nisin variants are termed CCCCA and CCCAA respectively, based on the number of cysteines still present (Figure 2). Furthermore, we constructed two truncated variants; one where the last six amino acids are missing termed nisin$_{1\text{-}28}$. One thing important to mention here is that although some amino acids are missing, all the lanthionine rings, A-E are still intact (Figure 2). The other variant is nisin$_{1\text{-}22}$ variant, which contained a stop codon at position 22 and its expressed prenisin variant contained the lanthioinine rings A-C, but lacks the rest of the C-terminus (Figure 2).

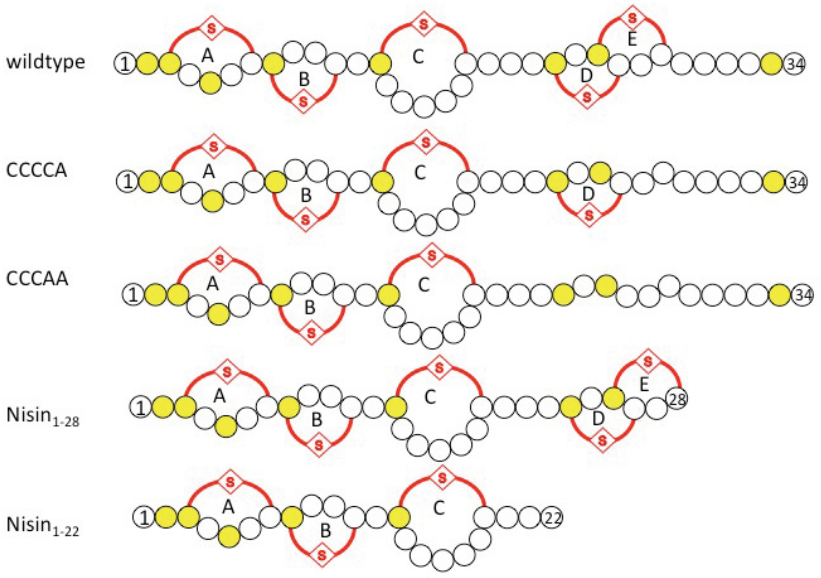

Figure 2. Nisin variants used in this study. Schematically shown are the structures of the wild-type nisin peptide and the variants CCCCA, CCCAA, nisin$_{1\text{-}28}$ and nisin$_{1\text{-}22}$. Highlighted in yellow are the dehydrated residues and the lanthionine rings are highlighted with a red line. The rings are labeled as A, B, C, D and E. This figure was taken from Chapter IV

The CCCCA and CCCAA variants, displayed IC$_{50}$ values of 74 ± 1.7 nM and 182 ± 8 nM respectively, against the sensitive NZ9000Erm strain (chapter IV and Table 3) which when compared to the wild-type nisin, resemble an 8-9-fold and a 20-fold reduction, respectively.

The truncated variants also showed a reduced activity. The nisin$_{1-28}$ variant which lacks the last six amino acids showed an IC_{50} value of 177 ± 15nM (20-fold decrease) whereas the nisin$_{1-22}$ variant displayed an IC_{50} value of 224 ± 15 nM (decrease of 25-fold) (Chapter IV and Table 3).

Table 1. IC_{50} values of wild-type nisin and its variants against the NZ9000Erm strain

	Nisin	**CCCCA**	**CCCAA**	**Nisin$_{1-28}$**	**Nisin$_{1-22}$**
NZ9000Erm	9 ± 0.7 nM	74 ± 1.7 nM	182 ± 8 nM	177 ± 15nM	224 ± 15 nM

Additionally, to visualize the activity of the full immunity system against these nisin variants, their IC_{50} values, when incubated with the NZ9000NisIFEG strain, were also determined. An IC_{50} value was observed for wild-type nisin as 990 ± 10 nM when using the NZ9000NisIFEG strain (see above). An almost 100-fold increase when compared to the NZ9000Erm strain. This directly showed the synergistic or cooperative effect of NisI and NisFEG in the membrane of *L. lactis*. Both the CCCCA and CCCAA variants showed lower IC_{50} values against the NZ9000NisIFEG strain, *i.e.,* 629 ± 71 nM and 882 ± 32 nM, respectively, which are contrary to the higher IC_{50} values obtained with the sensitive NZ9000 strain (see above).

A similar effect was also observed with the truncated variants. The IC_{50} values of Nisin$_{1-28}$ and Nisin$_{1-22}$ were 816 ± 43 nM and 491 ± 83 nM, respectively, against the NZ9000NisIFEG strain (Table 2). Although, these observed IC_{50} values are just slightly higher than the ones observed for the CCCCA and CCCAA variants, they still belong to the same range.

Table 2. IC_{50} values of wild-type nisin and its variants against the NZ9000NisIFEG strain.

	Nisin	**CCCCA**	**CCCAA**	**Nisin$_{1-28}$**	**Nisin$_{1-22}$**
NZ9000NisIFEG	990 ± 10	629 ± 71	882 ± 32	816 ± 43	491 ± 83

The *L. lactis* cells stop growing at lower concentrations when incubated with the nisin variants, suggesting that the variants becoming more active. This is intriguing since one would expect exactly the opposite. As shown earlier, the immunity provided by the NZ9000NisIFEG strain is 100-fold (see above) which is inline with the fold of immunity mediated by NisI and NisFEG when expressed together in *B. subtilis* (Stein et al., 2003)

As observed in Chapter IV, the nisin variants when incubated with NZ9000NisFEG, displayed increased IC_{50} values, implying that although NisFEG is still recognizing the nisin variants, it looses some of its immunity activity. It was further observed that the last six amino acids as well as ring E are especially important for NisFEG to fully explore its nisin expelling capacity. Therefore, this 'activation' of the nisin variants observed with the NZ9000NisIFEG strain is likely resulting from the expression of NisI.
To further investigate this, the IC_{50} values for the nisin variants were also determined with the NZ9000NisI strain where only NisI is expressed and the NisFEG protein is lacking.

