



Lantibiotic Nisin and Its Detection Methods

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Abstract

The type A lantibiotic nisin produced by several *Lactococcus lactis* strains, and one *Streptococcus uberis* strain is a small antimicrobial peptide that inhibits the growth of a wide range of gram-positive bacteria, such as *Bacillus*, *Clostridium*, *Listeria* and *Staphylococcus* species. It is nontoxic to humans and used as a food preservative (E234) in more than 50 countries including the EU, the USA, and China. National legislations concerning maximum addition levels of nisin in different foods vary greatly. Therefore, there is a demand for non-laborious and sensitive methods to identify and quantify nisin reliably from different food matrices.

The horizontal inhibition assay, based on the inhibitory effect of nisin to *Micrococcus luteus* is the base for most quantification methods developed so far. However, the sensitivity and accuracy of the agar diffusion method is affected by several parameters. Immunological tests have also been described. Taken into account the sensitivity of immunological methods to interfering substances within sample matrices, and possible cross-reactivities with lantibiotics structurally close to nisin, their usefulness for nisin detection from food samples remains limited.

The proteins responsible for nisin biosynthesis, and producer self-immunity are encoded by genes arranged into two inducible operons, *nisA/Z/QBTCIPRK* and *nisFEG*, which also contain internal, constitutive promoters P_{nisI} and P_{nisR} . The transmembrane histidine kinase NisK and the response regulator NisR form a two-component signal transduction system, in which NisK autophosphorylates after exposure to extra cellular nisin, and subsequently transfers the phosphate to NisR. The phosphorylated NisR then relays the signal downstream by binding to two regulated promoters in the nisin gene cluster, i.e the *nisA/Z/Q* and the *nisF* promoters, thus activating transcription of the structural gene *nisA/Z/Q* and the downstream genes *nisBTCIPRK* from the *nisA/Z/Q* promoter, and the genes *nisFEG* from the *nisF* promoter.

In this work two novel and highly sensitive nisin bioassays were developed. Both of these quantification methods were based on NisRK mediated, nisin induced Green Fluorescent Protein (GFP) fluorescence. The suitabilities of these assays for quantification of nisin from food samples were evaluated in several food matrices. These bioassays had nisin sensitivities in the nanogram or picogram levels. In addition, shelf life of nisin in cooked sausages and retainment of the induction activity of nisin in intestinal chyme (intestinal content) was assessed.

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List of original publications

This thesis is based on the following publications:

- I **J. Reunanen & P. Saris. 2003.** Microplate bioassay for nisin in foods based on nisin induced GFP-fluorescence. *Applied and Environmental Microbiology* **69**(7): 4214-4218.
- II **J. Reunanen & P. Saris. 2004.** Bioassay for nisin in sausage; a shelf life study of nisin in cooked sausage. *Meat Science* **66**(3): 515-518.
- III **J. Hakovirta, J. Reunanen & P. Saris. 2006.** Bioassay for nisin in milk, processed cheese, salad dressings, canned tomatoes, and liquid egg. *Applied and Environmental Microbiology* **72**(2): 1001-1005.
- IV **J. Reunanen & P. E. J. Saris. 2007.** Shelf life of nisin in intestinal environment. Submitted to *Applied and Environmental Microbiology*.

The publications are referred to in the text by their roman numerals.

Abbreviations

aa	amino acid
ABC	ATP-binding cassette
Abu	aminobutyric acid
Amp	ampicillin
ATP	adenosine triphosphate
Cam	chloramphenicol
CD	circular dichroism
Dha	didehydroalanine
Dhb	didehydrobutyrine
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
e.g.	<i>exempli gratia</i> , for example
ELISA	enzyme-linked immunosorbent assay
Erm	erythromycin
et al.	<i>et alii</i> , and others
etc.	<i>et cetera</i> , and so on
GRAS	generally regarded as safe
i.e.	<i>id est</i> , that is
IU	international unit
LAB	lactic acid bacteria
Lan	lanthionine
LD	lethal dose
LF-NisI	lipid free NisI
MALDI-TOF	matrix assisted laser desorption ionization time of flight mass
MeLan	methyllanthionine
NMR	nucleomagnetic resonance
PC	phosphatidylcholine
PCR	polymerase chain reaction
PG	phosphatidylglycerol
PMF	proton motive force
RNA	ribonucleic acid
RP-HPLC	reversed phase high performance liquid chromatography spectrometry
RT-PCR	reverse transcriptase polymerase chain reaction
SDS	sodium dodecyl sulphate

Review of the literature

1. Bacteriocins

It has been estimated, that in the US there are 76 million cases of food-borne illness each year, of which some 5000 result in death (Mead et al., 1999). The annual cost of food-borne diseases related to *Campylobacter jejuni*, *Clostridium perfringens*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella enterica*, *Staphylococcus aureus*, and *Toxoplasma gondii* is between \$6.5 and \$34.9 billion (Buzby and Roberts, 1997). Hence, there is still a huge demand to improve current methods of food preservation and thus food safety. However, the increasing awareness of the adverse effects of chemical preservatives, such as salts and antibiotics, has created a request for more “natural” food. As a result, naturally produced antimicrobial compounds, especially those produced by bacteria, the bacteriocins, have received great attention from both the food industry and food scientists.

Bacteriocins are ribosomally synthesized peptides, that exert their antimicrobial activity against either strains of the same species as the bacteriocin producer (narrow range), or to more distantly related species (broad range). The first report of bacteriocins in scientific literature dates back to 1925, when Gratia described antagonism between different strains of *Escherichia coli*. The inhibiting substances were characterized as proteins and named colicins, indicating the species they were originally found from. Only three years later, at the same time as Fleming discovered penicillin, Rogers and Whittier (Rogers, 1928; Rogers and Whittier, 1928) published their observation that lactic streptococci inhibited the growth of other lactic acid bacteria. Five years later Whitehead (1933) isolated the inhibitory molecule and showed it to be proteinaceous. In 1947, this antagonistic peptide was named nisin, or “group *N* inhibitory substance”, the suffix “-in” denoting antibiotic properties (Mattick and Hirsch).

The usefulness of bacteriocins in food protection is due to several reasons: firstly, bacteriocins are natural substances, and as proteins they are biodegradable. Secondly,

Table 1. Comparison of bacteriocins versus antibiotics. Adapted from Cleveland et al., 2001.

Characteristics	Bacteriocins	Antibiotics
Application	Food	Clinical
Synthesis	Ribosomal	Secondary metabolite
Activity	Narrow spectrum	Varying spectrum
Producer immunity	Yes	No
Target cell resistance or tolerance	Usually adaptation affecting cell membrane	Usually a genetically transferable determinant
Interaction requirements	Sometimes docking molecules	Specific target
Mode of action	Mostly pore formation	Cell membrane or intracellular targets
Toxicity/side effects	None known	Yes

being ribosomally synthesized molecules, it is possible to modify the bacteriocins by genetic engineering. Thirdly, each different bacteriocin has its own unique and rather narrow killing spectrum, thus allowing manipulation of food microbial ecosystems. Fourthly, bacteriocins can be distinguished from antibiotics by several criteria that are outlined in Table 1.

1.1 Bacteriocins produced by lactic acid bacteria

Perhaps the most interesting bacteriocins are those produced by lactic acid bacteria (LAB). The LAB bacteriocins are natural ingredients found in virtually all fermented foods and dairy products, and have thus been consumed unknowingly by humans for thousands of years. Due to their origin, some LAB bacteriocins meet the legislations concerning food supplements and can be added to fermented foods in the form of producing starter cultures or culture medium filtrates.

The bacteriocins of LAB comprise a large and heterogeneous group of antimicrobial peptides and proteins, and their classification is under constant revision. In the most well known and accepted classification, presented in Table 2 and originally suggested by Klaenhammer (1993), the LAB bacteriocins are divided into three main groups, based on their amino acid sequence, mode of action, heat tolerance, biological activity,

Table 2. Classification of bacteriocins according to Klaenhammer (1993).

Class I.	Lantibiotics
	I A: nisin-like, elongated, screw-shaped, cationic molecules I B: duramycin-like, globular molecules with low net negative charge
Class II.	Non-lantibiotics
	II A: pediocin-like antilisterial bacteriocins II B: two-peptide bacteriocins
Class III.	Large heat-labile proteins

Table 3. Classification of bacteriocins suggested by Cotter et al. (2005).

Classification	Remarks	Examples
<u>Class I</u>		
Lanthionine containing bacteriocins/lantibiotics	Includes both single- and two-peptide lantibiotics	Single peptide: nisin Two peptide: lacticin 3147
<u>Class II</u>		
Non-lanthionine-containing bacteriocins	Heterogeneous group of small peptides	Pediocin PA1, lactococcin A
<u>Bacteriolysins</u>		
Non-bacteriocin lytic proteins	Large heat-labile proteins	Lysostaphin, enterolysin A

presence of modified amino acids, and secretion mechanism. The classes I and II are further divided into subgroups, and the members of these classes are the most studied because they are so widespread among the LAB and due to their heat stability. The class III bacteriocins are heat-labile and therefore less interesting in the terms of food processing and protection.

Quite recently a new classification has been proposed by Cotter et al. (2005). In this scheme the most dramatic change is the removal of class III bacteriocins to their own group of “bacteriolycins”, hence making the group of bacteriocins smaller and more strictly defined (Table 3).

1.2 Lantibiotics

Lantibiotics are polycyclic LAB bacteriocins having intra-chain sulphur bridges and unusual thioether amino acids, such as lanthionine (Lan) and β -methyllanthionine (β -MeLan) (*lanthionine containing antibiotics*, Schnell et al., 1988). The lantibiotics also often possess α,β -unsaturated amino acids, for instance didehydroalanine (Dha) and didehydrobutyrine (Dhb) (Kellner and Jung, 1989) (Figure 1). The unusual amino acids result from post-translational enzymatic modifications. Lantibiotics are ribosomally synthesized as inactive prepeptides, which contain an amino terminal leader peptide and a carboxy terminal propeptide, the prolantibiotic (Jung, 1991, Sahl et al., 1995). The leader peptide is proteolytically removed from the propeptide either inside the producing cell or during/after transport out of the cell. This proteolytical cleavage releases the mature, biologically active lantibiotic (McAuliffe et al., 2001).

