NisB is required for the dehydration and NisC for the lanthionine formation in the post-translational modification of nisin

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Nisin produced by *Lactococcus lactis* subsp. *lactis* is a 34-residue antibacterial polypeptide and belongs to a group of post-translationally modified peptides, lantibiotics, with dehydrated residues and cyclic amino acids, lanthionines. These modifications are supposed to be made by enzymes encoded by *lanB* and *lanC* genes, found only in biosynthetic operons encoding lantibiotics. To analyse the extent of modification, His-tagged nisin precursors were expressed in *nisB* and *nisC* mutant strains. The His-tagged nisin precursors were purified from the cytoplasm of the cells, as lack of NisB or NisC activity impaired translocation of the nisin precursor. The purified His-tagged polypeptides were analysed with trypsin digestion followed by nisin bioassay, SDS-PAGE, N-terminal sequencing and mass spectroscopy. According to the results, nisin precursors from the strain lacking NisB activity were totally unmodified, whereas nisin precursors from the strain lacking NisC activity, but having NisB activity, were dehydrated and devoid of normal lanthionine formation. This is the first experimental evidence showing that NisB is required for dehydration and NisC for correct lanthionine formation in nisin maturation.

Keywords: lantibiotic, dehydroalanine, dehydrobutyrine, nisin biosynthesis

INTRODUCTION

Antimicrobial peptides produced by bacteria can be classified into several groups, one of which is lantibiotics (Nes *et al.*, 1996). These peptides are post-translationally modified, yielding mature peptides containing non-typical amino acids such as dehydroalanine, dehydrobutyrine, lanthionine and β -methyllanthionine (Sahl *et al.*, 1995). The most prominent member of this group is nisin, produced by some *Lactococcus lactis* strains. Nisin is an approved food additive (E234) used in various food products (Delves-Broughton *et al.*, 1996). The 11 genes involved in nisin biosynthesis, regulation and self-protection have been cloned and sequenced (Kuipers *et al.*, 1993; Engelke *et al.*, 1994; Ra *et al.*, 1996; Immonen *et al.*, 1998). Similar characterization work has been done for other linear lantibiotics, such as subtilin, epidermin, gallidermin and Pep5 (McAuliffe *et al.*, 2001), of which the first three share structural similarities with nisin. Comparison of these genes with

each other and to genes of known function in addition to functional analysis identifies two genes, *lanB* and *lanC*, found only in gene clusters needed for the biosynthesis of lantibiotics. These genes potentially encode the enzymes involved in the unique reactions of lantibiotic biosynthesis, i.e. the dehydration of serines and threonines of the precursor molecule, leading to dehydroalanine and dehydrobutyrine, which are essential for inhibition of spore outgrowth in the case of nisin and subtilin (Liu *et al.*, 1993; Chan *et al.*, 1996). Some of these modified amino acid residues are intermediate structures in the formation of lanthionine and β -methyllanthionine as a result of the addition of cysteine thiol groups to the unsaturated side groups.

Experimental evidence for the importance of *lanB* and *lanC* genes in the dehydration of serine and threonine, and lanthionine formation has accumulated to some extent. Pep5 precursors from *pepB* and *pepC* mutant strains have been purified (Meyer *et al.*, 1995). Analysis of these precursors showed that lack of PepB activity resulted in lack of dehydration, whereas lack of PepC activity yielded secreted precursors that had been correctly dehydrated but contained only one lanthionine

Abbreviation: MALDI-TOF, matrix-assisted laser desorption ionization/time-of-flight.

out of three. These results showed that PepC is not required for the dehydration reaction but seems to be involved in correct lanthionine formation. Whether or not these results can be extrapolated to the biosynthesis of other lantibiotics remains to be seen. Results of Sen *et al.* (1999) suggested that NisB is involved in the dehydration reaction of the nisin precursor. In their experiments, overexpression of the *nisB* gene increased the efficiency of dehydration. Thereby, the serine at position 33 of nisin, which in engineered nisin variants [Trp30] nisin A and [Lys27, Lys31] nisin A partly escaped dehydration, could be fully dehydrated.

In this study, His-tagged nisin precursors from *nisB* and *nisC* mutant strains were purified and analysed, providing evidence that NisB is required for the dehydration reactions and that NisC is needed for correct lanthionine formation in the biosynthesis of nisin.

