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## Distinct Contributions of the Nisin Biosynthesis Enzymes NisB and NisC and Transporter NisT to Prenisin Production by *Lactococcus lactis*<sup>∇</sup>

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**Several *Lactococcus lactis* strains produce the lantibiotic nisin. The dedicated enzymes NisB and NisC and the transporter NisT modify and secrete the ribosomally synthesized nisin precursor peptide. NisB can function in the absence of the cyclase NisC, yielding the dehydrated prenisin that lacks the thioether rings. A kinetic analysis of nisin production by *L. lactis* NZ9700 demonstrated that the prenisin was released from the cell into the medium before the processing of the leader sequence occurred. Upon the deletion of *nisC*, the production of prenisin was reduced by 70%, while in the absence of *nisB*, the production of prenisin was nearly completely abolished. In cells lacking *nisT*, no secretion was observed, while the expression of *nisABC* in these cells resulted in considerable growth rate inhibition caused by the intracellular accumulation of active nisin. Overall, these data indicate that the efficiency of prenisin transport by NisT is markedly enhanced by NisB, suggesting a channeling mechanism of prenisin transfer between the nisin modification enzymes and the transporter.**

In 1928, Rogers and Whittier reported “the formation of a definite substance inhibiting growth” during lactic acid fermentation (23). Two decades later, the growth inhibitory substance was identified and named nisin, for Lancefield group N inhibitory substance (15). The elucidation of the molecular structure of the 3.4-kDa nisin peptide (4) revealed the presence of one lanthionine residue and four β-methylanthionine residues making up five intramolecular ring structures. Antibiotic peptides containing lanthionine residues are now collectively known as lantibiotics (1, 26). Nisin is used in the food industry as a preservative. Its antimicrobial action is directed predominantly against gram-positive food-borne pathogens like *Listeria* species. This activity is based on two features, i.e., nisin binds to the pyrophosphate moiety of lipid II and removes lipid II from its functional location, thereby inhibiting cell wall synthesis, and it induces the formation of lipid II-nisin hybrid pores in the cytoplasmic membrane (5). Nisin biosynthesis and immunity require 11 genes (2, 3, 6, 11, 27, 30) organized in a cluster, *nisABTCIPRKFEFG*. *nisA* encodes the nisin precursor peptide containing an N-terminal leader sequence of 23 amino acids. NisB dehydrates serine and threonine residues in the nisin precursor peptide, yielding 2,3-didehydroalanine and 2,3-didehydrobutyrine, respectively. Next, the cyclase NisC couples the free thiol group of the cysteines to the dehydroresidues in a region-specific manner. The modified nisin precursor containing five lanthionine rings is secreted from the cell by the

ABC-type transporter NisT. Finally, on the outside of the cell, the leader sequence is removed by the protease NisP and biologically active nisin A is released (30). NisI and NisFEG are involved in immunity to the lantibiotic nisin produced by the cells (29). The histidine kinase NisK senses extracellular nisin, and upon autophosphorylation, the phosphate group is transferred to the transcriptional activator NisR (10). Activated NisR promotes the transcription of the *nis* gene cluster (with the exception of *nisRK*). The regulatory system NisRK induces expression in a dose-dependent manner; therefore, the expression of the gene cluster leads to an autoinduction cycle (10). The ability of extracellular nisin to induce the expression of the *nis* genes in a dose-dependent manner has been employed in the successful and widely used nisin-controlled gene expression (NICE) system (16). The most commonly used host of the NICE system is the *Lactococcus lactis* strain NZ9000. This strain does not contain the nisin gene cluster; only the *nisRK* genes are integrated into the genome, and the gene or genes of interest are typically expressed from a plasmid.