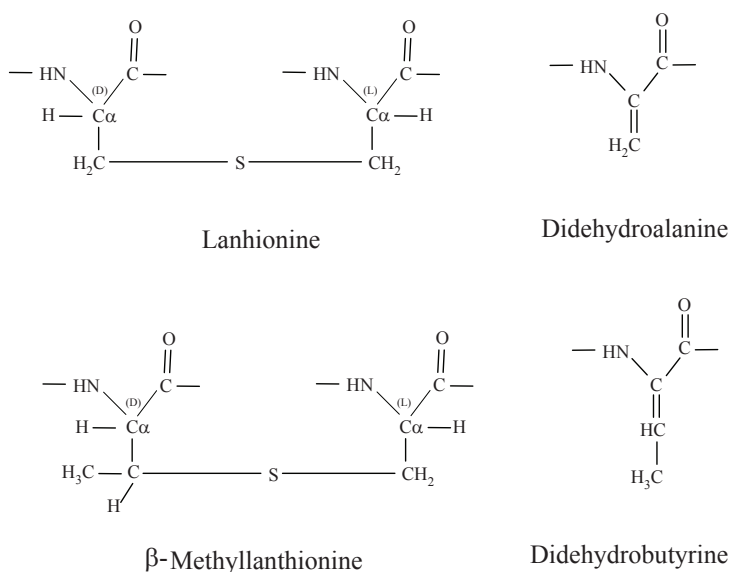


Figure 1. Amino acids characteristic for lantibiotics.

The lantibiotics have been divided into two separate groups according to their structure and mode of action, to the nisin-like (type A) and to the duramycin-like (type B). The type A lantibiotics are cationic, linear and screwed in shape, and they inhibit the growth of bacteria by depolarizing the cell membranes of target cells. Well known representatives of type A lantibiotics are nisin (Gross and Morell, 1971), subtilin (Gross and Kiltz, 1973), Pep5 (Kellner et al., 1989), epiderminin (Allgaier et al., 1986), and gallidermin (Kellner et al., 1988). The type B lantibiotics are globular molecules and have a low negative net charge (Klaenhammer, 1993). The peptides of this group act as enzyme inhibitors, and include among others mersacidin and actagardine (Ross et al., 2002).

To date, approximately 50 lantibiotics are known, and their number continues to grow (Patton and van der Donk, 2005). The DNA sequences for the regulons containing the structural genes have been characterized for many lantibiotics. The lantibiotic regulons have been found to enclose several conserved genes, and it is supposed, that the translation products of these conserved genes perform same activities in the biosynthesis of different lantibiotics. In 1995 de Vos et al. constructed a common nomenclature for these gene products, which include: i) the prepeptide LanA (e.g. NisA and Pep5) ii) enzymes LanM or LanB and LanC responsible for post-translational modifications iii) the LanP that proteolytically cleaves the leader peptide iv) the LanT, transfers the lantibiotic out of the cell v) regulators of biosynthesis, LanK and LanR vi) the mediators of immunity, LanI and LanFEG (de Vos et al., 1995).

2 Nisin

Not only is nisin the best understood and most thoroughly characterized lantibiotic, but it is also the first lantibiotic mentioned in the scientific literature. Nisin is non-toxic to humans and animals; its toxicity was comparable to that of a common table salt, when orally administrated to rats (LD_{50} 7 g/kg body weight) (Hurst, 1981). Of the known lantibiotics, nisin still is the only one with substantial industrial use. In fact, to date nisin is the only lantibiotic allowed as a food supplement. In 1969 the FAO/WHO Expert Committee on Food Additives stated nisin to be safe and natural food additive (FAO/WHO, 1969). Some fifteen years later nisin was commercially used in at least 39 countries (Hurst, 1983). In 1983 nisin was incorporated to the EEC food additive list and given the designation E234 (EEC, 1983). In the US, the Food and Drug Agency gave nisin a GRAS status (Generally Regarded As Safe) in 1988 (Federal Register, 1988). By the year 1996 nisin was allowed as a food additive in more than 50 countries, including the EU, China, and the US (Delves-Broughton et al., 1996).

Many gram-positive bacteria are sensitive to nisin, but on the growth of gram-negative bacteria, yeast, or fungi nisin has little or no effect (De Vuyst and Vandamme, 1994). Especially susceptible to nisin are the gram-positive spore-forming bacteria, such as species of *Bacillus* and *Clostridium*, whose spores are even more vulnerable to nisin than the vegetative cells (De Vuyst and Vandamme, 1994). As a result, nisin is mostly used as a preservative in heat sensitive foods, which instead of sterilization can only be pasteurized, as pasteurization is ineffective in inactivation of bacterial spores. Nisin is

also utilized to prevent the growth of undesirable LAB in acidic foods that can not be heat sterilized, for instance in salad dressings, beers, ciders, and wines. Furthermore, as yeasts are insensitive to nisin, it can be applied during alcoholic fermentation to control the growth of spoilage LAB. Also, Abee et al. (1994) have shown nisin to be toxic for *Listeria monocytogenes* grown at +4 °C. *L. monocytogenes* is a common pathogen in dairy products, and its ability to grow at low temperatures makes it difficult to suppress its outgrowth. Moreover, *L. monocytogenes* infection can be fatal for infants, pregnant, and elderly people.

2.1 Structure and chemical characteristics

The molecular weight of nisin is approximately 3350 Daltons, depending on the variant (see section 2.1.1). Nisin is soluble and highly stable at acidic solution; at aqueous solution of pH 2, the solubility of nisin is 57 mg/ml, and nisin retains its biological activity even if it is autoclaved (Hurst, 1981). However, at alkaline pH the solubility decreases dramatically and nisin becomes biologically inactive, probably due to chemical modifications (Liu and Hansen, 1990).

2.1.1 Primary structure

To date, four natural nisin variants have been described: nisin A (Gross and Morell, 1971), nisin Z (Graeffe et al., 1991; Mulders et al., 1991), nisin Q (Zendo et al., 2003), and nisin U (Wirawan et al., 2006). With the exception of nisin U, which is produced by *Streptococcus uberis*, all the other nisin variants are produced by *Lactococcus lactis*.

Nisins A, Z, and Q comprise 34 amino acids, of which 8 are posttranslationally modified. These mature molecules each contain one lanthionine, four methyllanthionines, two didehydroalanines, and one didehydrobutyrine (Gross and Morell, 1971; Graeffe et al., 1991; Mulders et al., 1991; Zendo et al., 2003). The lanthionines form five ring structures (designated as rings A, B, C, D, and E), and two of these rings (D and E) are fused together to establish a double ring structure. The structure of nisin U is essentially the same, though it is composed of 31 amino acids and has only one didehydroalanine but two didehydrobutyrines instead (Wirawan et al., 2006).

The differences in amino acid sequences of nisin variants are shown in Figure 2.

2.1.2 Three dimensional structure

Several research groups have studied the three dimensional structure of nisin during the late 1980's and early to mid 1990's using H-resonance NMR-spectroscopy (nucleomagnetic resonance) and CD-spectroscopy (circular dichroism). In 1989 three groups reported their results: Slijper et al. used water whereas Chan et al. both water and dimethylsulfoxide (DMSO) as a solvent. Palmer et al. focused on studying chemically synthesized, individual nisin rings A and B in DMSO. The biological target for nisin, however, is the cell membrane (Henning et al., 1986; Kordel and Sahl, 1986; Ruhr and Sahl, 1985); thus the conformation of nisin has also been examined in models mimicking biological membranes (van den Hooven et al., 1996)

The structure of nisin is rather elastic, and therefore the molecule does not possess a well defined tertiary structure. Instead, nisin can be depicted to consist of two amphipathic domains, that both contain several secondary structures, and a flexible hinge domain or

