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.
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*

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**Table 1. Strains and plasmids used in this study**

<table>
<thead>
<tr>
<th>Strains or plasmid</th>
<th>Relevant properties</th>
<th>Reference</th>
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<tr>
<td><strong>Strains</strong></td>
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<tr>
<td><em>E. coli</em> DH5α</td>
<td>Host strain for plasmid constructions</td>
<td>Hanahan (1983)</td>
</tr>
<tr>
<td><em>M. luteus</em> A1 NCIMB 86166</td>
<td>Nisin-sensitive indicator strain</td>
<td>National Collection of Industrial and Marine Bacteria</td>
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<td><strong>L. lactis</strong></td>
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<td>N8</td>
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<td>M61614</td>
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<td>LAC104</td>
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<td>This study</td>
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<td>LAC208</td>
<td>NZ9800 with pLEB561</td>
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<td>LAC212</td>
<td>LAC104+pLEB563</td>
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<td>pNZ8010nisB</td>
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<td>pK601-3</td>
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<td>Invitrogen</td>
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<td><em>L. lactis</em> expression vector with <em>Pₚₛₐₙ</em> promoter</td>
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<td>pLEB384</td>
<td>pLEB124+<em>cat</em></td>
<td>Qiao et al. (1996)</td>
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<td>pLEB563</td>
<td>pLEB384+His-tagged nisZ</td>
<td>This study</td>
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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'–GCTCTAGATTTGCTTACGTGAACTACA-3'). Primers used in amplification of the nisC gene were N17 (5'–GAACTTTATATTACAGGC-3') and NIS41 (5'–TCAGTTAATCATTTTCCTTCCCTTCTTTCA-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 Immobilin 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). Purification of the His-tagged nisin precursor. 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 nisin 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 69 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 nisin 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.
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 nisC 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 _HinClI_ 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 pHUH15 with _HindIII/BamHI_ 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 pNZ8010nisB (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 _BamHI_ 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
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 sequencing, 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 69 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 lanthion bic enzymes 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 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 (Siegers
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 which would suggest that cleavage of the leader should result in active nisin. Therefore, NisC is needed for correct lanthionine formation.

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