The *nisB*, *nisT*, and *nisC* genes are jointly transcribed as part of an operon (19). The products of these three genes, NisB, NisT, and NisC, were suggested previously to be part of a membrane-associated multimeric complex in the nisin-producing *L. lactis* strain KS100 (28). A gene disruption study with the related *L. lactis* strain NZ9700 demonstrated that *nisB* and *nisT* are essential for nisin production and that *nisC* is essential for maturation (18). However, since the disruption of these genes breaks the cycle of autoinduction, nisin-induced genes are no longer expressed and the effects observed may indirectly affect biosynthesis. In a previous study using the NICE system and the host *L. lactis* NZ9000, the expression of *nisABT* and *nisAT* resulted in the production of dehydrated prenisin and unmodified prenisin, respectively (8). This outcome demon-

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TABLE 1. Expression plasmids used in *L. lactis* NZ9000

| Plasmid               | Description  | Reference  |
|-----------------------|--|------------|
| pNZnisA-E3            | Carries <i>nisA</i> and erythromycin resistance gene   | 8          |
| pNZnisAtrunc-E3       | Carries truncated <i>nisA</i> encoding NisA amino acids -23 to 22 [NisA( $\Delta$ 23-34)]; pNZnisA-E3 derivative | This study |
| pNGnisT               | Carries <i>nisT</i> and chloramphenicol resistance gene  | 8          |
| pIL3BTC <sup>a</sup>  | Carries <i>nisBTC</i> and chloramphenicol resistance gene  | 20         |
| pIL3hpTC <sup>a</sup> | <i>nisTC</i> ; pIL3BTC derivative  | This study |
| pIL3hpBC <sup>a</sup> | <i>nisBC</i> ; pIL3BTC derivative  | This study |
| pIL3hpBT <sup>a</sup> | <i>nisBT</i> ; pIL3BTC derivative  | This study |
| pIL3hpT <sup>a</sup>  | <i>nisT</i> ; pIL3hpBT derivative  | This study |

<sup>a</sup> hp, hairpin; inverted repeat located upstream of the *nisB* gene.

strated that the NisB and NisT activities are independent of complex formation per se, but the study did not investigate the efficiency of prenisin production. An extensive range of prenisin mutant forms with mutations in the first two ring structures on the amino-terminal side, as well as a range of medically relevant peptides fused to the nisin leader peptide, can be produced by *L. lactis* cells expressing *nisBTC* (7, 21). These peptides contain dehydroresidues and thioether bridges, suggesting that the nisin biosynthesis enzymes and transporter are capable of producing and secreting a broad variety of modified peptides (20). Here, we now report the effect of each of the NisB, NisC, and NisT proteins on the relative production efficiency of the nisin precursor peptide. The data indicate that NisT efficiently excretes both fully modified and dehydrated prenisin and that the unmodified peptide is secreted poorly. These data suggest the channeling of prenisin from NisB to NisT to ensure efficient production.

#### MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** The expression strain *L. lactis* NZ9000 (12), the nisin-producing strain *L. lactis* NZ9700 (11), and the nonproducing strain *L. lactis* NZ9800 (11) were grown at 30°C in chemically defined medium (CDM) (14) with 1% (wt/vol) glucose as a carbon source. When appropriate, chloramphenicol and erythromycin (both at 4  $\mu$ g/ml) were used for plasmid selection. Plasmids used in this study are listed in Table 1. In plasmid pNZnisA-E3, the codons ATACGA, encoding amino acids T46 and A47 of NisA, were replaced by site-directed mutagenesis with the codons TGATCA, introducing a stop, to yield plasmid pNZnisAtrunc-E3. Plasmids pIL3hpTC, pIL3hpBC, and pIL3hpBT were created by PCR amplification of plasmid pIL3BTC from the start codon of *nisT* until the start codon of *nisB*, from the start codon of *nisC* until the start codon of *nisT*, and from the stop codon of *nisT* until the stop codon of *nisC*, respectively. PCR amplification was done with 5'-end-phosphorylated PCR primers and Phusion DNA polymerase (Finnzymes, Finland). Linear PCR products were ligated using T4 DNA ligase (Roche). Plasmid pIL3T was created in the same way as pIL3TC by using pIL3BT as a template.