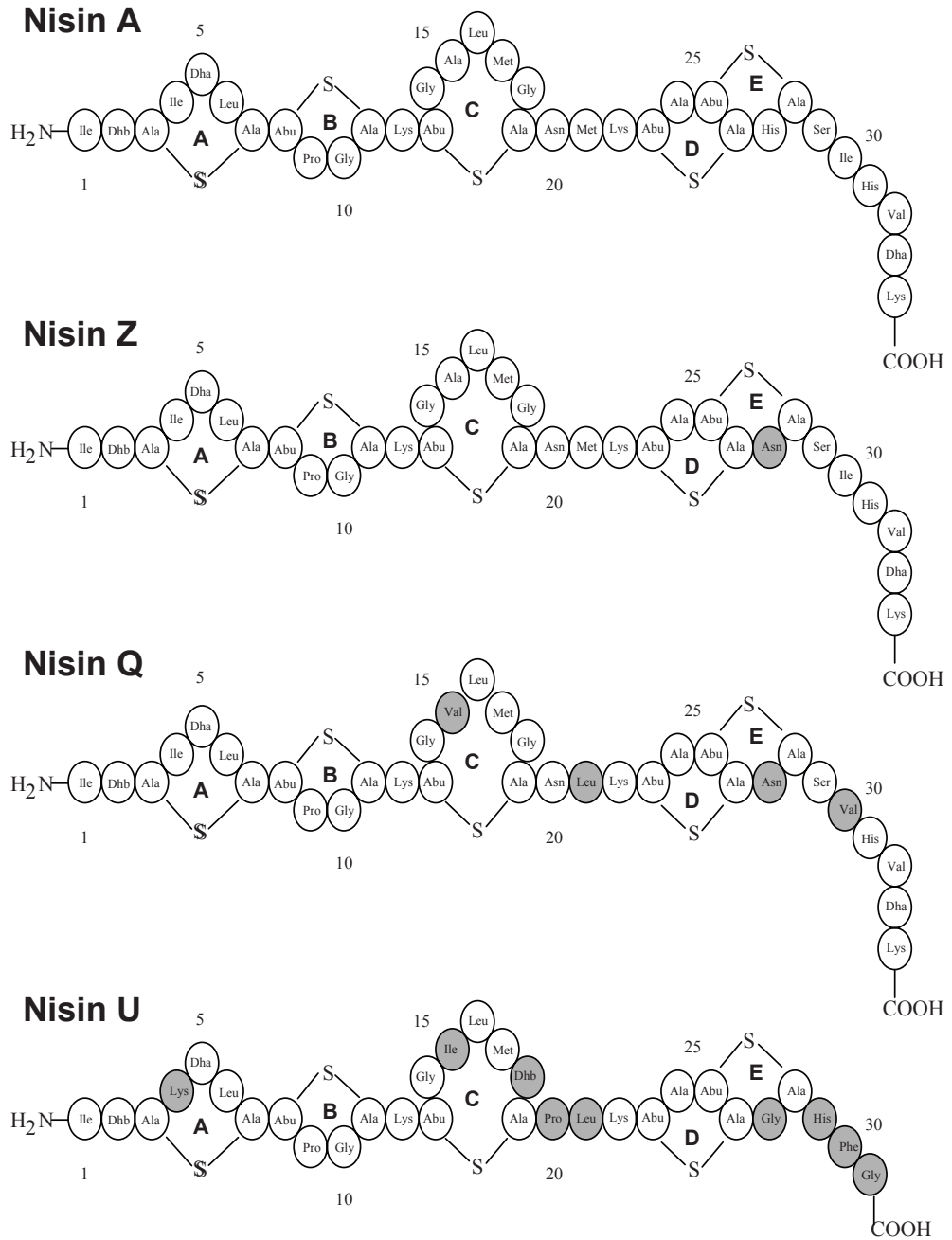


Figure 2. Primary structures of natural nisin variants. Dha, didehydroalanine; Dhb, didehydrobutyrine; Ala-S-Ala, lanthionine; Abu-S-Ala, methyllanthionine. The amino acids differing from those in nisin A are shaded in gray.

region connecting them. The amino terminal domain is composed of the lanthionine rings A, B, and C, and the carboxyl terminal domain is formed of the intertwined lanthionine rings D and E. The four-residue rings B, D, and E are in the β -turn configuration, which is fixed by the thioether bond in the lanthionine structures. Of these, the rings B and D appear to be type II β -turns, whereas ring E resembles type I β -turn. The amino acids preceding ring D (residues 21 and 22) form a type II β -turn together with residues 23 and 24 of the ring D, and this β -turn is the only secondary structure outside the lanthionine rings. Accordingly, the carboxyl terminal domain is composed of three consecutive β -turns (van den Hooven et al., 1996). The rings A and C have shown substantial variation in structural studies, and thus they can be considered lacking secondary conformations. The three dimensional structure of nisin is very similar in water and in micelles; the only observed conformational difference is located to the ring A: in water the amide proton of didydroalanine (residue number 5) is oriented outward from the centre of the ring, and is thus in contact with water; in micelles this same proton is projecting inward the centre of ring A. This difference results from a 180° change in the bond angle of both peptide bonds in the didydroalanine (van den Hooven et al., 1996). Nisin is a water soluble molecule, but obviously it also has to be able to bind to cell membranes. It has been shown in micellar systems, that the didydroalanine and leucine of ring A (residues 5 and 6) will insert themselves to the lipid phase (van den Hooven et al., 1996). Thus, the energetically most favourable conformations of the ring A differ considerably in water and lipid phases.

The both domains of a nisin molecule are amphipathic: in the amino terminal domain the hydrophilic lanthionines and the side chain of Lys12 are located on the same face of the domain, whereas the hydrophobic residues Ile4, Dha5, Leu6, Ala15, Leu16, and Met17 are situated on the opposite side. Analogously, in the carboxyl terminal domain the side chains of positively charged amino acids (Lys22 and His27) can be found from the opposing face as compared to the location of the hydrophobic Met21 and Ala24. Nisin is also amphipathic in a second respect: the charged and hydrophilic residues mainly reside on the carboxyl terminal domain, while there is only one charged residue on the amino terminal domain, the rest of the residues being mainly hydrophobic (van den Hooven et al., 1996).

2.2 Nisin genes and biosynthesis of nisin

Nisin is synthesized at ribosomes as an inactive prepeptide composed of two domains: the leader peptide that retains the molecule inactive, and the propeptide, of which the biologically active nisin is enzymatically formed from. In generic nomenclature, the prenisin is also often called prepeptide. The nisin prepeptide contains 57 amino acids, of which the first 23 amino terminal residues form the leader peptide. The leader peptide is charged and hydrophilic, and contains a FNLD consensus sequence; van der Meer et al. have shown, that even conservative point mutations in the this region abolish the formation of mature nisin (1994). The scheme for chemical pathway leading to formation of dehydroamino acids and lanthionine structures was originally proposed by Ingram in 1969. However, not until very recently have the enzymes involved in and the molecular details of nisin biosynthesis been unravelled.

2.2.1 Nisin regulon

The genes needed for biosynthesis of nisin variants A, Z, and Q and producer self immunity are organised as regulons composed of two nisin inducible operons, i.e. *nisA/Z/QBTCIPRK* and *nisFEG* (de Ruyter et al., 1996; Kuipers et al., 1995). The operon *nisA/Z/QBTCIPRK* contains an internal constitutive *nisRK* promoter (de Ruyter et al., 1996). Quite recently there was an observation, that *nisI* mRNA could be detected without nisin induction, thus suggestin that the *nisA/Z/QBTCIPRK* operon might contain another internal and constitutive promoter (Li and O'Sullivan, 2006). The *nsu* genes in nisin U producing *Streptococcus uberis* are arranged somewhat differently, i.e. *nsuPRKFEGABTCI* (Wirawan et al., 2006). Nisin regulons are located on large transposons (~70 kb), which also contain the genetic determinants of sucrose metabolism. Examples of these transposons include Tn5276 (Rauch and de Vos, 1992), Tn5301 (Dodd et al., 1990), Tn5307 (Buchman et al., 1988), and Tn5481 (Immonen et al., 1998). The general structure of a nisin A/Z/Q regulon is presented in Figure 3.

2.2.2 NisB

NisB is a hydrophilic protein, which also contains several amphipathic α -helices. It resides on the cell membrane, but it is not clear whether the membrane association is integral or peripheral (Engelke et al., 1992). It has been long assumed, that NisB has a pivotal role in the post-translational modification reactions. The first report supporting this assumption came, when Engelke et al. showed (1994), that no active nisin appeared before NisB could be detected. Just two years later Siegers et al. published their article entitled “*Biosynthesis of lantibiotic nisin- Posttranslational modification of its prepeptide occurs at a multimeric membrane- associated lanthionine synthetase complex*” (1996). In this publication, which can be regarded as a landmark paper that started the era of resolving the questions of nisin biosynthesis, they showed among other things the interaction of prenisin with NisB by the yeast two-hybrid system and co-immunoprecipitation. Later on, the loss of nisin production or synthesis of unmodified prenisin was demonstrated in studies with *L. lactis* strains with inactivated NisB genes (Ra et al., 1999; Koponen et al., 2002). In parallel, dehydrated prenisin has been produced in strains expressing the *nisABT* (Kuipers et al., 2004). Furthermore, with *L. lactis* strains engineered to produce nisin mutants only half of the Ser33 had been dehydrated to Dha33; sole over expression of NisB was sufficient to reverse the phenotypes of these strains to produce fully dehydrated, mature nisin (Karakas, 1999). Finally, in 2007 Cheng et al. successfully

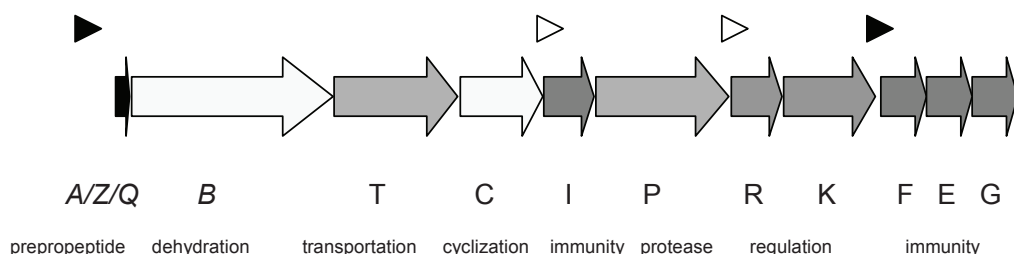


Figure 3. Schematic presentation of nisin A/Z/Q regulon. Filled triangles denote nisin inducible promoters and open triangles constitutive ones.

reconstituted nisin biosynthesis *in vitro* by applying PCR-products of *nisA*, *nisB*, and *nisC* to an *E. coli* rapid transcription/translation system (Roche), followed by trypsin treatment to cleave the leader peptide. The outcome of this *in vitro* reaction was fully mature, biologically active nisin, and thus the activity of NisB was undoubtedly shown to be the dehydration of serine and threonine to didehydroalanine and didehydrobutyrine, respectively (Cheng et al., 2007).

2.2.3 NisC

In 1996 Siegers et al. showed by yeast two-hybrid system and co-immunoprecipitation that prenisin interacts with NisC. In this study the authors also demonstrated by immunoblot analysis the membrane association of NisC, and that disruption of the *nisC* gene abolishes nisin production. In addition, it was observed that a whole prenisin molecule was needed for the recognition of prenisin by NisC, since neither the leader peptide nor the propeptide alone was able to be bound by NisC. It was not until 2002, when Koponen et al. purified His-tagged nisin precursor from a *L. lactis* strain with inactivated *nisC* gene. This strain had an intact *nisB* gene, and the purified nisin precursor was shown to be dehydrated but devoid of lanthionine structures. Furthermore, the investigators were able to restore the production of biologically active nisin by complementing the *nisC* deficient strain with a NisC producing plasmid. One year later NisC was identified as a zinc protein, and it was suggested, that the metal atom might activate cysteine thiols of the dehydrated prenisin toward intramolecular Michael addition to the didehydroalanines and didehydrobutyrines (Okeley et al., 2003). Very recently, Li et al. (2006) produced NisC heterologously in *E. coli*, and incubated the purified NisC together with dehydrated prenisin; the resulting reaction mixture was subsequently treated with trypsin in order to remove the leader peptide, and the final product proved to be biologically active nisin. Moreover, when the leader peptide was cut off before NisC reaction, no mature nisin was produced, confirming that the leader peptide is a necessity for the interaction of nisin precursor with NisC, as originally suggested by Siegers et al. (1996). Therefore, it can be stated that this *in vitro* reconstitution of NisC activity together with the results of Cheng et al. (2007) closes the ring concerning the formation of nisin's lanthionine structures.