Activity of the nisin variants against the NZ9000NisI strain

As previously shown in the chapter III, the NZ9000NisI strain displayed a 8-9 fold increase in the IC_{50} values when compared to the NZ9000Erm strain. Even though the NZ9000NisIΔ22strain was less active, it still showed a 2-3 fold immunity due to the binding of the nisin molecules to the expressed NisI deletion mutant suggesting that one could observe a similar increase for the nisin variants when the mutation does not influence the activity of NisI or NisIΔ22 protein.
In case the NisI protein does not recognizes the nisin variants any more, the IC_{50} value should drop to the same value observed when these variants are incubated with the NZ9000Erm strain which allows us to theoretically calculate a range of the expected IC_{50} values for the NZ9000NisI and the NZ9000NisIΔ22 strain (Table3). So for the CCCCA and CCCAA variants with incubated with the NZ9000NisI strain, this range would be 74-629 nM and 182-1547 nM, respectively. Similarly, for $nisin_{1-28}$ and $nisin_{1-22}$, the expected range of IC_{50} values would be 177-1504 nM and 224-1904 nM, respectively. Since NisIΔ22 protein displayed lower but still significant 2-3 fold immunity, a theoretical range could also be calculated and are shown in Table 3
Interestingly, on comparing to the variants incubated with the NZ9000Erm strain, all the nisin variants incubated with the NZ9000NisI strain showed lower IC_{50} values rather than higher values. The CCCCA and CCCAA variants displayed an IC_{50} value of 40 ± 7nM and 116± 13 nM, respectively, against the NZ9000NisI strain. Even the truncated mutants, $Nisin_{1-28}$ and $Nisin_{1-22,}$ showed a lower IC_{50} value of 90± 12 nM and 54 ± 6 nM, respectively against the NZ9000NisI when compared to the IC_{50} values of the variants where incubated with the NZ9000Erm strain (Figure3 and Table 3).

Table 3. IC_{50} values of the nisin variants against the NZ9000NisI and NZ9000NisIΔ22 strains. Shown are the theoretically calculated range of the IC_{50} values based on the 7-8 and 2-3 fold of immunity displayed by the NZ9000NisI and NZ9000NisIΔ22 strains, respectively, against wild-type nisin (highlighted in *italic*).

NZ9000NisI					
	Nisin (nM)	**CCCCA (nM)**	**CCCAA(nM)**	**$Nisin_{1-28}$ (nM)**	**$Nisin_{1-22}$ (nM)**
Theoretical	-	*74-629*	*182-1547*	*177-1504*	*224-1904*
Observed	74 ± 3	40 ± 7	116 ± 13	90 ± 12	54 ± 6
NZ9000 NisIΔ22					
	Nisin (nM)	**CCCCA (nM)**	**CCCAA(nM)**	**$Nisin_{1-28}$ (nM)**	**$Nisin_{1-22}$ (nM)**
Theoretical	-	*74-435*	*182-455*	*177-442*	*224-560*
Observed	25 ± 1.7	56 ± 6	48 ± 9	36 ± 2	53 ± 2

A similar effect was also observed for the NZ9000NisIΔ22 strain when incubated with the nisin variants. In this case, the wild-type nisin showed an IC_{50} value of 25 ± 1.7 nM, which is almost a 3-fold reduction when compared to the NZ9000Erm strain. The mutants CCCCA and CCCAA displayed IC_{50} values of 58 ± 15 nM and 80 ± 27 nM, respectively. $Nisin_{1-28}$ and $nisin_{1-22}$ displayed an IC_{50} values of 36 ± 2 nM and 44 ± 12 nM, respectively (Table 3 and Figure 3)

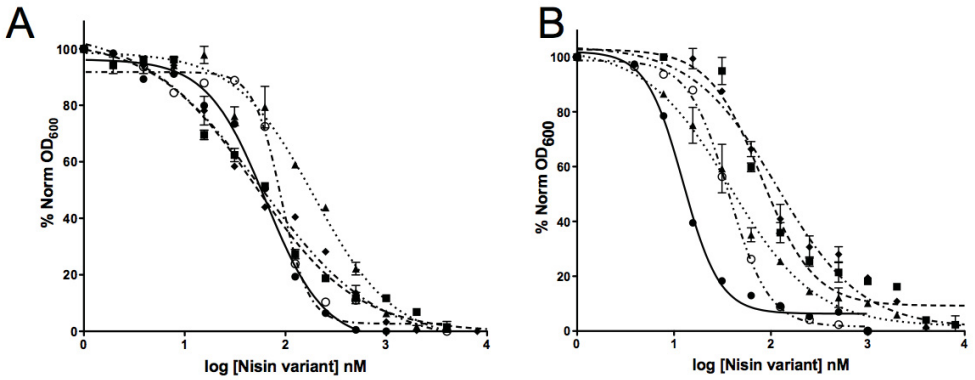

Figure 3. IC_{50} determination of the nisin variants against the NZ9000NisI and NZ9000NisIΔ22 strains. Growth inhibition experiments were performed (at least in triplicates) with wild-type nisin and its variants against the (A) NZ9000NisI and (B) NZ9000NisIΔ22 strains. (●): wild-type nisin; (■): CCCCA variant; (▲): CCCAA variant; (◆): Nisin1-22; (o) Nisin1-28. Data was fitted and evaluated according to equation (1).

Therefore, no immunity was observed for the NZ9000NisI and the NZ9000NisIΔ22 strains which would have been indicated by a shift in the IC_{50} value towards a higher number, in contrast to the presently observed lower IC_{50} values of the nisin variants in the presence of NisI or even NisIΔ22. The curves displayed by the NZ9000NisI as well as the NZ9000NisIΔ22 strains appear to be less steep when compared to the wild-type nisin curves. Similar observation was made in all the IC50 curves where the nisin variants were incubated with strains expressing NisI or its C-terminal truncated mutant indiacting that may be the cells are not instantly killed by these nisin variants but rather slowly stop growing.

Discussion

Lantibiotics are small post-translationally modified peptides, which display a high antimicrobial activity against numerous Gram-positive bacteria. The best-characterized lantibiotic is nisin, which is produced by several *L. lactis* strains (Piper *et al.*, 2011). This, 3.4 kDa antimicrobial peptide comprises of five lanthionine rings (ring A-E) in its fully active conformation, specifically introduced by two enzymes (Koponen *et al.*, 2002). These lanthionine rings are crucial for the high antimicrobial activity as well as for the protection against proteolytic degradation (Chatterjee *et al.*, 2005a). To confer immunity against nisin, the producer *L. lactis* strain co-expresses the membrane associated protein systems NisI and NisFEG (Kuipers *et al.*, 1993). The expression of these genes is regulated by a two-component system, consisting of NisR and NisK, which senses the external nisin concentration present in the habitat (Ra et al., 1996).