**In vivo protein labeling.** Fresh CDM was inoculated with samples from stock cultures frozen at -80°C, and the cells were grown overnight. Each culture was then 11-fold diluted in fresh CDM and incubated for approximately 2 h until it reached an optical density at 660 nm ( $OD_{660}$ ) of 0.3 to 0.6. Nisin (Sigma) was added to obtain a concentration of 10 ng/ml, unless stated otherwise, and the culture was then incubated for half an hour. After cooling of the cultures on ice, cells were harvested by centrifugation and washed with ice-cold CDM lacking methionine and containing 10 ng of nisin/ml, unless stated otherwise. After centrifugation, cells were resuspended in the same medium to an  $OD_{660}$  of 0.5 and kept on ice. The cell suspension was incubated in a water bath at 30°C. After 5 to 10 min of preincubation, a 1/250 volume of Redivue Pro-mix L-<sup>35</sup>S in vitro cell-labeling mix (GE Healthcare) was added (final concentration of ~40  $\mu$ M, with 1.48 MBq of [<sup>35</sup>S]methionine/ml). In the prolonged prenisin production studies, the suspension was supplemented with unlabeled methionine at a concentration of 0.1 mM. Samples were taken at regular time intervals, and reaction mixtures were chased with 20 mM unlabeled methionine. Cells and medium were separated by centrifugation. Cell pellets were lysed using 5 mg of lysozyme

(Sigma)/ml in the presence of a small amount of DNase I (Sigma) in 100 mM HEPES-KOH, pH 7.0. Protein was precipitated from medium fractions by the addition of a 1/4 volume of 1-g/ml trichloroacetic acid (TCA; Sigma) and then incubated for 30 min on ice and subsequently subjected to centrifugation. Protein pellets were washed with acetone (-20°C) and resuspended in 1 M Tris-HCl, pH 8.45. Medium and cell protein samples were analyzed by Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Tricine-SDS-PAGE; 16% total acrylamide, of which 5% was bisacrylamide) (25) using a Mini-Protein II electrophoresis system (Bio-Rad). Gels were dried on a model 583 gel dryer (Bio-Rad) and imaged using a Cyclone Plus phosphorimager (PerkinElmer), and protein band intensities were quantitated. Nisin precursor NisA harbors three methionines: M23, M17, and M21. The relative intensities of the prenisin bands were corrected for the absence of the first methionine in 60% of the prenisin secreted by *nisBTC*-expressing cells as determined by mass spectrometry. A similar production experiment was performed using unlabeled methionine. At the time points indicated (see Fig. 3A), samples were taken and processed as described above. Intracellular prenisin was subsequently detected by immunoblotting using antibodies directed against the nisin leader sequence (8).

**Specific growth rate determination.** Specific growth rates were determined based on growth in 96-well microtiter plates under semianaerobic conditions. Cells inoculated into fresh medium from overnight cultures were grown to mid-exponential phase. The cultures were diluted 20-fold in fresh medium, and nisin was added. Aliquots of 200  $\mu$ l of the cell suspension were transferred into sterile low-protein-binding 96-well microplates (Greiner). Silicone oil (50  $\mu$ l) was pipetted onto the top of the sample to prevent evaporation. Cells were grown at 30°C in the absence or presence of various inducing amounts of nisin, and the cell density was monitored by measuring the  $OD_{660}$  every 3 min for 14 h with a multiscan photometer (Titertek Multiscan MCC/340 MKII). The natural log of the relative  $OD_{660}$  [ $\ln(OD_{660,t=n}/OD_{660,t=0})$ , where  $t$  is time,  $OD_{660,t=n}$  is the  $OD_{660}$  at time point  $n$ , and  $OD_{660,t=0}$  is the  $OD_{660}$  at time zero] was plotted against the time, and the slope of the curve (the maximum specific growth rate,  $\mu$ ) during the exponential growth phase was determined (32).

**MALDI-TOF mass spectrometry.** Fresh CDM was inoculated with aliquots from -80°C frozen stock cultures and grown overnight. Cultures were then diluted 11-fold in fresh CDM and incubated for 2 h until an  $OD_{660}$  of 0.3 to 0.6 was reached. Nisin (Sigma) was added to a concentration of 10 ng/ml, and the cultures were incubated for 4 h. Cells and medium were separated by centrifugation. Protein was precipitated from medium fractions by the addition of a 1/4 volume of 1-g/ml TCA (Sigma), overnight incubation on ice, and centrifugation. The protein pellet was washed with acetone (-20°C) and resuspended in demineralized water. Trifluoroacetic acid (TFA) was added to the protein samples to a final concentration of 0.1% (vol/vol). Samples for matrix-assisted laser desorption-time-of flight (MALDI-TOF) mass spectrometry were obtained by purification with a ZipTip<sub>PC18</sub> pipette tip (Millipore). ZipTips were activated and equilibrated with 50% (vol/vol) acetonitrile, followed by demineralized water. A TFA-acidified protein sample was then applied to the ZipTip, which was subsequently washed with a 0.2% (vol/vol) TFA solution. Bound proteins were eluted using a 50% (vol/vol) acetonitrile solution containing 0.1% (vol/vol) TFA. Eluted proteins were vacuum dried and stored at -20°C. Prior to mass spectrometry analysis, the dried eluents were dissolved in 50% (vol/vol) acetonitrile containing 0.1% TFA and mixed with  $\alpha$ -cyano-4-hydroxycinnamic acid matrix solution (Sigma) in a 1:1 ratio (final volume, 10  $\mu$ l). Samples (1.2  $\mu$ l) were then spotted onto the MALDI target and allowed to dry. Mass spectra were recorded with a Voyager-DE PRO MALDI-TOF mass spectrometer (Applied Biosystems). Spectra were recorded in linear mode. In order to maintain a high level of sensitivity, an external calibration was performed.