2.2.4 NisT

The gene encoding NisT was first described by Engelke et al. in 1992. From the gene the researchers deduced the protein product to be a 600 amino acid protein sharing homology with several ATP-dependent transport proteins. Four years later Siegers et al. (1996) showed an interaction between the carboxyl terminal domain of NisT and NisC with yeast two-hybrid system. They also suggested, that at least two NisT molecules form a complex, which is in agreement with the general view of bacterial ABC-transporters (Higgins, 1995). At the same year, Qiao and Saris demonstrated, that a mutant *L. lactis* with a deletion in the *nisT* gene did not secrete nisin. However, upon lysis of these cells nisin could be detected. Furthermore, complementation of this strain with a NisT encoding plasmid was sufficient to restore the nisin secreting phenotype. In general, the bacterial type ABC-transporters are assumed to have stringent substrate specificity. This,

however, does not hold the truth with NisT: Kuipers et al. have quite recently shown, that NisT can export not only fully modified prenisin, but also unmodified as well as dehydrated prenisin. Moreover, and most surprisingly, they also established that even non-lantibiotic peptides can be secreted by NisT, given these peptides are fused to the nisin leader peptide (Kuipers et al., 2004).

2.2.5 NisP

NisP was first mentioned in 1993, when van der Meer et al. cloned and partially characterized a 12 kb DNA fragment of the conjugative transposon Tn5276. One of the open reading frames found was shown to encode for a protein sharing similarities with the subtilisin-like serine proteases, which all possess an amino terminal secretory signal sequence, a potential signal peptidase cleavage site (proposed to be between Gly-22 and Glu-23 in the case of NisP), a catalytic site, and an LPXTG consensus sequence commonly found in the carboxyl terminus of cell surface proteins of Gram-positive bacteria (Siezen, 1999). Van der Meer and colleagues, however, did not restrict their studies to the DNA level: they also cloned and over expressed NisP in *E. coli*, and were able to show that a cell extract of this *E. coli* cleaved purified nisin precursor, thus liberating mature, biologically active nisin. Furthermore, parallel activity was observed with whole cells of *L. lactis* containing Tn5276, from which the *nisA* gene had been inactivated, whereas membrane-free cell extract of this strain did not produce active nisin, suggesting evidence for membrane location of NisP, as predicted from sequence analysis (van der Meer et al., 1993). Similar results were obtained by Qiao et al. (1996) with a *L. lactis* strain deficient in *nisP* gene.

To provide insight for the biological role of the leader peptide, Kuipers et al. (1993) fused the leader peptide sequence of subtilin, the structurally closest lantibiotic analogue of nisin, to the propeptide sequence of nisin Z. This hybrid was expressed in nisin A producing *L. lactis* strain, and a simultaneous production of nisin A and an approximately 6 kDa sl-nisin Z (derived from 'subtilin-leader/nisin Z hybrid protein) was observed. The secreted sl-nisin Z was at least 200-fold less efficient in antimicrobial activity compared to nisin A. These findings implied, that i) NisP is protease with (rather narrow) specificity to prenisin ii) the function of the leader peptide is (at least) to keep the fully modified prenisin inactive.

The importance of specific amino acids in nisin prepeptide for NisP function on the close vicinity of the cleavage site was shown in another survey: changing the Arg⁻¹ to Gln (as was the case with sl-nisin Z) or Ala⁻⁴ to Asp led to the production of fully modified prenisin with leader peptide still attached. Furthermore, even conservative point mutations in the strongly conserved FNLD lantibiotic leader peptide region (residues -18 to -15 in prenisin) resulted in a total loss of biosynthesis of nisin or its precursors (van der Meer et al., 1994). This indicates that the leader peptide has a function not only to keep the modified prenisin inactive prior secretion, but also during early biosynthetic steps, as suggested by Siegers et al. (1996).

The prerequisites on the propeptide side of prenisin for NisP activity are largely unknown. However, neither unmodified nor dehydrated prenisin can be cleaved by NisP, as shown by Kuipers et al. (2004). This proposes, that one or more lanthionine rings are essential for NisP catalysed cleavage.

2.2.6 Regulation of biosynthesis

According to the generally accepted view, the proteins NisR and NisK form a two-component signal transduction system, in which the histidine kinase sensor NisK is located in the cytoplasmic membrane. The extracellular nisin binds to the NisK, which upon interaction with nisin autophosphorylates at a conserved His-238 residue. Subsequently, the phosphate moiety is transferred from NisK to the NisR, which in its activated state acts as a transcriptional activator, thus leading to the transcription of the genes downstream from the nisin-inducible promoters, namely the *nisA/Z/Q/U* and the *nisF* promoters.

The *nisR* gene was first identified in 1993 by van der Meer and colleagues, and the predicted NisR amino acid sequence shared similarity with the family of transcriptional regulatory proteins, members of two-component signal transduction systems. In addition, the researchers demonstrated that disruption of the *nisR* gene abolished the production of nisin precursor. Interestingly, when the direction of the *nisR* gene in the plasmid was inverted, the prenisin producing phenotype was retrieved (van der Meer et al., 1993). The missing counterpart, the sensory histidine kinase NisK was discovered one year later by Engelke et al. (1994).

It had for a while been known, that an intact structural *nisA* gene is a prerequisite for nisin biosynthesis, since Kuipers et al. had shown (1993), that a 4 bp deletion in the middle of the *nisA* gene (Δ *nisA*) halted transcription of the gene Δ *nisA*. The role for nisin as an inducer molecule of its own biosynthesis started to emerge, when the same group noted that adding sublethal concentrations of nisin to the growth medium recovered Δ *nisA* transcription. By Northern blotting they showed, that the level of transcription was dependent on the amount of nisin added, and that no Δ *nisA* transcript could be detected when the *nisK* gene had been inactivated (Kuipers et al., 1995). Next, the researchers studied whether mutated nisin analogues and several lantibiotics other than nisin possessed this induction activity. The outcome was, that nisin mutants retained varying (or even increased) levels of inductory capacity, whereas other lantibiotics, i.e. subtilin, lactacin 481, and Pep5 failed to initiate Δ *nisA* transcription. In order to figure out, whether the NisRK system could be used to produce heterologous proteins in *L. lactis*, and to attain a more quantitative assay system, the group fused the *gusA* gene from *E. coli* to the *nisA* promoter (P_{nisA}). The results were similar to those obtained with Δ *nisA* by Northern blotting. And finally, Kuipers et al. studied in this landmark survey the structural requirements for nisin to elicit the β -glucuronidase activity via the NisRK pathway: by using synthetic nisin A fragments, the essential part of nisin molecule to gain minimal induction capacity was shown to reside in the amino acids 1 to 11, which form the nisin rings A and B (2 % induction compared to nisin A). Supplementation with the third ring (ring C) enhanced induction to 8-30 %, whereas fragments composed of rings B and C or D and E totally lacked the induction capability. Furthermore, a severe or total loss of induction was established, when the amino terminal Ile-1 and didehydrobutyrine-2 were not present in synthetic nisin fragments (Kuipers et al., 1995). Therefore, the most probable site in nisin A for interaction with NisK resides in the residues 1 to 11.

At the same year, Siegers and Entian reported of another, possibly nisin-inducible promoter upstream of the gene *nisF* in the nisin regulon (1995). However, they did not offer any experimental data. The next year Ra et al. (1996) published a study, in which

they showed by RT-PCR and Northern blots that the genes of the nisin biosynthetic machinery are arranged as a regulon composed of two nisin inducible operons, the *nisZBTCIPRK* and *nisFEG*. Similar conclusions were drawn by de Ruyter et al. (1996), who fused the *gusA* gene to the *nisF* promoter (P_{nisF}), and to the *nisR* promoter (P_{nisR}) recently described by Kuipers et al. (1995). They showed the P_{nisF} to be nisin-inducible, though less efficient in transcription initiation than the P_{nisA} , whereas the P_{nisR} turned out to function in a constitutive manner (de Ruyter et al., 1996).

However, no direct in vitro evidence for neither interaction of nisin with NisK nor binding of the phosphorylated NisR to nisin inducible promoters P_{nisA} and P_{nisF} has been published to date.

2.3 Antimicrobial mechanisms

Most of the research done this far has been concentrated on the ability of nisin to form pores to membranes. The process of pore formation triggers a rapid efflux of small molecules and metabolites, e.g. ions, nucleotides, and amino acids, and dissipates the proton motive force (PMF), thus ceasing all cellular biosynthetic processes (Ruhr and Sahl, 1985). The dispersion of small molecules, however, was only attained with whole cells and membrane vesicles prepared from nisin susceptible bacteria: nisin could not affect the integrity of liposomes made of L- α -phosphatidylcholine from soybeans. Based on this observation the authors stated a hypothesis, which was verified not earlier than some 15 years later: “*The inability of these peptides (nisin, Pep5, and colicin V) to influence soybean phospholipid vesicles could point to a need for an integral membrane component which could serve as a mediator for nisin binding to membranes and which is lacking in nonbacterial membrane extracts. In this respect, the murein precursors could facilitate nisin interaction with the cytoplasmic membrane resulting in the membrane disintegration demonstrated by our results.*” (Ruhr and Sahl, 1985).