The proteins of the immunity system, NisI and NisFEG, act cooperatively, due to the fact that the full immunity is only observed when both the proteins are present simultaneously (Kuipers et al., 1993, Stein et al., 2003). This was shown by knockout studies in *L. lactis* itself (Ra et al., 1996) and by heterologous expression in *Bacillus subtilis* (Stein et al., 2003). These strains gained immunity against nisin even at high concentrations whereas the single expression of one of the genes reduced this nisin resistance drastically (Stein et al., 2003).

A similar observation was made when NisI or NisFEG was expressed alone in *L. lactis* (Chapter III and IV). Here, NisI displayed an IC_{50} value of 74 nM, however, this is contradicting since at concentrations above this value the *L . lactis* cells are not dying, rather they form long chains of cocci. One could term this effect a “sleeping mode” of the

cells as they fully recover and start growing again when the concentration of nisin drops again which was shown by a regrowth experiment (chapter III). NisFEG, in contrast, appears to be a classical ABC exporter, expelling nisin from the membrane and thereby lowering the overall nisin concentration because of which more nisin molecules are required to kills the NisFEG strain which is reflected by the IC_{50} value of 64 nM.

The expression of NisI and NisFEG together on one plasmid in the NZ9000 *L. lactis* strain resulted in full immunity against wild-type nisin which was further confirmed by IC_{50} determination and by comparing it to the value obtained with the nisin producer NZ9700 *L. lactis* strain. This highlights that when both immunity proteins are expressed at the same time in the nisin non-producer strain, the natural high level of immunity is reached.

Surprisingly, the immunity the NZ9000NisIFEG strain did not show immunity against the nisin variants. A very huge difference was observed between the IC_{50} values obtained with the sensitive strain for the nisin variants depicting a mere 2-5 fold immunity as compared to the 100-fold full immunity observed with the wild-type strain (Table 2).

In chapter IV, the same mutants were used to detect the substrate specificity of NisFEG. It was observed that a reduced but still significant immunity mediated by NisFEG against the variants. This implicates that the low fold of immunity shown in the NisIFEG expressing strains, against the nisin variants, is likely due to the presence of NisI. Therefore, the variants were further tested against strains expressing the NisI or its C-terminal truncation NisIΔ22 protein. Here, the effect became more pronounced and all the cells displayed an IC_{50} value in range of 40-116 nM depending on the nisin variant used. The experiment using a NisIΔ22 expressing strain also displayed similar values. However, one has to take into account that the NisI expressing cells are not killed by nisin, rather, they stopped growing which appears to be a distinct feature of NisI. As shown in chapter III, this activity is not located at the C-terminus of NisI. So likely the variants are still able to induce this long chain formation and the cells stop growing.

This seems interesting and suggests that NisFEG has the ability to lower the nisin concentration in the membrane to roughly untill 60 nM. If the concentration further raises, the NisI protein induces a new morphology of the *L. lactis* cells as observed by long chain formation. Together this allows the *L. lactis* cells to survive high nisin concentrations, up to 1000 nM. However, further experiments are needed to support and confirm the same. These might include using SYTOx green assays, which was

described in Chapters III and IV , to clearly identify whether the NZ9000NisIFEG strain suffers from pore formation at concentrations above the determined IC_{50} value. Creation of other variants of nisin like CCCAA, CCAAA and CAAAA to analyse which part of nisin is actually important for inducing long chain cell formation and would shed light on whether a lanthionine ring is really important or not.

The presence of NisI in the nisin gene cluster is very crucial in preventing pore formation by nisin. All lantibiotic producer strains express immunity proteins that belong either to the LanI lipoprotein family, like NisI for the lantibiotic nisin in *L. lactis* and SpaI for the lantibiotic subtilin *in B. subtilis;* or an ABC transporter called LanFEG, which in *L. lactis* is NisFEG and SpaFEG in *B. subtilis*. A detailed analysis of these systems revealed that the presence of only LanI or LanFEG or both together, correlates with the mode of action of the corresponding lantibiotic. Additionally, Chatterjee *et al.* classified lantibiotics in different classes and a identified a pattern between LanI/FEG and their lantibiotic (Chatterjee et al., 2005b). When the lantibiotic is exhibiting pore forming activity, like nisin, subtilin, pep5 or epicidin 280, there is a LanI protein present (Heidrich *et al.*, 1998, Parisot *et al.*, 2008, Alkhatib et al., 2012). However, only LanFEG homolog is present when the lantibiotic activity involves binding to Lipid II or its precursors, thereby solely inhibiting the cell wall synthesis. Examples include the strains expressing mersacidin and lacticin 481 (Alkhatib et al., 2012, Brotz *et al.*, 1995). On the contrary, when the lantibiotic comprises of a combined mode of action of inhibiting cell wall synthesis as well as pore formation, both LanI and LanFEG homologs can be found in the producer strain, as observed in the case of nisin, subtilin, streptin, epidermin (Alkhatib et al., 2012).

In this study here, it was clearly shown that NisI and likely the other LanI homologs are important for the inhibition of pore formation of their corresponding lantibiotic. This would lead to the identification of the mode of action of newly found lantibiotics, based on the presence of either of the two immunity protein systems, LanI and LanFEG.

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DISCUSSION

The model lantibiotic Nisin

Active nisin is a 3.4 kDa peptide, consisting of 34 amino acids and contains five lanthionine-based rings. The first three rings (A, B, and C) are separated from the intertwined rings four and five (D and E) by a flexible hinge region (Figure 10). It is ribosomally synthesized; post-translational modified peptide secreted in a non-active form. After secretion it is activated by cleaving off the leader peptide, which is attached to its N-terminal. The genes which are responsible for the synthesis, modifications and secretions of nisin are organized in an operon as shown in Figure 14.