METHODS

Bacterial strains, plasmids, media and growth conditions.

The bacterial strains and plasmids used in this study are presented in Table 1. Strains and plasmids, which need a more detailed description, are also described below. The nisin

producer *Lactococcus lactis* N8 (Graeffe *et al.*, 1991) was used as the host strain for making the *nisC* mutant strain. The non-nisin producer and plasmid-free *L. lactis* MG1614 (Gasson *et al.*, 1983) was used as a host strain for constructed plasmids. *Micrococcus luteus* A1 NCIMB 86166 (National Collection of Industrial and Marine Bacteria) was used as a nisin-sensitive indicator strain in nisin bioassays. Plasmid pLEB22 consisted of pUC6S (Viera & Messing, 1991) with an erythromycin-resistance gene, *erm* (Axelsson *et al.*, 1988), functional in *L. lactis*. Plasmid pKTH1980 (Graeffe *et al.*, 1991) served as template for amplifying the *nisZ* gene. *L. lactis* expression vectors pLEB124 (Qiao *et al.*, 1995) and pLEB384 (Qiao *et al.*, 1996) were used as vectors for the construction of His-tagged prenisin production constructs. *L. lactis* cells were grown at 30 °C without shaking in M17 (Terzaghi & Sandine, 1975) supplemented with 0.5% (w/v) glucose and 0.5% sucrose (M17GS). *Escherichia coli* cells were grown at 37 °C with shaking in Luria broth. When needed, media were supplemented with antibiotics in the following concentrations: 30 µg ampicillin ml⁻¹, 200 µg erythromycin ml⁻¹ (*E. coli*), 5 µg erythromycin ml⁻¹ (*L. lactis*) and 10 µg chloramphenicol ml⁻¹.

DNA manipulations. Plasmids were isolated by alkaline lysis followed by further purification using the Magic Miniprep kit (Promega). Chromosomal DNA was isolated by the method of Marmur (1961). Established protocols were followed for molecular biology techniques (Maniatis *et al.*, 1982). *L. lactis*

Table 1. Strains and plasmids used in this study

Strain or plasmid	Relevant properties	Reference
Strains		
<i>E. coli</i> DH5α	Host strain for plasmid constructions	Hanahan (1983)
<i>M. luteus</i> A1 NCIMB 86166	Nisin-sensitive indicator strain	National Collection of Industrial and Marine Bacteria
<i>L. lactis</i>		
N8	Nisin Z producer	Graeffe <i>et al.</i> (1991)
M61614	No plasmids, no nisin genes	Gasson <i>et al.</i> (1989)
NZ9800	<i>nisA</i> mutant	Kuipers <i>et al.</i> (1993)
LAC53	<i>nisB</i> mutant	Qiao <i>et al.</i> (1996)
LAC71	<i>nisP</i> mutant	Qiao <i>et al.</i> (1996)
LAC104	<i>nisC</i> mutant	This study
LAC166	LAC104 + pLEB563	This study
LAC208	NZ9800 with pLEB561	This study
LAC214	LAC53 + pLEB563	This study
LAC212	LAC104 + pLEB563	This study
Plasmids		
pNZ8010 <i>nisB</i>	P _{<i>nisA</i>} <i>nisB</i> , the <i>nisB</i> complementation plasmid	Kuipers <i>et al.</i> (1993)
pLEB22	<i>erm</i>	Per Saris, Helsinki
pLEB36	' <i>nisTCIP</i> '	Immonen <i>et al.</i> (1995)
pK601-3	His-tag	Kari Keinänen, Helsinki
pKTH1980	<i>nisZ</i>	Graeffe <i>et al.</i> (1991)
pCRII	T/A cloning vector	Invitrogen
pLEB124	<i>L. lactis</i> expression vector with P ₄₅ promoter	Qiao <i>et al.</i> (1995)
pLEB384	pLEB124 + <i>cat</i>	Qiao <i>et al.</i> (1996)
pT <i>Clux</i> Hb	Vector used for the <i>nisC</i> complementation plasmid	Jacob <i>et al.</i> (1995)
pLEB406	<i>erm</i> , ' <i>nisC</i> ', integration plasmid	This study
pLEB507	P ₄₅ + <i>nisC</i> , the <i>nisC</i> complementation plasmid	This study
pLEB544	His-tagged <i>nisZ</i>	This study
pLEB561	pLEB124 + His-tagged <i>nisZ</i>	This study
pLEB563	pLEB 384 + His-tagged <i>nisZ</i>	This study