Several mutually controversial studies of the pore formation mechanism of nisin were published in the middle of the 1990's. The first report came from Driessen et al., who analyzed the nisin induced release of 6-carboxyfluorescein from phosphatidylcholine (PC) and phosphatidylglycerol (PG) vesicles, and concluded that nisin induces the release of 6-carboxyfluorescein and other fluorescent anionic dyes from zwitterionic PC liposomes, but not from anionic PG vesicles (Driessen et al., 1995). In contrast, in January 1996 Demel et al. published their lipid monolayer study, in which both nisin A and nisin Z showed high affinity to anionic lipids, whereas little or no interaction was observed with zwitterionic lipids. In addition, their results indicated, that it is primarily the amino terminal part of the nisin molecule (residues 1-22) that penetrates into the lipid phase (Demel et al., 1996). Just six month later, Martin et al. published their paper describing an interaction of a nisin variant (Ile-31 to Trp) with vesicles differing in lipid compositions (Martin et al., 1996). The conclusions were in severe disagreement with the previous study, since Martin et al. demonstrated that, it is the carboxyl terminal which enters the lipid phase. Eventually, Breukink et al. (1998) analysed the orientation of nisin in membrane systems composed of DOPC (zwitterionic) and DOPG (anionic) in different ratios. In this study three unique nisin mutants were used, having tryptophan residues at positions 1, 17, and 32, respectively. The authors suggested a somewhat consensus orientation for nisin in membranes, i.e. nisin appeared to adopt an overall

parallel orientation in the membrane with respect to the membrane surface. Furthermore, both ends of the nisin molecules were observed to insert to the lipid phase, the depth of penetration depending on the amount of negatively charged lipids (Breukink et al., 1998). However, the question of why micromolar concentrations of nisin were needed to induce leakage in artificial membrane systems, whereas only nanomolar nisin concentrations were sufficient enough to kill viable nisin sensitive bacteria remained unanswered.

As early as 1973 Linnet and Strominger described an *in vitro* inhibition of cell wall synthesis by nisin, and it was seven years later shown to be due to the formation of complexes between nisin and cell wall precursors lipid I and lipid II (Reisinger et al., 1980). Nevertheless, for some reason these observations were disregarded for some twenty five years, despite the above mentioned hypothesis presented by Ruhr and Sahl in 1985. It was not until 1998, when Brötz et al. showed, that lipid II functions as a specific docking molecule for nisin and facilitates pore formation. However, the magnitude by which lipid II reduces the amount of nisin needed for pore formation, from micromolar to nanomolar concentration, was resolved by Breukink et al. one year later (1999). They also showed, that nisin has a high affinity for lipid II, since only one lipid II molecule per 1500 phospholipid molecules in liposomes was enough to markedly increase the nisin induced leakage in model membranes. The researchers also revealed, that the degree of pore formation was related to the lipid II concentration in the membranes in the range of 0,001 to 0,1 % of total lipid content, thus explaining the diverse sensitivities of nisin susceptible bacteria (Breukink et al., 1999). To gain insight to the nisin/lipid II recognition, Hsu et al. (2002) studied this interaction in SDS micelles with high-resolution NMR spectroscopy, and observed large chemical shift perturbations in the nisin rings A and B, whereas the carboxyl terminal part of nisin remained unaffected, suggesting the interaction to involve the amino terminal part of the nisin molecule (Hsu et al., 2002). At the same year van Heusden et al. published their paper, in which the topology of nisin in lipid II containing membranes was analysed with site-directed tryptophan spectroscopy (using the same tryptophan nisin mutants as Breukink et al., 1998): according to the results, the researchers proposed lipid II to induce a change in the orientation of nisin from parallel to perpendicular with respect to the membrane surface. They also observed, that the amino terminus of nisin resided in close vicinity of the lipid II head group, whereas the carboxyl terminus was most probably located near the interface between the acyl chain region and the lipid II headgroups (van Heusden et al., 2002).

Now it was shown that lipid II not merely functions as a docking molecule, but that it also orientates nisin to a membrane crossing direction. However, the definite composition of the pore assembly still remained unknown. To unravel this question, Breukink et al. used pyrene labelled lipid II and demonstrated by fluorescence measurements, that lipid II actually is an integral part of the nisin pore (Breukink et al., 2003). In this article, the authors suggested the first described model for stabile membrane penetrating pores composed of five to eight nisin molecules and an identical number of lipid II. A refined picture of the pore structure was gained by means of pyrene fluorescence and circular dichroism measurements conducted by Hasper et al. in 2004. In this study it was shown, that the nisin/lipid II pores are uniform in structure and consist of eight nisin and four lipid II molecules. The authors also verified the pores to be remarkably stable, since

destruction of the membranes with mild detergents such as Tween 20 left the pores intact, even after overnight incubation. In addition, the researchers also showed, that the hinge region of nisin is a necessity for the assembly of pore complexes (Hasper et al., 2004).

The pore forming mechanism of nisin with lipid II was thus resolved, but the early observation in lantibiotic literature that nisin terminates cell wall synthesis still remained without attention. In 1973 Linnet and Strominger wrote: “*Nisin inhibited synthesis of both lipid intermediate and peptidoglycan. There is no previous example of an antibiotic which does that, although some detergents do. Nisin could be an inhibitor of one of the early steps in peptidoglycan synthesis.*” Seven years later Reisinger et al. (1980) stated: “*Nisin inhibits murein biosynthesis with concomitant accumulation of undecaprenyl-pyrophospho-MurNAc (pentapeptide) (lipid intermediate I). This inhibition is caused by the formation of a complex between the antibiotic and lipid intermediate I. Undecaprenyl-pyrophospho-MurNAc (pentapeptide)-GlcNAc (lipid intermediate II) also forms a complex with nisin.*” Even with these hints in the literature, the interest in the role of nisin in cell wall synthesis sequestration was restored only after the identification of a baseball glove-like structure formed by the nisin rings A and B, which binds the pyrophosphate of lipid II (Hsu et al., 2004). Recently, a novel mechanism by which nisin (and several other lantibiotics) kills gram-positive bacteria, was described: in this study Hasper et al. (2006) showed, that nisin removes lipid II from its functional sites, i.e. from the sites of cell wall synthesis, and therefore blocks cell division. Thus, nisin can be considered as a molecule with dual lipid II -mediated killing functions, one acting via permeabilization of the cell membrane, and the other by cessation of cell wall synthesis.

2.4 Immunity

The first nisin immunity determinant to be described was the *nisI* gene from the nisin-sucrose transposon Tn5276 (Kuipers et al., 1993). The deduced amino acid sequence of NisI shared no similarity to any known proteins in the databases. However, a putative lipoprotein signal sequence could be predicted from the carboxyl terminal sequence of the NisI, suggesting NisI to be a peripheral membrane protein located at the outer membrane leaflet. Expression of NisI in a nisin sensitive *L. lactis* strain MG1614 led to a small but significant level of nisin immunity, proposing a role for NisI in immune development in nisin producing cells (Kuipers et al., 1993). Engelke et al. ended up with same conclusions with practically identical experimentation (1994). One year later Qiao et al. (1995) experimentally demonstrated the membrane localization of the NisI, and in 1999 Ra et al. showed, that an *L. lactis* with an in-frame deletion on the *nisI* gene had a markedly reduced nisin immunity compared to that of a wild type strain (Ra et al., 1999). Few years later Stein et al. reported the interaction between nisin and NisI produced heterologously in *E. coli* (2003). In 2004 Koponen et al. noted, that approximately 50 % of NisI could be found from culture supernatant and thus had been escaped from the lipid modification system. To study the role of the secreted NisI the same group expressed lipid-free NisI (LF-NisI), and showed it to provide a nisin sensitive *L. lactis* with immunity, although at low level (Takala et al., 2004). In this paper it was discussed, that an immunity protein of a bacteriocin producer might provide protection either by binding to the bacteriocin, or by binding to a cellular molecule required for bacteriocin activity, thus blocking the interaction between the particular molecule and the bacteriocin

(Takala et al., 2004). Recently, Takala et al. made serial deletions to the carboxyl terminus of the NisI, ranging from -5 to -74 amino acids. A 21 amino acid deletion was found to result into approximately 85 % loss of immunity, whereas deletions larger than this did not reduce nisin tolerance any further (Takala et al., 2006). This finding *per se* suggested the nisin binding site to be located at the carboxyl terminus of NisI, but the researchers went further and deleted a 21 aa fragment from the carboxyl terminus of the subtilin-specific immunity lipoprotein SpaI, and replaced it with the 21 aa fragment from NisI. The hybrid protein SpaI'-NisI was then expressed in nisin sensitive *L. lactis*, and a concomitant increase in nisin immunity was observed (Takala et al., 2006). This was the first time, when a lantibiotic immunity mediator was moved to another protein.

The genes required for nisin synthesis, however, are located in conjugative transposons that are horizontally transferred to new cells. Furthermore, the operons *nisA/Z/QBTCIPRK* and *nisFEG* require extracellular nisin to initiate their transcription. This raises the question of, how sufficient levels of immunity can be predisposed before the recipient cells encounter nisin in environment. Very recently this enigma was resolved by Li and O'Sullivan, who identified an internal promoter upstream of the *nisI* gene, and showed it to activate transcription of *nisI* independently of nisin in both *Enterococcus* sp. and *L. lactis* strains (2006).