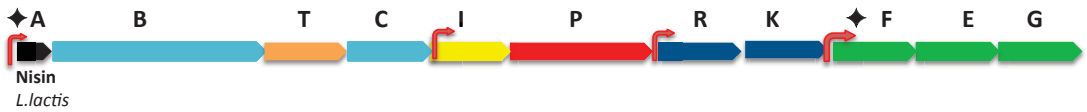

Figure 14. Gene operon of nisin biosynthesis represent genes expressing for nisin precursor NisA (Black), modification machinery NisB and NisC (Torques), transporter NisT (Orange), protease NisP (Red), regulatory system NisR and NisK (Dark blue), immunity system NisI (Yellow) and NisFEG (Green).

Those genes are expressing the following proteins. NisA is the nisin precursor, NisB is a dehydratase catalyzes dehydration of serine and threonine residues in the prepeptide of prenisin, which is the nisin precursor[41]. Then the dehydrated prenisin is modified by NisC which catalyzes the coupling of dehydrated residues to the C-terminal located cysteine residues to form lanthionine rings [42]. After that, NisT, the ABC transporter, is exporting the modified prenisin, which is still inactive, and then the anchored cell-membrane protease NisP cleaves the leader peptide of nisin to turn it into active nisin.

The mode of action of nisin has been thoroughly studied and it was shown that nisin has several modes of action (as shown in Figure 11): binding to Lipid II, which results in growth inhibition, sequestering Lipid II out of septum, which also inhibits the division and the growth of cells, and formation of pores in the target membrane, which cause the lysis of the target cell. All these modes of action rely on the binding of nisin to Lipid II, which is mediated by the first two lanthionine-rings [79]. The last two rings and the hinge region are able to flip into in the membrane and create a pore.

The pore formation mode of action of nisin in the membrane of Gram-positive bacteria was the focus of the research for decades as it enables nisin, as well as other lantibiotics, from being highly active in nanomolar range. It has been shown that nisin uses Lipid II as a 'docking molecule' to form pores with high efficiency. Subsequently a specific transmembrane orientation involving the C-terminal part of nisin[80] leads to very pronounced

and stable pores that cause the leakage of the small cytoplasmic compounds such as amino acids and ATP which leads to a rapid cell death.

The immunity system against nisin

L. lactis strains that produce nisin express NisI and NisFEG as an immunity system in order to protect their membranes from any possible activity of nisin.

Both NisI and NisFEG are important to provide full immunity. This was shown in *L. lactis* itself by knockout studies as well as by heterologous expression in *Bacillus subtilis*. Expression of only one of the two genes reduced nisin resistance drastically[45], however the exact function and role in immunity for both proteins is not well understood. In this thesis, an *in vivo* system of nisin immunity system was created in *L. lactis* in order to investigate the immunity mechanism against nisin.

***In vivo* system of the immunity system**

To investigate the role of each immunity protein in the nisin immunity, an *in vivo* system has been established. Each NisI, NisFEG or both, NisI and NisFEG were expressed homologously in *L. lactis* NZ9000, which is a nisin non-producer strain [102] resulting in NZ9000NisI, NZ9000NisFEG and NZ9000NisINisFEG, respectively. Another two strains, NZ9000NisIΔ22 where the last 22 amino acids of the C-terminal of NisI were deleted and NZ9000NisF_{H181A}EG, where the *nisF* is mutated and thereby the ABC transporter cannot hydrolyze ATP, were also cloned and, after transformation, there were expressed in the *L. lactis* NZ9000 strain. All the resulted strains are listed in Table 1.

Table 1. Cloned and used *L. lactis* strains in this study.

Strain	Plasmid	Characteristics	Reference
L. lactis NZ9000	-	$nisRK^+$	[103]
NZ9000Erm	pNZSV*nisA*	(Empty plasmid)	Chapter III
NZ9000NisI	pNZSV-*nisI*	NisI	Chapter III
NZ9000NisIΔ22aa	pNZSV-*nisIΔ22aa*	deletion of last 22aa of C-terminal of NisI	Chapter III
NZ9000NisFEG	pIl-*nisFEG*	NisFEG	Chapter IV
NZ9000NisF_{H181A}EG	pIl-*nisF$_{H181A}$EG*	ATP hydrolyse-deficient	Chapter IV
NZ9000NisIFEG	pNZ-*nisInisFEG*	Full immunity system	Chapter V
L. lactis NZ9700	-	*nisABTCIPRKEFG* (nisin producer)	[104]

The role of NisI in the immunity system

In the case of the control strain, NZ9000Erm, nisin exhibits a high activity (IC_{50}= 9.1 ± 0.7 nM), while the expression of NisI in the NZ9000NisI strain reduced the effect of nisin activity almost 8-10 fold as reflected by the IC_{50} value of 73.0 ± 10.2 nM (Table 2). This result is in line with other studies, which showed a 10 fold increase of immunity when NisI is expressed [100]. Comparing to the full immunity, NisI showed around 8 %, which is similar to previous studies where NisI has provided 4% of the maximum immunity obtained by the nisin producer strain [96].

In order to detect the mechanism of NisI in immunity, the different strains were incubated with nisin in the presence of SYTOx green dye, which binds the DNA of lysed cells. If pores in the cytoplasmic membrane of target cells are present, the dye will binds to the DNA of these cells. This assay has been used in other studies to visualize pore formation, for example for salivaricin 9 from *Streptococcus salivarius*, which is also a pore former lantibiotic [105]

Table 2. IC_{50} values of nisin incubated with NZ9000Erm, NZ9000NisI and NZ9000NisIΔ22aa (Chapter III)

	NZ9000Erm	**NZ9000NisI**	**NZ900NisIΔ22aa**
Nisin	9.1 ± 0.7 nM	73.0± 10.2 nM	25.3 ± 1.7 nM

As nisin is a pore forming antimicrobial peptide, which displays activity in the nM range, nisin was able to form pores at concentration above 10 nM in case of the nisin sensitive NZ9000Erm strain, while no pore formation was noticed at 10 nM or even at 30nM for NZ9000NisI (Chapter III). Interestingly, for NZ9000NisI no even signal of SYTOx was observed with a very higher concentration up to 1000 nM suggesting that nisin was not able to form pores. This concentration of nisin is a 15-fold higher than the IC_{50} value in the growth assay where no growth was observed for the NZ9000NisI cells above 70nM. The last 22 amino acids of the C-terminus of NisI showed to be important, since their deletion (NZ9000NisIΔ22) allowed nisin to form pores (IC_{50} value of 22 nM). This reflects the role of the C-terminus of NisI in the inhibition of pore formation. The remained activity of this NisI variant, as observed by the higher IC_{50} value when compared to the NZ9000Erm, is likely arising from a nisin binding event to the rest of the NisI protein. Due to this interaction, a higher amount of nisin is needed.