Fig. 1. Schematic presentation of the nisin operons of *L. lactis* N8 and LAC104 (the *nisC* mutant) and cloned nisin sequences. (A) The nisin operons of N8 (Immonen *et al.*, 1995; Immonen & Saris, 1998) and nisin regions used. The nisin-inducible promoters are shown in black and the hairpin loops represent the Rho-independent transcription stop signals. The constitutive *nisR* promoter is not shown. (B) The nisin operons of LAC104 with pLEB406 integrated into the *nisC* gene. The plasmid sequence is shown as dots and promoters, except *nisR*, are shown as black arrows. Only relevant restriction enzymes sites are shown. H2, *HincII*; H3, *HindIII*; N, *NcoI*.

cells were transformed by electroporation (Holo & Nes, 1989). DNA was cleaved, ligated and amplified according to the conditions recommended by the supplier of enzymes used (Promega). Oligonucleotide primers used in amplification of the *nisZ* gene were O423 (Graeffe *et al.*, 1991) and NIS123 (5'-GCTCTAGATTGCTTACGTGAATACTACA-3'). Primers used in amplification of the *nisC* gene were N17 (5'-GAACTTTATTATTCAGAGC-3') and NIS41 (5'-TCAGTTAATCATTTCTCTTCCCTCCTTTCA-3'). PCR was performed in a Perkin Elmer Cetus DNA thermal cycler using *Taq* polymerase (Promega).

Nisin bioassay. Antibacterial activity of nisin was determined as growth inhibition zones on M17GS agarose plates inoculated with *M. luteus*, a nisin-sensitive indicator strain. On the top of the agar surface, 3 µl of the sample or a streak of the bacterium to be tested was applied. The plates were read after growth of approximately 16 h at 37 °C. As positive control, a dilution series (0–10 µg ml⁻¹) of nisin (Sigma) was used. Trypsin treatment of the isolated nisin precursor prior to nisin bioassay was done as described previously (Qiao *et al.*, 1996).

Western analysis. Proteins were separated using 20% SDS-PAGE, and transferred to an Immobilon filter. The filter with the proteins was treated according to the instructions of the Protoblot (Stratagene) immunodetection kit. The specific antiserum used to detect the His-tag was the mouse IgG1 isotype RGS-His antibody (Qiagen).

Inactivation of the *nisC* gene. First, a *nisC* inactivation plasmid was constructed. The internal fragment (*HincII*–*NcoI*) of the *nisC* gene in plasmid pLEB36 was cloned into pLEB22, a pUC6S derivative not able to replicate in Gram-positive bacteria but containing a functional selection marker, *erm*, for *L. lactis*. This constructed plasmid pLEB406 was transformed into the nisin producer *L. lactis* N8 with erythromycin selection. Erythromycin-resistant transformants potentially contained plasmid pLEB406 integrated into the chromosome. If integration had occurred by recombination in the *nisC* sequence, the transformants would have two kinds of mutated *nisC* genes, one with a deletion in the 5' part (non-functional due to lack of RBS, initiation codon and the first 33 amino acids) and the other with a deletion in the 3' part (potentially non-functional due to deletion of the 88 amino acids from the C-terminus). Integration of plasmid pLEB406 into the *nisC* gene would place the *erm* gene, which does not contain a transcriptional terminator, in front of the *nisIPRK*

genes, ensuring transcription of these genes from the constitutive *erm* promoter (Fig. 1).