However, since the first articles describing the role of NisI in nisin immunity it was clearly evident, that there must be additional tolerance mechanism(s), since expression of NisI in nisin sensitive *L. lactis* strains could not provide the cells with levels of immunity comparable to that of wild-type nisin producers (Kuipers et al., 1993; Engelke et al., 1994). Reversibly, in-frame deletion of NisI was not sufficient to turn the phenotype into nisin vulnerable (Ra et al, 1999). Three more genes were characterized from the nisin gene cluster in 1995 by Siegers and Entian, namely the *nisFEG*. The homology analyses of the deduced amino acid sequences suggested the NisFE proteins to belong to the family of ATP-binding cassette (ABC) transporters, whereas the hydrophobic NisG shared similarity with immunity proteins described for colicins. Disruptions in either *nisF*, *nisE*, or *nisG* gene led to reduced nisin immunity (Siegers and Entian, 1995). This study, however, suffered from non-scientific reporting, since it was impossible to reason from the manuscript whether the gene disruptions made were in-frame or out-frame, and thus the possible polar effects cannot be ruled out. More trust-worthy experimental evidence supporting the assumption that NisFE might function as a membrane transporter came from the study of Immonen and Saris (1998), in which heterologously expressed NisF was found exclusively from the membrane fractions in *E. coli*. Furthermore, production of antisense-RNA to *nisG* and *NisEG* clearly diminished immunity in nisin producing strain N8 (Immonen and Saris, 1998). The role of NisFEG has been most thoroughly examined by Stein et al. (2003), who expressed NisFEG and NisI in different combinations in *Bacillus subtilis*. Highest level of immunity was attained, when all the four immune proteins (NisIFEG) were produced simultaneously. Takala et al. obtained similar results with secreted LF-NisI in NisFEG expressing *Lactococcus lactis* (2004). In addition, in peptide release assays nisin was found to be cell associated in NisI strain of *B. subtilis*, whereas with NisIFEG or NisFEG cells the amount of cell associated nisin was clearly decreased and the quantity of nisin in supernatant was increased. Therefore, a role in transporting nisin out of the cells was suggested for NisFEG (Stein et al., 2003).

Furthermore, in these peptide release studies more than 90 % of the added nisin could be recovered, as assayed by RP-HPLC and MALDI-TOF, thus indicating that nisin immune system does not act via degradation of nisin.

In conclusion, four genes related to nisin tolerance have been described, but a profound insight of how these proteins contribute to nisin immunity separately and in concert, remains still to be clarified. It seems, that NisI has a slightly dominant role in providing nisin producers with tolerance, since disruption of the *nisI* gene reduced immunity approximately by 80 % (Kuipers et al., 1993), suggesting that the NisFEG accounts only for 20 % immunity. Actually, Takala and Saris (2002) have constructed a food-grade cloning vector with *nisI* gene as a selection marker. A proline iminopeptidase *pepI* gene from *Lactobacillus helveticus* was expressed with this plasmid in both *L. lactis* and *Lactobacillus plantarum*, and the heterologous production was shown to result in 200-fold increased PepI activity as compared with plasmid-free hosts. Thus NisI was proven to be a suitable selection marker in food-grade systems instead of classical antibiotic resistance genes (Takala and Saris, 2002). Nevertheless, this clearly cannot be the whole picture, given that neither NisI nor NisFEG alone have been able to supply cells with immunity more than 25 % of that of a wild type. Thus, it seems justified to assume, that the immune systems NisI and NisFEG act in cooperation, and that full immunity can only be achieved, if both components are present in nisin producing cell.

2.5 Detection methods

2.5.1 International nisin activity unit, IU

A standard preparation of nisin was prepared in 1968 due to request of the WHO Expert Committee on Biological Standardization. The standard preparation, which by far is not pure nisin, is stored in the central laboratory of the Ministry of Agriculture, Weybridge, Surrey, Great Britain (WHO, 1969). One international nisin activity unit (IU) equals to the activity of one microgram of the standard preparation. The activity of the commercial nisin preparation, Nisaplin (Sigma), is adjusted to equal that of the standard preparation during manufacture. Of Nisaplin, 2,5 % is pure nisin: thus, 1 g of Nisaplin contains the activity of 10^6 IU, whereas 1 g of pure nisin has an activity of $40 \cdot 10^6$ IU. Hence, 1 IU equals to 25 ng pure nisin (WHO, 1969).

2.5.2 Chemical methods

Already in 1934 Cox published a method, that could be used to detect “inhibitory lactococci” from milk. The test was based on the ability of the micro-organisms present in milk to rapidly reduce methylene blue to a colourless form; the time required for this reduction reaction was substantially longer, if nisin producers were present in milk. The first quantitative chemical detection method was developed in 1950 by Hirsch, who utilized the ability of the rapidly growing and nisin sensitive *Lactococcus lactis* subsp. *cremoris* to reduce methylene blue. In this end-point method, the change in the growth-rate of the indicator strain was monitored as a function of the amount of a sample added via reduction of methylene blue. The use of such a fast growing and sensitive indicator strain, however, made the analysis difficult: if the dilution range of the unknown sample was reasonably narrow in respect to precision and sensitivity of the test, the end-point at

which no reduction occurred was easily lost (Hirsch, 1950). The method of Hirsch was modified to be faster and more reproducible and accurate by Friedmann and Epstein, who replaced methylene blue with resazurin; they also determined the optimal growth phase for the indicator strain in the test, and were able to measure 0,5 IU/ml with a standard error of approximately 10 % (Friedmann and Beach, 1950; Friedmann and Epstein, 1951). Later on, Kalra et al. changed both the indicator strain and the molecule to be reduced. The reducer in this assay with a detection limit of 2,5 IU/ml was *Enterococcus faecalis*, and the substrate for the colorimetric reduction reaction was triphenyltetrazolium chloride (Kalra et al., 1973).

2.5.3 Methods based on growth inhibition

2.5.3.1 Methods with liquid media

The first nisin quantification method, in which the amount of nisin was assayed solely by growth inhibition of the indicator bacteria without chemical colour reactions, was described by Mattick and Hirsch in the same article that nisin was given its name (1947). In the Mattick and Hirsch assay different dilutions of the analyte were added to tubes inoculated with *Streptococcus agalactiae*, and the tubes were incubated for 16-20 hours at +37 °C. The amount of nisin was expressed as a reciprocal of the smallest dilution inhibiting the growth of the indicator strain (Mattick and Hirsch, 1947). Hirsch continued with nisin assay development, and noted three years later that sublethal nisin concentrations prolonged the lag phase of growth of *S. agalactiae*, and that the delay was linearly dependent of the nisin concentration in the range of 5-10 IU/ml (Hirsch, 1950). The turbidity of a *S. agalactiae* culture at logarithmic growth phase was also utilized by Berridge and Barret (1952). This method was developed further by Hurst (1966), who used *L. lactis* subsp. *cremoris* as an indicator strain. The bacteria were grown with different amounts of nisin for 2,5-3 hours, until the growth was terminated with thiomersalate. The growth was determined by measuring the optical densities of the cultures at the wavelength of 600 nanometres. A standard curve was obtained by plotting the optical densities against the logarithms of the nisin concentrations. The method of Hurst was rapid and sensitive, having a detection range of 0,04-0,4 IU/ml, although its use was limited to clear sample solutions (Hurst, 1966).

2.5.3.2 Methods with solid media

The use of solid culture medium in nisin quantification was first described by Hirsch in 1950. In this plate-count assay the amount of viable *S. agalactiae* cells after exposure to nisin was calculated (Hirsch, 1950). The first assay utilizing vertical diffusion of nisin in solid medium was first introduced by Friedmann and Beach in 1950, who let nisin to diffuse overnight at +4 °C to agar cast in a test tube. The diffusion properties of nisin were improved by supplementing the agar with 0,1 % Crill 20. After the diffusion step the tubes were transferred to a temperature of 37 °C, at which the inoculated indicator strain *S. agalactiae* started to grow. With this method the authors obtained a linear relation between nisin concentration and the logarithm of the depth of the inhibition zone with nisin concentrations in the range of 100-5000 IU/ml, the standard error being 15-20 % (Friedmann and Beach, 1950). Stumbo et al. (1964) developed their own version of

the vertical diffusion assay, in which heat treated spores of *Bacillus stearothermophilus* were used as an indicator, and they reported this assay to be able to quantify levels of 0,3 IU/ml with good accuracy.

The first assay based on the growth inhibition of horizontally diffused nisin was developed by Mocquot and Lefebvre in 1956. In this method the weak diffusion properties of nisin were enhanced by addition of 0,3 % Tween 80 to the agar, and by letting nisin to diffuse to agar overnight at +4 °C. After this prediffusion step the plates were removed to a temperature of +30 °C, at which the indicator (*Lactobacillus delbrueckii* subspecies *lactis* or *Lactococcus lactis* subspecies *cremoris*) strain was able to grow. This method was rather sensitive, since amounts of 10 IU/g in cheese gave clearly visible inhibition zones. (Mocquot and Lefebvre, 1956). The assay, however, still remained slow to perform due to the prediffusion step.

Tramer and Fowler (1964) improved the method of Mocquot and Lefebvre by adding 1 % Tween 20 to the agar, and were thus able to eliminate the prediffusion step, thereby shortening the overall time needed for nisin analysis by one day. In this assay *Micrococcus luteus* is used as an indicator strain, and the diameter of the inhibition zone is directly related to the logarithm of nisin concentration in the range of 0,5-10 IU/ml. The method is especially well suited for analysis of nisin from food samples, since for the first time in the scientific literature concerning nisin, an extraction method was described, that allowed researchers efficiently to extract nisin from other proteins bound to nisin, by boiling the food samples at pH 2. Furthermore, a well recognized problem, the presence of other inhibitory substances than nisin in food samples, was elegantly solved by inclusion of a novel control sample: a portion of the sample to be analyzed was removed, and the pH was elevated to 11, followed by incubation at +63 °C for 30 minutes, after which the pH was set to 2. After this alkaline heat treatment the control sample did not contain nisin activity anymore. By using an unknown sample treated this way as a solvent for preparation of positive control samples containing known amounts of nisin, the effect of other inhibitory substances than nisin to the sizes of inhibition zones could be excluded (Tramer and Fowler, 1964). In the same article the authors presented another, a semiquantitative nisin assay, suitable for screening of large amounts of samples. In this method heat treated spores of *Bacillus stearothermophilus* were poured on agar lacking any nutrients. The unknown and positive control samples were prepared similarly as in the *M. luteus* assay. Special nutrient discs were then immersed to the sample extracts, after which the discs were placed on agar plates containing the *B. stearothermophilus* spores. The plates were incubated overnight at + 55 °C. During this overnight incubation the nutrients and nisin possibly present in the food material diffused to the plates, and thus it was possible to semiquantitatively observe inhibition zones from the plates, had the food extracts contained nisin in the concentrations of 0,5-10 IU/ml (Tramer and Fowler, 1964).