Microscopic analysis of the NZ9000NisI cells showed a special clustering of cells, while the NZ9000Erm cells are normal cocci. This chain formation is directly correlated to the concentration of externally added nisin. Increasing the nisin concentration, leads to chains consisting of more cells (up to 20 cells were observed in one chain) and normal chains of the NZ9000NisI strain (2 cells per chain) were observed when no nisin was added. These double

cocci are also observed in the NZ9000Erm strain which does not express NisI This suggest that the chaining is correlated with the presence of both NisI as well as nisin.

Interestingly, this chaining event, or morphology change, is reversible. Upon the removal of nisin, the NZ9000NisI cells start growing again, albeit with a delay time, and the cells are organized in double cocci again. It is worth mentioning that this chaining event is also observed with the NZ9000NisIΔ22 strain, but only at concentration below the IC_{50} value, since this NZ9000NisIΔ22 were suffering from pore formation above the IC_{50} value as shown with the SYTOx green assay. This leads to the conclusion that this special morphology effect is not induced by the C-terminal part of NisI, it rather will be localized somewhere else in the NisI protein.

A recovery assay was performed in order to determine if the NZ9000NisI cells, which were exposed to high nisin concentration, were dead or they simply stopped cell division. Thereby NZ9000Erm, NZ9000NisI and NZ9000NisIΔ22 were exposed to a very high concentration of nisin, 10 fold higher than the corresponding IC_{50}. Only the cells of the NZ9000NisI strain were able to re-grow after removal of the growth-inhibitory concentration of nisin. Here, the observation was made that the incubation time with nisin was directly correlated to the number of cells surviving. After 1 hour of treatment 10 time more cells started growing again when compared to the number found after 2 hours.

Altogether, this allows the hypothesis that the NisI-expressing cells, when treated with nisin, are not killed. The IC_{50} determination however showed that they stopped growing and form long chain of cells, which allows the *L. lactis* cells to survive high concentrations of nisin for a certain period of time. Once the concentration of nisin drops again some of the cells survived and start to grow again.

As pore formation requires the binding of nisin to Lipid II, one of the possible explanations for the obtained results is that the C-terminal part of NisI protects or shields Lipid II thereby ensuring that nisin cannot reach Lipid II. Lipid II is crucial for cell wall synthesis and the binding of the C-terminus of NisI to Lipid II will lead to reduced growth or even complete inhibition of growth of the cells. The NZ9000NisI strain is growing comparable to the NZ9000Erm strain without adding nisin, thus the C-terminus seems not be influencing the growth behavior when no nisin is present. Or to put this in other words, the C-terminus is only shielding the Lipid II molecules when nisin is present. This leads to the conclusion that nisin is binding to NisI and thereby triggers a conformational change of at least the C-terminus which then binds Lipid II and inhibits pore formation of nisin. At concentration below 70 nM nisin this conformational change is not triggered.

Inhibition of the nisin-Lipid II binding has been indirectly observed when vancomycin was added prior to nisin to the nisin-sensitive cells. Since lipid II was occupied with vancomycin, which does not have any pore formation activity, nisin was not able form pores [23]. This could be the case of NisI itself. As the C-terminus of NisI is binding to Lipid II, no nisin-Lipid II complex can be formed and thus immunity can be achieved.

The role of NisFEG in the immunity against nisin

NisFEG was expressed in *L. lactis* and the expressing strain NZ9000NisFEG conferred 6-7 fold higher levels of immunity (see Chapter IV) and 6% of the full immunity. In another study, it was shown that a disruption in the *nisI* gene in the nisin producer strain resulted in an immunity level of 10-20% of the maximum immunity of the wild-type nisin-producing strain, provided by the NisFEG[96].

The $NisF_{H181A}EG$ strain, where the histidine residue of the H-loop was mutated, showed almost no immunity as reflected by an IC_{50} value of 13 ± 0.3 nM, indicating that ATP hydrolysis is crucial for the NisFEG ABC transporter to function. This was indirectly postulated by the expelling function of NisFEG [45].

Since NisI is able to inhibit pore formation at nisin concentration above the IC_{50} value, experiment were performed to investigate whether NisFEG also is able to achieve this. So the pore forming activity of nisin for NZ9000Erm, NZ9000NisFEG and NZ9000$NisF_{H181A}EG$ was monitored via the SYTOx green assay, using three different nisin concentrations, 10, 30 and 100 nM respectively (Chapter III). No pore formation was observed from NZ9000NisFEG strain at 30 nM nisin, implying that NisFEG to protect the cells from pore formation at this concentration. This is in line with the IC_{50} data since 30 nM is below the IC_{50} value obtained for this strain. In contrast at 30 nM pore formation was observed for the NZ9000$NisF_{H181A}EG$ strain, as the 30 nM of nisin is above the IC_{50} value determined with this strain.

The NZ9000NisFEG strain suffered from pore formation when nisin was added at a concentration above the IC_{50} value. Here, 100nM nisin was used and appeared to be sufficient to create pores in the membrane of this strain. This shows that, in contrast to NisI protein, NisFEG is not able to confer a protection at elevated nisin concentrations

To conclude, the NisFEG is able to protect the cells from the activity of nisin up to 60nM. When the concentration of nisin becomes higher, nisin is able to attack the NZ9000NisFEG cells and can form pores in the membrane as shown by the SYTOx green assay. While NisI is able to inhibit nisin mediated pore formation even at very high concentrations up to 1000nM, a 15-fold higher concentration than the corresponding IC_{50}, NisFEG was able to inhibit pore formation only up to the IC_{50} value.