Purification of the His-tagged prenisin. *L. lactis* strains LAC214 and 212 were first grown in 2 l M17G until OD₆₀₀ reached 0.2, followed by addition of nisin to a final concentration of 25 ng ml⁻¹ to induce the nisin operons and the production of the His-tagged nisin precursor. Cultivation was then continued for 4 h and the cells were collected by centrifugation. Cells containing the His-tagged prenisin were digested with 4 mg lysozyme ml⁻¹ in phosphate buffer, pH 7.4, for 1 h at 37 °C, followed by freezing and sonication to disrupt the cells. The unbroken cells and cell debris was removed by centrifugation (30000 g, 30 min). The supernatant was filtered using a MillexHV 0.45 µm filter. Imidazole was added to the supernatant to a final concentration of 140 mM and loaded to a 5 ml HisTrap column (Pharmacia). Washing and elution of the HisTrap column was done according to the instructions of the supplier. After a lyophilization step and resuspension in water (adjusted with HCl to pH 2.5), samples of the fractions were analysed using 20% SDS-PAGE. The fractions containing the highest amount of the expected product (expected migration according to a size of approximately 6.9 kDa) were further purified using a reverse-phase column DeltaPak C4 (3.9 mm, 150 mm and 5 µm) with the HP1090 Liquid Chromatograph model 1040A (Hewlett Packard). A 3–60% gradient of acetonitrile for 20 min was used for elution.

N-terminal amino acid sequencing and mass spectrometry. Confirmation of the identity of the putative His-tagged prenisin from the last purification step was done by N-terminal sequence analysis in a gas/pulsed liquid sequencer (Kalkkinen & Tilgman, 1988). The mass of the His-tagged nisin precursor was analysed using matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) MS on a Biflex time of flight instrument (Bruker-Franzen Analytik) equipped with a laser operating at 337 nm as described previously (Saarinen *et al.*, 1999).

RESULTS

Inactivation of the *nisC* gene

The aim of this study was to analyse the role of NisB and NisC in the modification of nisin by purification and analysis of modification intermediates from strains

Fig. 2. Complementation of the *nisC* mutation. Bacterial streaks 1 (*L. lactis* N8, wild-type nisin producer), 2 (*L. lactis* LAC104, *nisC* mutant) and 3 (*L. lactis* LAC104, containing plasmid pLEB507 with an intact *nisC* gene) on agarose with a lawn of *M. luteus*. The dark zone around streaks 1 and 3 indicate inhibition of *M. luteus* by nisin produced by the bacteria in the streaks. The experiment was repeated three times with similar results.

lacking NisB or NisC activity. Therefore, *nisB* and *nisC* mutant strains having the other nisin genes transcribed were needed. We have previously constructed a *nisB* mutant strain, LAC53, in which the downstream genes (*nisTCIPRK*) are transcribed from the promoter of the *erm* gene located downstream of the *nisB* mutation (Qiao *et al.*, 1996). This strain could be used for analysis of NisB function. A *nisC* mutant strain had to be constructed because previously described *nisC* mutants (Siegers *et al.*, 1996; Ra *et al.*, 1999) were either polar (Ra *et al.*, 1999) or no data regarding the polar effect on the *nisIPRK* genes downstream of the *nisC* gene were available. The putative *nisC* mutants obtained by transformation of plasmid pLEB406 into *L. lactis* N8 were tested for nisin production and none of the transformants produced nisin. This indicated that plasmid pLEB406 had integrated into the *nisC* gene because *nisC* mutants do not produce nisin (Siegers *et al.*, 1996; Ra *et al.*, 1999). To confirm the site of integration, one transformant, named LAC104, was analysed further. The integrated plasmid pLEB406 with flanking DNA was excised with *HindIII* out of the isolated chromosomal DNA of LAC104 (Fig. 1B), circularized with ligase and transformed into *E. coli* where this plasmid, named pLEB407, can replicate. Analysis of the flanking DNA of pLEB407 by restriction enzymes verified that pLEB406 had integrated into the *nisC* gene in the LAC104 strain as the flanking DNA upstream of the *HincII* site contained the 5' sequence of the *nisC* gene not present in pLEB406 (results not shown). A *nisC*-expressing plasmid was constructed (pLEB507) and

transformed into the LAC104 strain for complementation studies, yielding strain LAC166. Plasmid pLEB507 was constructed by cloning a *nisC* gene containing PCR fragment, generated with primers NIS17 and NIS41 using *L. lactis* N8 chromosomal DNA as template, into plasmid pTCluxHb with *HindIII*/*Bam*HI ends. Strain LAC166 harbouring this plasmid gained the capability to produce nisin (Fig. 2). This showed that the intact *nisC* gene in the pLEB507 plasmid could complement the *nisC* mutation and that the other genes in the nisin operons were functional and expressed to levels needed for nisin production. Therefore, the LAC104 strain fulfilled all the requirements for the planned analysis. The *nisB* mutant strain LAC53 has been previously constructed (Qiao *et al.*, 1996) and the *nisB* gene complemented with plasmid pNZ8010*nisB* (Kuipers *et al.*, 1993) (results not shown). Therefore, also this strain fulfilled all the requirements for the planned analysis.