The Tramer-Fowler method is still the most used method for analysing nisin from food samples. With few improvements, such as inclusion of the nisinase treatment (a didehydroalaninereductase), which allows for distinction in between nisin and other antimicrobial substances used in food manufacture (Fowler 1975), the Tramer-Fowler method has been assigned as the standard nisin measurement method in the Great Britain (British standard, 1974).

However, not all the variables affecting the measurement results can be eliminated using the same solvent to dilute the known and unknown samples. For example, the extraction efficiency of nisin from meat is dependent on the fat content of the meat product (Bell and De Lacy, 1986). Furthermore, the acidities of fermented products might *per se* produce larger inhibition zones than nisin present in these products (Wolf and Gibbons, 1996). Thus, some changes have been suggested to the Tramer-Fowler method: Calderon et al. improved the quantification of nisin in meat products (1985), and De Vuyst and Vandamme (1992) in fermentation broths. Also, novel horizontal diffusion assays have been developed: Odgen and Tubb (1985) described a method to characterize nisin sensitivities of beer spoilage LAB; Rogers and Montville (1991) restored the prediffusion step and used *Lactobacillus sake* as an indicator strain; Wolf and Gibbons (1996) on their behalf reduced the concentration of agar, buffered the agar with phosphate salts, and returned *Micrococcus luteus* as a nisin sensing organism.

2.5.4 Immunological methods

The first immunological nisin assay, the enzyme-linked immunosorbent assay (ELISA), was developed by Falahee et al. in 1990. With polyclonal antibodies raised against nisin in sheep, they were able to measure 0,5 ng/ml pure nisin and 231 ng/ml, when nisin was spiked in cheese. Even though this enzyme immunoassay was more sensitive for pure nisin than the Tramer-Fowler method, it was clearly less sensitive for analysis of nisin in food samples than the agar diffusion assay. Furthermore, both these methods failed to differentiate nisin from subtilin, the closest structural analogue of nisin (Falahee et al., 1992; Fowler et al., 1975). Suárez et al. published two competitive direct ELISA assays: in the first method, based on polyclonal mouse antibodies, the detection limit for nisin was 5-10 ng/ml (Suárez et al., 1996a); in the second system utilizing monoclonal mouse antibodies concentrations above 10 ng/ml were measurable (Suárez et al., 1996b). In 1998 Bouksaim et al. described a chemiluminescence assisted immunodot assay for nisin, in which serum from rabbit immunized with nisin Z was used to detect nisin. The authors reported this method to have detection limits for nisin Z 3 ng/ml in pure solution, and 155 ng/ml, when nisin was spiked to milk or whey (Bouksaim et al., 1998). The next publication from the same authors, however, was moving on the boundaries of scientific ethics: the authors described the detection limits to be 0,75 ng/ml in buffer, 1,7 ng/ml in milk, and 3,5 ng/ml in complex medium (Bouksaim et al., 1999). In this article the authors presented a standard curve, in which the linear dose-response area for nisin in buffer is in the range of 0,4-7,8 ng/ml. However, both milk and whey were initially spiked with 5 µg of nisin per ml, and prior to ELISA these nisin spiked solutions were diluted with ELISA buffer. From this high dilution factor (in the order of 1:1000) two conclusions can be drawn: the ELISA itself was highly sensitive, not only for detecting pure nisin from a buffer solution, but also to the other components than nisin present in milk and whey. Therefore, milk and whey samples had to be extensively diluted with the ELISA buffer, and thus the initial level of nisin in milk or whey had to be at least 5 µg/ml to be detectable with this ELISA assay.

Flow-injection immunoassay systems have been proposed as technology to quantify nisin in real-time in a fermentation process: Nandakumar et al. reported to be able to measure nisin concentrations of 20-300 ng/ml in a fermentation broth with their flow-

injection systems (2000). The first method to distinguish between nisin A and nisin Z was developed by Dadoudi et al. in 2001. The monoclonal antibodies raised against nisin Z could be used to quantify nisin Z with detection limits of 78 ng/ml in pure solution, 87 ng/ml in fermentation broth, 106 ng/ml in milk, and 90,5 ng/ml in whey. The antibodies developed in this study did not react with nisin A (Dadoudi et al., 2001).

2.5.5 Other methods

The only electrophoretical method for nisin quantification has been published by Rossano et al. in 1998. Their capillary zonal electrophoretical assay was used to analyze nisin from milk, and a linear response to nisin concentration was achieved in the range of 10 to 100 µg of nisin per ml milk (Rossano et al., 1998).

The biosynthetic machinery of nisin itself has been utilized as well. In 1999 Wahlström and Saris developed a plasmid, in which the genes of the two-component signal transduction system NisRK were expressed constitutively. In the same plasmid the bioluminescence genes *luxAB* from *Xenorhabdus luminescens* were placed under the nisin inducible *nisF* promoter. This plasmid was transformed into the non-nisin producer *L. lactis* MG1614. The resulting indicator strain LAC182 was able to produce bioluminescence as a result of extra cellular nisin stimulation and subsequent addition of substrate for the luciferase. This assay was the most sensitive of known nisin quantification methods at the time of its publication, having detection limits of 0,0125 ng/ml for pure nisin, and 1 ng/ml in milk. As compared to immunological methods, the major improvement with this assay was that, no pretreatment of the milk samples was needed. However, the drawback in this luciferase method was the addition of the substrate (*n*-decyl-aldehyde) for the luciferase: the indicator cells had to be in the same energetic state in all samples, thus requiring stringent timing for substrate addition and subsequent bioluminescence measurement. Therefore, the amount of samples to be processed simultaneously remained limited (Wahlström and Saris, 1999).

Very recently, an improved luciferase assay was introduced by Immonen and Karp (2007). In this assay the whole luciferase operon from *Photobacterium luminescens*, containing all the five luciferase genes (*luxABCDE*), was cloned under the control of the *nisA* promoter. The presence of all the luciferase genes rendered the addition of the luciferase substrate unnecessary, thus greatly improving the number of samples to be analysed at the same time. Furthermore, a new record in sensitivity was achieved, being 0,1 pg/ml for nisin in pure solution, and 3 pg/ml in milk. Most importantly, the overall time needed for nisin quantification was reduced to three hours only (Immonen and Karp, 2007).

Aims of this study

Nisin is allowed as a food supplement in more than 50 countries, including the EU, the US, and the peoples' republic of China. National legislations concerning levels of nisin in foods, however, vary markedly. At the present, the most widely used method to quantify nisin from food samples, the agar diffusion assay, is based on the inhibitory effect of nisin to a given indicator organism. The agar diffusion method, however, is laborious to perform and vulnerable to interfering substances present in food samples. The aims of this study were:

1. to develop simple, efficient, and pluripotential nisin quantification methods, based on NisRK signal transduction system coupled to Green Fluorescent Protein (GFP) production
2. to analyze the shelf life of nisin in a food material using the novel bioassays developed in this study, cooked sausages being a representative of food material
3. with the GFP-assays to study the possibility to use nisin as an inductory molecule in intestinal environment to achieve controlled and time dependent production of biomolecules in the intestinal tract.

Materials and methods

The plasmids used in this study are shown in Table 4, bacterial strains in Table 5, and sequences of oligonucleotide primers are given in Table 6. A schematic representation of the nisin analysis procedure used throughout this study is presented in Figure 4 (Section Results and discussion). The methods used in this study are described in more detail in the materials and methods sections in the publications I-IV.

Table 4. Plasmids used in this study.

Plasmid	Antibiotic Resistance	Relevant characteristics	Reference/used in
pCR4-TOPO	Amp, Kan	T/A cloning	Invitrogen/III
pGFPuv	Amp	<i>GFPuv</i>	Clontech/III
pKPSpgfp	Amp	P11 mutant <i>GFP</i>	2)/I
pLEB338	Erm	<i>nisRK</i> , P _{<i>nisF</i>}	I
pLEB599	Erm	<i>nisRK</i> , P _{<i>nisF</i>} , P11 mutant <i>GFP</i>	I, II, IV
pLEB651	Cam	P _{<i>nisA</i>} , <i>GFPuv</i>	III
pNZ8048	Cam	P _{<i>nisA</i>}	1)/III

References within this table: 1) Kuipers et al., 1998 2) Scott et al., 1998.

Table 5. Bacterial strains used in this study.

Strain	Relevant characteristics	Reference	Used in
<i>L. lactis</i> LAC240	indicator strain		I, II, IV
<i>L. lactis</i> LAC275	indicator strain		III
<i>L. lactis</i> MG1614	plasmid free host	1)	I
<i>L. lactis</i> NZ9000	<i>nisRK</i>	2)	III

References within this table: 1) Gasson, 1983 2) Kuipers et al., 1998.

Table 6. Sequences of oligonucleotide primers used in this study.

Primer name, restriction sites	Sequence 5'→3'	Used in
<i>Mlu</i> I- <i>Sac</i> I linker forward, <i>Mlu</i> I <i>Sac</i> I	CGCGTGGGCCCGGGTCTAGAGCT	I
<i>Mlu</i> I- <i>Sac</i> I linker reverse, <i>Mlu</i> I <i>Sac</i> I	CTAGACCCGGGCCCA	I
G0575	AGCTGCATGTGTCAGAGGTTTTCA	III
G0576, <i>Bsp</i> HI	AGAAATCATGAGTAAAGGAGAAGAAC	III

Results and discussion

1. Performance of the GFP-bioassays

Two novel and highly sensitive nisin bioassays were developed in this study. In the first measurement method the red-shifted P11 mutant of the *GFP* gene was cloned under the control of the nisin inducible *nisF* promoter in the plasmid pLEB599, which also encodes the genes *nisR* and *nisK* in a constitutive manner. The plasmid pLEB599 was transformed to a plasmid free *Lactococcus lactis* MG1614 strain, and the resulting indicator strain was designated LAC240 (I).