Substrate specificity of NisI and NisFEG

Since the C-terminal part of nisin is responsible for pore formation [106], different nisin mutants were created at this part.Two different variants, missing the lanthionine rings at the C-terminus, were genetically constructed by replacing the last or the last two cysteines. This resulted in two nisin variants in which ring E (CCCCA) or rings D and E (CCCAA) were missing (Figure 15). Those variants were called "ring mutants". Another two variants were cloned in which amino acids of the C-terminal part of nisin were missing. One variant ($Nisin_{1-28}$) lacked the last six, C-termianl amino acids and in the other variant, a truncation of last 14 amino acids was introduced to result in a nisin variant covering just the first three rings ($Nisin_{1-22}$). Those variants were called " truncated mutants". The four variants were purified and activated as shown in Chapter (IV) and then incubated with the different strains in order to study the interaction of both, NisI and NisFEG, with the nisin variants and to detect weather any part of the C-terminus of nisin is interacting with NisI and NisFEG.

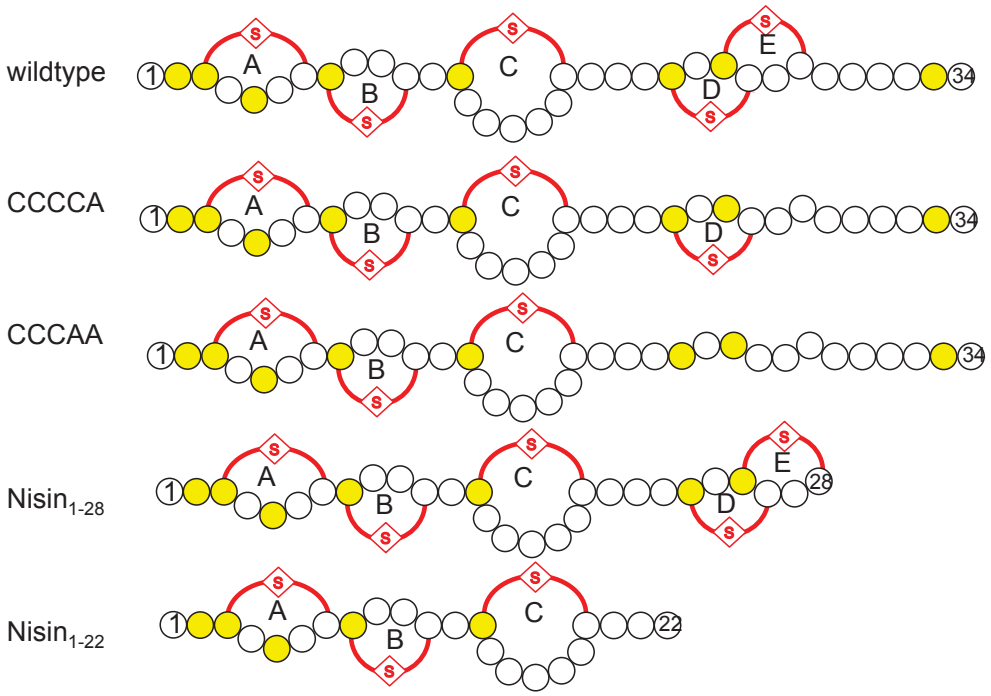

Figure15. Nisin variants used in this thesis. Wildtype nisin, CCCCA, CCCAA, $Nisin_{1-28}$ and $Nisin_{1-22}$.

The nisin CCCCA and CCCAA variants displayed an 8- 9 fold and 12 fold reduction, respectively, when compared to the wildtype nisin. This is in line with previous studies highlighting that the C-terminal part of nisin is crucial for its activity in the low nM range. Similarly, the truncated mutations also showed a significantly reduced activity. Both $Nisin_{1-28}$ and $Nisin_{1-22}$ variants showed 20 and 25 fold reduction of activity which was also shown in previous studies (16-100 fold reduction) [84,86].

Incubating each variant with each NisI and NisFEG expressing cells in a liquid assay and calculating the IC_{50} were used for the comparison between the different strains. When the mutation is not affecting the interaction between the nisin and the immunity protein, it is expected to create an increase in immunity of the same magnitude which observed for wildtype nisin. If the mutation had a direct effect on this interaction, a different magnitude or no immunity at all should be observed.

Different folds of immunity were determined for the different variants of nisin when using the NZ9000NisFEG strain. When incubating the NZ9000NisFEG strain with the CCCCA, CCCAA, $Nisin_{1-28}$ and $Nisin_{1-22}$ variants, 3.6-fold, 3.4-fold, 3.8-fold and 2.5-fold increases in immunity were observed. So all the mutations of the C- terminus of nisin had an influence on the nisin-NisFEG interaction, although the highest affect was observed for the removal of the last 6 amino acids and the ring E. Their deletion lowered the fold of immunity by a factor of 2. Almost the same fold of immunity was observed after removing the C-terminal 14 amino acids which displayed a 2.5 fold immunity or ring D-E which displayed a 3.4 fold immunity (Table 3).

Since no further reduction of the fold of immunity is observed when one more lanthionine ring is missing or when a nisin variant with a larger deletion of the C-terminus is used, the conclusion can be made that the last 6 amino acids of the C-terminal part of nisin and the ring E are the most important for activity of NisFEG. In table 3, the corresponding IC_{50} values are summarized.

Table 3. IC_{50} values of nisin and its variants with NZ9000Erm and NZ9000NisFEG strains and the folds of immunity of NisFEG when incubated with these variants (Chapter IV).

	NZ9000Erm	**NZ9000NisFEG**	**Fold of immunity**
Nisin	9 ± 0.7 nM	59 ± 3.7 nM	6.5
CCCCA	74 ± 1.7 nM	237±32 nM	3.6
CCCAA	182 ± 8 nM	624 ± 87nM	3.4
$Nisin_{1-28}$	177 ± 15nM	678± 70nM	3.8
$Nisin_{1-22}$	224 ± 15 nM	578 ± 63nM	2.5

The results of NZ9000NisI when incubated with nisin variants were unexpected. The nisin variants showed however a lower IC_{50} value when using the NZ9000NisI strain when compared to the nisi sensitive NZ9000Erm strain At a first glance this suggests that the nisin variants become activated in the presence of NisI. However, NisI is not manipulating the nisin molecule, by cleaving or changing therefor the “apparent activation” is likely due to another mechanism.