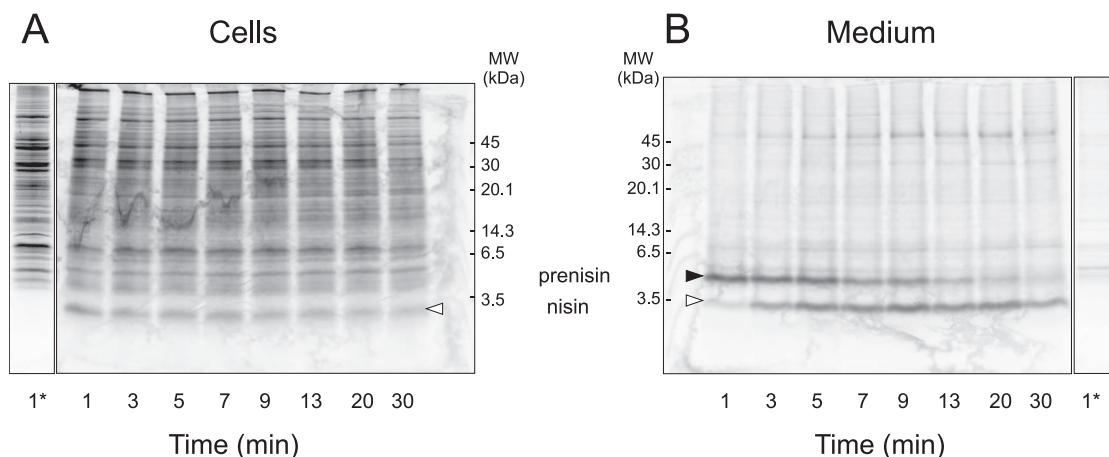


FIG. 1. Prenisin secreted by *L. lactis* NZ9700 is slowly processed into mature nisin. *L. lactis* NZ9700 cells were pulse-labeled with [<sup>35</sup>S]methionine. Samples were taken at the indicated time points after the addition of [<sup>35</sup>S]methionine. Cells were lysed, and medium components were precipitated with TCA. A fraction containing 10% of the lysed cells (A) and the complete precipitated medium fraction (B) were loaded onto a Tricine-SDS-16% polyacrylamide gel. Molecular sizes (MW) of marker proteins are indicated in between the panels; prenisin and mature nisin are indicated by black and white arrowheads, respectively. \*, samples from a similarly labeled *L. lactis* NZ9800 culture.

**Nisin bioactivity assay.** Protein from medium fractions was prepared essentially as described for mass spectrometry analysis. TCA-precipitated protein was washed thoroughly with ice-cold ( $-20^{\circ}\text{C}$ ) acetone and then dried using a Speed-Vac (30 min at  $45^{\circ}\text{C}$ ) and subsequently dissolved in 25  $\mu\text{l}$  of 100 mM HEPES-KOH (pH 7) buffer. Aliquots were diluted twofold with the same buffer with or without 20 mg of trypsin/ml. A high concentration of trypsin was used to ensure the complete removal of the nisin leader sequence. Samples were incubated for 30 min at  $37^{\circ}\text{C}$  and subsequently cooled down on ice. The specific growth rate of the indicator strain *L. lactis* LL108(pORI280) was determined with a 100- $\mu\text{l}$  culture volume in the presence of 4  $\mu\text{l}$  of the protein samples.