Construction and evaluation of the functionality of a His-tagged nisin

For analysis of nisin precursors expressed in the NisB and NisC mutant strains, these precursors had to be purified. A His-tag coding sequence was added to the *nisZ* gene to aid the purification step. For this construction the *nisZ* gene was amplified using PCR with primers O423 and NIS123 and plasmid pKTH1980 as template. NIS123 was designed such that an *XbaI* site was created before the stop codon of the *nisZ* gene. The amplified fragment was first cloned into the pCRII T/A vector and from there as an *EcoRI*-*XbaI* fragment into plasmid pK601-3, resulting in plasmid pLEB544 containing the *nisZ* gene with a His-tag coding region in-frame fused to the 3' end of the *nisZ* gene. To be able to express the His-tagged nisin precursor in *L. lactis*, the gene was removed from pLEB544 as a *Bam*HI fragment and cloned into the expression vectors pLEB124 and pLEB384, resulting in pLEB561 and pLEB563. The only difference between these two plasmids is that the latter also contains a chloramphenicol marker selectable in *L. lactis*. Plasmid pLEB561 was transformed into the *nisA* mutant strain *L. lactis* NZ9800 (Kuipers *et al.*, 1993), resulting in LAC208. The NZ9800 host strain does not produce nisin but can be complemented by an intact *nisZ* gene (Kuipers *et al.*, 1993), a natural variant of the *nisA* gene with only one nucleotide difference (Graeffe *et al.*, 1991; Mulders *et al.*, 1991). If the His-tagged nisin precursor encoded by the pLEB561 plasmid were a functional substrate for the nisin maturation machinery, then LAC208 should be able to produce active nisin. Without nisin induction the cells of LAC208 did not produce nisin, but induction with 25 ng nisin ml⁻¹ resulted in secretion of active nisin (Fig. 3). This result showed that the His-tagged nisin precursor is a functional substrate for the nisin modification enzymes and transport protein, but not a functional inducer of the positively autoregulated nisin operons (Kuipers *et al.*, 1995; Qiao *et al.*, 1996; Ra *et al.*, 1996). Therefore, plasmid pLEB563, identical to pLEB561 except for the chloramphenicol-resistance marker, was transformed

Fig. 3. Verification of the functionality of His-tagged prenisin for the nisin modification machinery of *L. lactis* NZ9800. Spots: 1, growth supernatant of *L. lactis* N8, wild-type nisin producer; 2, nisin (25 ng ml⁻¹)-induced growth supernatant of *L. lactis* LAC208, the *nisA* mutant NZ9800 containing plasmid pLEB561 with the gene encoding His-tagged nisin; 3, growth supernatant of *L. lactis* LAC208 without nisin induction. The dark spots indicate inhibition of *M. luteus* on the agar surface by nisin produced into the growth supernatants. The MIC value for *M. luteus* in this assay is approximately 50 ng ml⁻¹. The experiment was repeated three times with the same result.

Fig. 4. Western analysis of proteins produced by *L. lactis* LAC214 and LAC212 using a His-tag-specific antiserum. Lanes: 1, cells of LAC214; 2, cells of nisin-induced LAC214; 3, cells of LAC212; 4, cells of nisin-induced LAC212; 5, growth supernatant of nisin-induced LAC214 cells; 6, growth supernatant of nisin-induced LAC212 cells. The experiment was repeated three times with similar results.

Fig. 5. SDS-PAGE analysis of His-tagged prenisin purified from the *nisB* and *nisC* mutants by His-Trap purification. Lanes: 1, molecular mass marker; 2, His-tagged prenisin isolated from *L. lactis* LAC214; 3, nisin; 4, molecular mass marker; 5, His-tagged prenisin isolated from the LAC212 strain. The experiment was repeated three times with the same result.