As observed in Chapter III, the NisI expressing cells did not suffer from pore formation at concentration above the determined IC_{50} value. Furthermore, a new phenotype was observed

where the cells cluster in long chains, which was achieved by the addition of nisin, and occurs at concentration around 70 nM nisin.

All nisin variants when incubated with the NZ9000NisI strain all displayed IC_{50} values in between 40-116 nM nisin. Here, the growth was inhibited by 50%. From this it can be concluded that the nisin variants induced the cells to stop growing, similar to the wildetype nisin. Since the variants used in this study were all localized at the C-terminus of nisin and this cell growth inhibition was observed for all the variants (Chapter V and Table 4), the triggering factor must lay in the N-terminal part of nisin. Presumably the first two rings (ring A and B) play a role here, since they are also crucial for the initial binding of nisin to Lipid II.

Table 4. IC_{50} values of nisin and its variants with NZ9000Erm and NZ9000NisI strains.

	NZ9000Erm	**NZ9000NisI**
Nisin	9 ± 0.7 nM	74 ± 3 nM
CCCCA	74 ± 1.7 nM	40 ± 7 nM
CCCAA	182 ± 8 nM	116 ± 10 nM
$Nisin_{1-28}$	177 ± 15nM	90 ± 12 nM
$Nisin_{1-22}$	224 ± 15 nM	54 ± 12 nM

The full immunity system NisI / NisFEG

To gain a view on the full immunity system a plasmid was created where both NisI and NisFEG were expressed simultaneously (Chapter V). Here, the full immunity was observed resulting in an IC_{50} of 990 ±10 nM. This is a 100-fold increase of the IC_{50} value when compared with the nisin sensitive NZ9000Erm strain. More importantly this is in the same range as the value obtained for the nisin producer strain, NZ9700, where a value of 1100 ±10 nM was observed. This shows that both NisI and NisFEG are expressed at similar levels as in the NZ9700 strain. The created NZ9000NisIFEG strain has a major advantage when compared to the NZ9700 strain. Here, the NZ9000NisIFEG strain is not producing any nisin molecules, and therefor the effects observed are solely due to the externally added nisin molecules. Especially when using nisin variants, the exclusion of any effects of naturally expressed nisin is crucial.

For the nisin variants, one would also expect a 100-fold by the NZ9000NisIFEG strain when the variants do not effect both proteins. The fold of immunity observed with all the nisin variants were only 2-8 fold (see Table 5). This is only 2-8% of the fold immunity observed with wildtype nisin. This is a result of the combined expression and function of NisFEG and NisI.

NisFEG alone was able to expel the nisin variants up to a specific concentration, almost 2-3 fold immunity (Chapter IV), which is 30-40 % of the fold immunity observed for the

wildtype nisin. NisI when expressed alone, displayed a lower IC_{50} value since the cells when incubated by the nisin variants stopped growing and form long cell chains.

This suggests that the NZ9000NisINisFEG cells stopped growing at a certain nisin concentration due to the activity displayed by NisI resulting in a low fold of immunity for the nisin variants.

Table 5. IC_{50} values of nisin and its variants with NZ9000Erm and NZ9000NisINisFEG strains and the folds of immunity of NisINisFEG when incubated with these variants (Chapter V).

	NZ9000Erm	NZ9000NisINisFEG	Fold of immunity
Nisin	9 ± 0.7 nM	990 ±10 nM	100
CCCCA	74 ± 1.7 nM	629 ± 71 nM	8.5
CCCAA	182 ± 8 nM	882 ± 60 nM	4.8
$Nisin_{1-28}$	177 ± 15nM	816 ± 65 nM	4.6
$Nisin_{1-22}$	224 ± 15 nM	491 ± 83 nM	2.2

Model of the nisin immunity system

Depending on the data provided in this thesis and the results of previous published studies, a model of the nisin immunity system can be proposed.

Initially, when nisin is produced, (very) low concentrations are reaching the exterior and the membrane of the *L. lactis* strain. Here, NisFEG is able to expel nisin away from the membrane (Figure 16A). Thereby nisin cannot reach its dockin partner Lipid II. Thus, the *L. lactis* cells do not require any function of NisI. When the nisin concentration reaches 60-70 nM, NisFEG becomes saturated and is not able to expel more nisin molecules. This allows nisin to reach the membrane and display its antimicrobial activity (Figure 16B). At this point the *L. lactis* cells would not be immune anymore against nisin and need a second mechanism of immunity. This is mediated by the presence of NisI. At 60-70 nM nisin, NisI is binding nisin molecules, which induces a conformational change at the C-terminus of NisI. (Figure 16C-I). This C-terminus is triggered and binds or shields Lipid II by which the nisin molecules can not reach their docking molecule in the membrane anymore. This results in an extremely potent immunity, where even at 1000 nM nisin no pore formation can be observed or detected.

Besides this conformational change, the binding of nisin to NisI also results in cells which stopped growing (Figure 16C-II). This mechanism is intriguing although it is not clear how NisI exactly induces this phenotype.

Over time the nisin molecules will diffuse away into the media and thereby the concentration at the membrane will be lowered. Once the concentration is decreased to a level below 60 nM, NisI releases its bound nisin and thereby the C-terminus is released from the membrane and

is no longer shielding lipid II (Figure 16-D). This can be very nicely observed when performing a regrowth assay with the NisI expressing strain (Chapter III).

At this point NisFEG is able to expel nisin from the membrane and no further immunity system is needed. The immunity system is so to speak back to its initial configuration (Figure 16-A).