In the second quantification method the *GFPuv* gene was placed under the control of the *nisA* promoter, and the resulting plasmid pLEB651 was transformed to the *L. lactis* strain NZ900, which harbours the *nisRK* genes in its chromosome. This new nisin sensing strain was named LAC275 (III).

The both indicator strains were tested for their abilities to sense extra cellular nisin derived from various food matrices, and to transduce it to GFP fluorescence. The general concept of the both assays is outlined in Figure 4, and the linear dose-response areas for nisin in different food systems for both strains are presented in Table 7.

As can be seen from Figure 4, both bioassays are extremely simple to perform, and being on a microplate format they allow one to analyze hundreds of samples simultaneously. In the EU and the USA the allowed addition levels of nisin in foods vary from 3 μg to 250 μg per gram of food (Anonymous, 2002) The sensitivity of the indicator strain LAC240 is in the range of nanograms, whereas that of LAC275 is in the

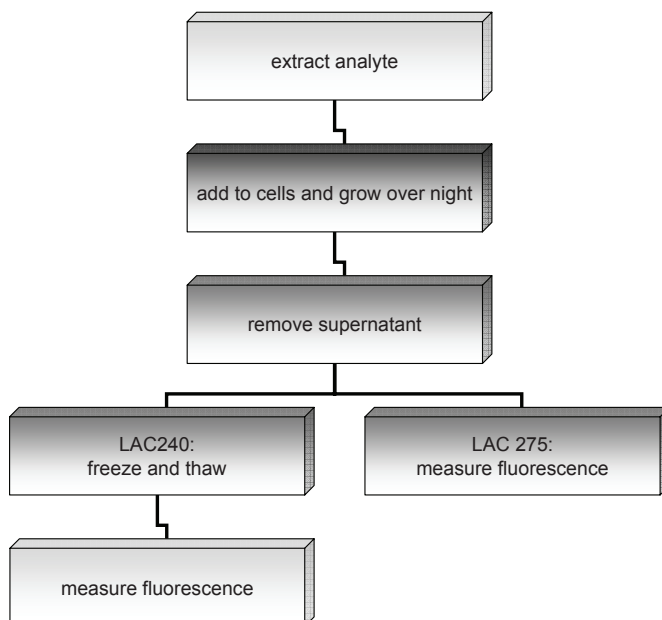


Figure 4. Schematic presentation of the essential steps of the bioassays developed in this study.

range of picograms. Thus, food materials can be extensively diluted prior to analysis with the nisin bioassays developed in this study, and therefore the possible adverse effects caused by interfering agents present in food matrices can be eliminated. The minimum detectable levels of nisin in food products of the most sensitive published nisin detection methods are summarized in Table 8. From Table 8 it can be clearly seen, that both the LAC240 and LAC275 based bioassays are among the most sensitive published nisin quantification methods, and most importantly, the bioassays developed in this study are the most versatile and flexible ones in respect to different food matrices they allow to be analyzed.

Table 7. Ranges of nisin concentrations detectable in the bioassays developed in this study, given as final assay concentrations, or as a concentration in the corresponding food product (μg or ng per g or ml , marked with *).

Analyte matrix	LAC240 (I, II)	LAC275 (III)
Pure solution	2,5-20 ng	10-70 pg
Cheese	5-20 ng	20-100 pg
Milk	5-20 ng	50-100 pg
Salad dressings	1-5 μg^*	1-18 ng^*
Sausage	5-15 ng	
Liquid egg		20-180 pg
Canned tomatoes		1-15 ng^*
Chyme	2,5-10 ng	

Table 8. Comparison of modern nisin bioassays. Detection limits for nisin in foods are given as ng per g or ml in the corresponding food product.

Food	Falahee 1992	Suárez 1996b	Bouksaim 1998	Bouksaim 1999	Wahlström 1999	I, II	III	Immonen 2007
Cheese	1250	50				900	3,6	
Milk			155	5000	1	45	0,9	0,003
Salad dressing						1000	1	
Sausage						900		
Canned tomatoes							1	
Liquid egg							9	

2. Shelf life studies

The GFP-bioassay developed in publication I was used to analyze the shelf life of nisin in cooked sausages (II) and in jejunal chyme (intestinal content) obtained from fistulated dogs (IV). The sausages were prepared such that nisin was added to the sausage mass to a concentration of 11,25 mg/kg prior to cooking. After cooking the sausages were vacuum packed and stored at 6 °C for 28 days. Samples were taken from the sausages at days 0, 6, 10, 14, 21, and 28, and analysed for nisin content with the GFP-bioassay. After cooking at day zero, 91 % of the added nisin activity could be detected, indicating that nisin tolerates well the cooking process of the sausage manufacture. After 10 days storage at 6 °C, 55 % of the added activity could be measured, but beyond that time point the amount of detectable nisin increased reaching 68 % of the originally added level (Figure 5). The possible explanation for this observation might be the liberation of fatty acids, and thus increased solubility of nisin, through the action of lipases present in the meat material. The surveillance of the antimicrobial activity of nisin, however, could not be assessed in this study due to the presence of nitrite in the sausages, which is frequently used as a preservative in meat products. Nevertheless, since nisin fragments have been shown to elicit only a 2-30 % induction level compared to intact nisin, it is reasonable to assume that most of the nisin signal observed in this study was derived from intact nisin molecules. Thus it can be assumed, that when nisin is added to cooked meat products, it persists there for a substantial period of time.

Genetically engineered LAB have received great attention as orally administrable vaccine carriers and producers of therapeutic peptides for treatment of intestinal malfunctions. However, in medical applications it is vitally important to be able to control the place, time, and amount of the drug release. Nisin might prove to be useful as an inducer of controlled protein production in the intestine by the genetically engineered LAB via the NisRK system. However, very little is known about the faith of nisin in intestine. The only study focused on nisin in intestine, revealed that nisin is quite rapidly degraded in the gastrointestinal tract (Bernbom et al., 2006). In that study rats were fed with high amounts of nisin (60 mg/rat, whereas the maximum allowed level

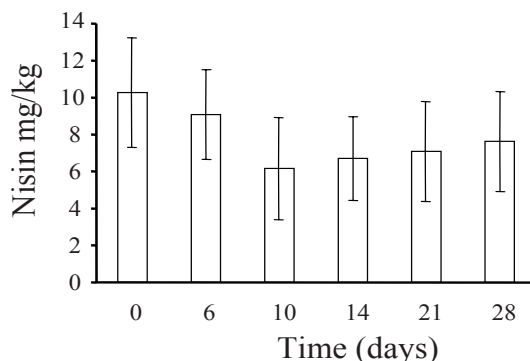


Figure 5. The amount of detectable nisin in vacuum packed cooked sausages after varying times of storage at 6 °C.

in food in the EU is 12,5 mg/kg), and three hours after ingestion the nisin content from duodenal and ileal samples was analysed by immunoassay and inhibition assay. The researchers were only able to detect nisin amounts in the range of micrograms per gram intestinal chyme, indicating that most of the nisin was degraded within three hours in the intestine (Bernbom et al., 2006). The weaknesses of that study are obvious: the amount of administered nisin was far beyond the maximum allowed levels of nisin in foods; as shown by Immonen and Karp (2007), the time needed for nisin induced protein production is only ten minutes, and thus the time period of three hours clearly was too long to assess the suitability of nisin as an induction molecule within the intestine. Therefore, the shelf life of the inductive activity of nisin in jejunal chyme was determined in this study (IV). Nisin was spiked to chyme to a final concentration of 6,75 µg/g, a concentration well correlated with allowed addition levels, and the chyme was incubated at 37 °C for one hour, samples being withdrawn every 15 minutes. After 30 minutes incubation 66 % of the added nisin induction activity could be detected, and even after one hour 17,5 % induction activity could be obtained (Figure 6). These results strongly suggest, that nisin makes a strong candidate molecule to achieve controlled production of bioactive proteins in the intestine by genetically engineered LAB.

In the future the *in vivo* inductive activity of nisin will be assessed. In that study rats will be fed with the *luxABCDE* expressing *L. lactis* strains (Immonen & Karp, 2007), and the possible resulting intestinal bioluminescence after oral administration of nisin will be followed in real-time with *in vivo* luminescence imaging.

In summary, of the methods published to date, only those involving the use of the NisRK pathway can be assumed to be exclusively nisin selective, since: i) neither the chemical methods nor the methods based on the inhibitory action of nisin against a sensitive indicator organism can differentiate between nisin and other bacteriocins ii) antibodies utilized in immunological assays might cross-react with other lantibiotics besides nisin iii) the NisRK system has been shown to be nisin specific. However, as shown by Kuipers et al. in 1995, activation of the NisRK signal transduction does not necessarily require intact nisin molecules; in fact, amino terminal nisin fragments composed of nisin rings A and B were able to initiate, although to a small extent, the NisRK signal cascade (Kuipers et al., 1995). Therefore, there is still a demand for the future research to develop more reliable and exclusive nisin quantification methods.

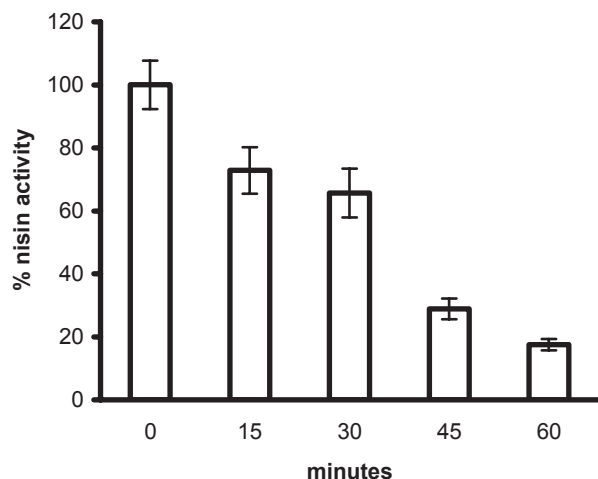


Figure 6. Retainment of the induction activity of nisin in chyme.

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