into the *nisB* and *nisC* mutant strains LAC53 and LAC104, resulting in LAC214 and LAC212, in order to produce partially modified nisin for analysis of the function of the NisB and NisC enzymes. Growing the LAC214 and LAC212 cells in M17GS supplemented with 25 ng nisin ml⁻¹ induced the expression of His-tagged nisin precursor. No nisin activity was observed in the growth media or cells of the LAC214 and LAC212 strains, whereas nisin-induced polypeptides were observable from cells using Western analysis with a His-tag-specific antiserum (Fig. 4). These polypeptides were purified and analysed by N-terminal amino acid sequen-

Fig. 6. Mass spectrometry analysis of His-tagged prenisin from *L. lactis* LAC214 and LAC212 by MALDI-TOF MS. (a) His-tagged prenisin from LAC212 (NisB is functional, but not NisC); 100% corresponds to 2750 absolute intensity. (b) His-tagged prenisin from LAC212 (NisC is functional but not NisB); 100% corresponds to 1250 absolute intensity. The relevant areas are indicated by bars, representing in (a) the expected size distribution (6736–7006 Da, all potential residues dehydrated to no dehydration) of differently dehydrated His-tagged nisin precursors. The bar in (b) represents the expected size distribution (7006–7120 Da) of the unmodified His-tagged nisin precursor and different salt adducts thereof.

cing, SDS-PAGE and mass spectrometry analysis (Figs 5 and 6). The result of the SDS-PAGE (Fig. 5) showed that the purified putative His-tagged nisin precursor migrated as a polypeptide of approximately 6.9 kDa, indicating that the nisin leader had remained uncleaved. The N-terminal sequencing of the purified His-tagged nisin precursor verified that the purified polypeptide was the His-tagged nisin precursor with the N-terminal leader and that no modified amino acid residues were present in the leader (results not shown). The C-terminus was likely to be intact as the polypeptide could be purified using the HisTrap column and detected using Western analysis with His-tag-specific antiserum. The result of the mass spectrometry analysis is shown in Fig. 6.

The major signal in the MALDI analysis of the His-tagged nisin precursor isolated from the LAC214 strain was 7044 Da, which possibly represents disodium (+44 Da) and potassium (+38 Da) adducts of the His-tagged nisin precursor (expected mass 7006 Da). The mass (6732 Da) of the polypeptides in the peak representing the lightest polypeptides purified from the

LAC212 strain corresponded to the mass (6736 Da) of a His-tagged nisin precursor with serine and threonine residues dehydrated similarly as in wild-type nisin. Polypeptides with slightly larger mass were also detected with mass differences (mean 17.3 Da) close to the mass of water. The purified nisin precursor isolated from LAC212 could potentially contain all lanthionines typical for active nisin. Such precursors would be activated if the leader were digested with trypsin (van der Meer *et al.*, 1993). Therefore, the isolated nisin precursor (1 µg) and, as a control growth supernatant of LAC71, a *nisP* mutant strain secreting nisin precursors that can be activated with trypsin (Qiao *et al.*, 1996), were treated with trypsin. Nisin activity was observable only in the control sample.

DISCUSSION

To study the function of the putative nisin-modifying enzymes NisB and NisC, several approaches are evident. The purification of LanB and LanC enzymes for enzymic studies has not been successful (Kupke & Götz, 1996), but isolation and analysis of Pep5 lantibiotic precursors from *pepB* and *pepC* mutant strains has been successful (Meyer *et al.*, 1995). In this study a similar approach was used to experimentally verify the putative functions of NisB and NisC, e.g. dehydration and catalysis of lanthionine formation. We have previously constructed a *nisB* mutant strain having all other nisin genes intact and transcribed (Qiao *et al.*, 1996). A similar *nisC* mutant strain has not been available. Therefore, a *nisC* mutant strain with all other nisin genes functional was constructed. Second, purification of nisin precursors expressed in *nisB* and *nisC* mutant cells had to be made easy. For this purpose a His-tag was added to the C terminus of the nisin precursor. The functionality of this fusion protein for the nisin biosynthetic machinery had to be ensured. The results of production of this fusion protein in the *nisA* mutant strain showed that the His-tagged nisin precursor is a functional substrate for the NisB and NisC enzymes, the NisT transporter and the NisP protease, otherwise active nisin could not have been detected in the growth medium after nisin induction. This also showed that the His-tag did not impair the activity of nisin.