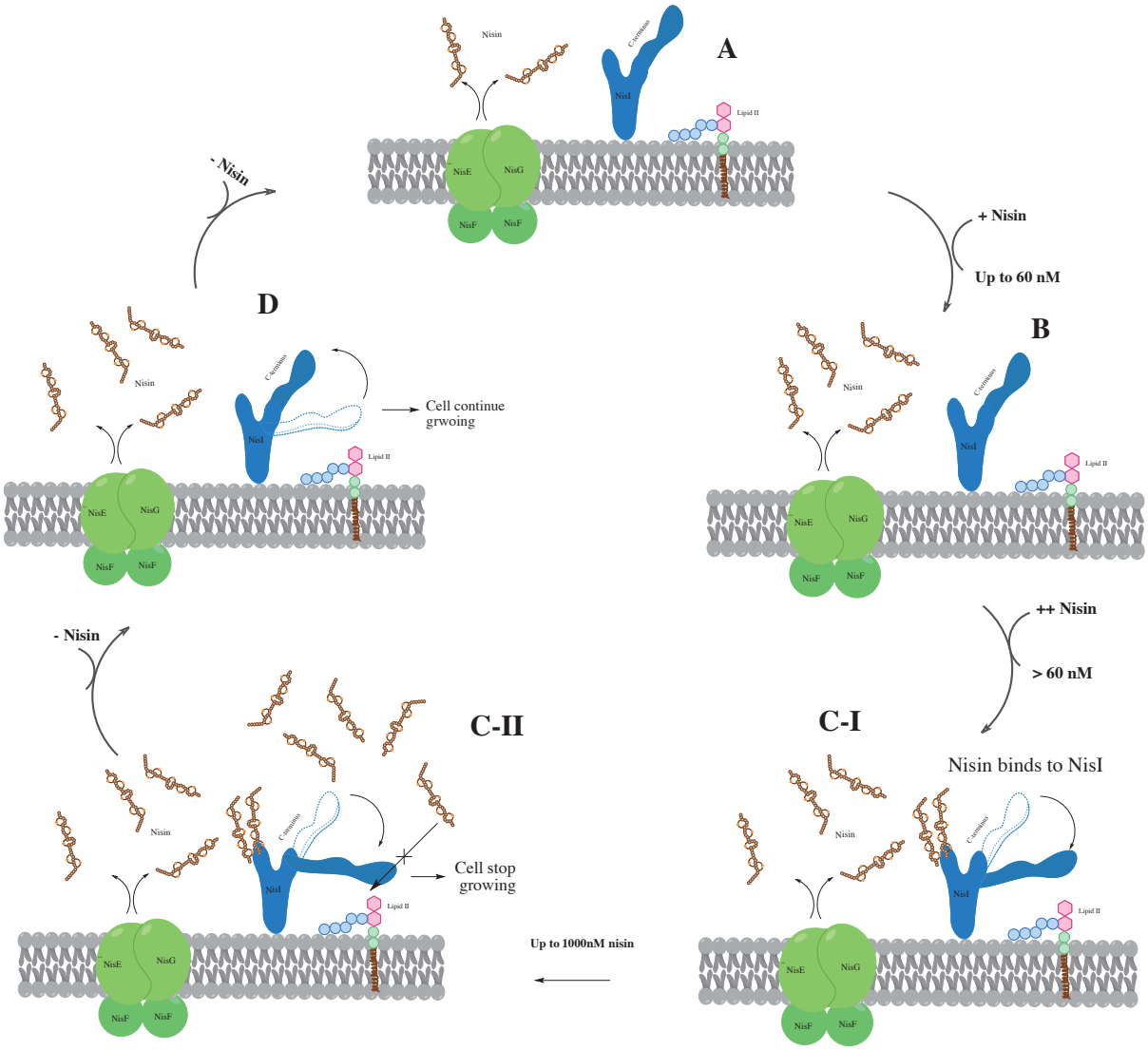

Figure 16. Model of the immunity system against nisin. Nisin is produced in low concentration into the media. The expressed NisFEG is expelling nisin away from the membrane (A). NisFEG is able to confer immunity up to 60 nM (B). The nisin concentration is increasing above 60nM and thus the extra nisin molecules are able to reach the membrane and bind to NisI. Upon this binding, the C-terminus of NisI is activated to shield Lipid II and inhibit any possible pore formation (CI). The nisin concentration reaches its maximum and the immunity system provides full immunity (CII). Afterwards, nisin concentration is decreasing and the C-terminus of NisI flips back to the normal position and the cells are able to grow again. Here the nisin-expelling mechanism of NisFEG is sufficient to provide the required immunity (D).

A similar system is found also in *Bacillus subtilis*, which produces subtilin. SpaFEG and SpaI confer immunity against subtilin. Subtilin is a pore former, which binds to Lipid II by its first two lanthionine rings and it is shown that its positive charges at the C terminus of subtilin could serve as binding site(s) for the highly negatively charged N-terminus of Spa I. This flexible N-terminus of Spa I folded upon Lipid binding [101]. In our system, the C-terminus of NisI, which upon induction of nisin, is able to provide high immunity and likely is binding Lipid II.

The lantibiotic, Nukacin ISK-1, produced by *Staphylococcus warneri*, also is able to form pores in the membrane of for example *streptococcin* FF22. The Nukacin ISK-1 producer strain is, like *L. lactis,* also expressing an immunity system consisting of two protein systems; NukFEG and NukH. Here, NukFEG is an ABC transporter and NukH is a lipoprotein similar to the organization of NisI and NisFEG. It was shown that NukH confers immunity against Nukacin ISK-1 and the authors suggested a possible binding between NukH and Nukacin ISK-1 which leads to inhibit them from being inserted into the cytoplasmic membrane and thus the survival of the NukH-expressing strains [93]

These two examples show that the immunity system of NisI and NisFEG is conserved and sequence alignments studies revealed that the lantibiotic producing strains have a similar set of proteins conferring immunity against their own lantibiotic (Chapter I).

These immunity genes are expressing either LanI lipoprotein or encoding ABC transporter; LanFEG. This presence of one of these genes or both might correlated with the mode of action of the corresponding lantibiotic. It can be observed that when the lantibiotic is exhibiting a pore-forming activity like nisin, subtilin, pep5 or epicidin 280 there is a need for a LanI and LanFEG protein, while when the lantibiotic activity is solely relying on the binding to Lipid II, thereby displaying solely inhibiting the cell wall synthesis, only a LanFEG homolog is present, as observed for strains expressing mersacidin and lacticin 481 where only the proteins MrsFEG and LctFEG are present [87,107].

NisI and NisFEG appear to work side by side. When NisFEG reaches its maximum velocity and cannot provide more immunity, NisI is able to increase the immunity further by two distinct mechanisms. This allows the survival of *L. lactis* cells at high concentrations of its own product, nisin.

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DECLARATION

The dissertation presented here has been made independently and without unauthorized assistance. The dissertation has not been submitted in this or any similar form to any other institution. I have not taken any unsuccessful promotion exams so far.

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