It is known that all lanthionines are needed for nisin activity and if the leader is not cleaved by NisP, the fully modified nisin precursor still containing the N-terminal leader is inactive (van der Meer *et al.*, 1993; Qiao *et al.*, 1995). Interestingly, the His-tag seemed to impair the inductive capacity of nisin as no active His-tagged nisin could be produced unless cells were induced with intact nisin. Nisin-induced expression of the His-tagged nisin precursor in the LAC214 and LAC212 strains did not yield any active nisin inside or outside the cells, as expected. Western analysis with the His-tag-specific antiserum showed that if NisB and NisC do not modify the nisin precursor, the precursor could not be transported. Therefore, it is unlikely that the nisin leader could be used for directing proteins other than fully

modified nisin or structurally very similar polypeptides out of the cells.

The mass spectrometry analysis, SDS-PAGE and N-terminal amino acid analysis of the His-tagged nisin precursors purified from the LAC214 and LAC212 strains showed that the N-terminal leader was not digested from the nisin precursor inside the cell. This indicates that the nisin precursor is protected from intracellular proteases as long as it is not completely modified, because in a *nisT* mutant strain, where transport of the modified nisin was blocked, the leader was digested and active nisin could be isolated from inside the cells (Qiao & Saris, 1996).

For every dehydration reaction the mass of the nisin precursor decreases by 18 Da. Therefore, mass spectrometry can be used to distinguish between a nisin precursor that is not dehydrated and one that is. The mass analysis of the His-tagged nisin precursor from the LAC214 strain (NisC functional but no NisB activity due to the mutation) showed that the mass corresponded to a His-tagged nisin precursor with none of the serine and threonine residues dehydrated. This shows that NisB is needed for the dehydration reaction to occur. The isolated nisin precursor was potentially a salt adduct in the MALDI analysis, explaining the difference in the expected (7006 Da) versus the observed (7044 Da) size. The nisin precursor has three negatively charged aspartate residues and can thereby attract one, two or three positively charged ions. The broadness of the mass peak (Fig. 6b) could be a result of a mixture of nisin precursors with different levels of either potassium or sodium, or mixtures thereof. The size range of the broad peak (approx. 7000–7120 Da) could include the plain nisin precursor (7006 Da), all of the intermediate forms and the heaviest one consisting of the nisin precursor salt with three potassium ions (7120 Da). The same analysis using the nisin precursor purified from the LAC212 strain (NisB functional but no NisC activity due to the mutation) showed that the His-tagged nisin precursor was not as heavy as when isolated from the LAC214 strain. The lightest mass peak corresponded to a His-tagged nisin precursor with serines and threonines dehydrated to an extent that occurs in wild-type nisin. The majority of the peptides had potentially fewer dehydrated residues, as indicated by the larger mass with differences close to 18 Da, the mass of water, which is removed by every dehydration reaction. This result clearly indicated that NisB seems to be responsible for the dehydration reaction and that NisB does not need NisC for the dehydration reaction. According to the results, NisB was not able to efficiently dehydrate all the serine and threonine residues as the majority of the nisin precursors were only partly dehydrated. The structural gene of the His-tagged nisin was located on a multicopy plasmid in the LAC214 strain, resulting in potentially too high levels of nisin precursor for the NisB enzyme to dehydrate all the potential sites. Another explanation for the partial dehydration is that lack of NisC has an effect on the activity of the NisB enzyme, which is known to form a complex with NisC and NisT (Siegiers

et al., 1996). Clearly, the observed inefficient dehydration by the NisB enzyme does not hinder the function of the nisin biosynthetic machinery as active His-tagged nisin could be secreted by the *nisA* mutant strain containing plasmid pLEB561 (Fig. 2). The nisin precursors isolated from strain LAC214 were not in the form of a salt adduct in the mass spectrometry analysis. This could be a reflection of the lower level of production of this precursor compared to precursor production of LAC212 cells, resulting in potential differences in salt concentration of the samples subjected to mass analysis. Another possibility is that dehydration of the nisin precursor results in a conformational change that makes the residues involved in adduct formation less available for salt formation to occur. By mass spectrometry analysis one cannot judge if any of the potentially dehydrated residues have reacted with cysteine to form lanthionine. However, if all lanthionines were formed, then cleavage of the leader should yield active nisin. We could not find any nisin activity after a trypsin treatment of the nisin precursor isolated from the LAC212 strain. Therefore, NisC is needed for correct lanthionine formation.

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