**Stability of secreted prenisin-derived peptides.** The stability of prenisin-derived peptides was evaluated by long-term incubation of the purified peptides in spent medium. The *L. lactis* NZ9000 strains harboring *nisAT*, *nisABT*, and *nisABTC* were used to produce unmodified, dehydrated, and fully modified prenisin, respectively. Cells were grown on minimal medium (7) containing 0.5% (wt/vol) glucose and induced with 10 ng of nisin (Sigma)/ml for 24 h. After centrifugation, the cell-free medium containing the peptide was subsequently diluted 1:1 with 100 mM lactic acid, filtered through a 0.2- $\mu\text{m}$ -pore-size filter (Whatman), and subjected to SP-Sepharose chromatography. Bound peptide was eluted with 50 mM lactic acid containing 1 M NaCl. Centriprep YM-3 (Millipore) centrifugal devices were used to remove the salt and to concentrate the peptides. Peptides were stored in 100 mM HEPES-KOH, pH 7, containing 10% (vol/vol) glycerol. For the peptide stability assay, spent medium was prepared from a culture of *L. lactis* NZ9000 cells grown overnight at  $30^{\circ}\text{C}$  in CDM containing 1% (wt/vol) glucose. Cells were removed by centrifugation and subsequent filtration through a 0.2- $\mu\text{m}$ -pore-size filter. Purified peptide and commercial nisin (Sigma) at a concentration of  $\sim 0.3$  mg/ml were incubated in the presence of 100 mM HEPES-KOH, pH 7, or spent medium at  $30^{\circ}\text{C}$ . After 0 and 4 h, samples were removed and analyzed by Tricine-SDS-16% PAGE. Proteins were stained with Coomassie brilliant blue. Blue-prestained protein standards (Invitrogen) were used as molecular size markers.

## RESULTS

**Nisin production by *L. lactis* strain NZ9700.** The nisin-producing strain *L. lactis* NZ9700 harbors a single chromosomal copy of the nisin-sucrose transposon Tn5276 containing the complete nisin biosynthesis gene cluster, including the leader peptidase gene *nisP* and the immunity genes *nisI* and *nisFEG* (11). To study the kinetics of the production of nisin, *L. lactis* NZ9700 cells were pulse-labeled with [<sup>35</sup>S]methionine, together with trace amounts of nisin to ensure the proper induction of the nisin gene cluster. The fate of radiolabeled prenisin

over time was monitored through the separation of cells from the medium fraction by centrifugation. Efficient protein labeling occurred within the first minute (Fig. 1A). Interestingly, prenisin was already detected in the medium fractions as the most prominent labeled protein 1 min after the addition of [<sup>35</sup>S]methionine (Fig. 1B). In time, the intensity of the prenisin band decreased while a protein band with higher-level mobility increased in intensity. A protein with similarly high-level mobility was associated with the cells (Fig. 1A). Based on comparison with the mobilities of the marker proteins, the molecular mass of approximately 3.5 kDa corresponded to the mature nisin (3,354 Da). In contrast, the prenisin and nisin were not observed in either the lysate or medium fraction from cells of *L. lactis* NZ9800, an *L. lactis* NZ9700 derivative that does not produce nisin (Fig. 1) (11). Moreover, in the absence of NisP, only the fully modified prenisin was observed in the medium fraction (see Fig. 2A). The data demonstrate that the prenisin is released from the cell into the medium before the processing of the leader sequence occurs. Thus, prenisin export and release are not coupled to leader cleavage, which is a slow process compared to secretion.

**Specific contributions of NisB, NisC, and NisT to prenisin production.** A recent study has shown that *L. lactis* NZ9000 cells expressing *nisABTC* from a plasmid secrete fully modified prenisin (8). In the absence of NisC or of both NisB and NisC, dehydrated prenisin without the thioether rings or unmodified prenisin can be detected in the medium, respectively, as evidenced by mass spectrometry. However, the efficiency of the secretion of unmodified or partially modified prenisin has not been studied. Here, the specific contribution of the enzymes NisB, NisC, and NisT to the efficiency of prenisin production was determined. Since secretion is not strictly coupled to the removal of the leader sequence, the expression strain NZ9000, which lacks the leader peptidase NisP, is a suitable host to study prenisin production. For this investigation, *nisA* was expressed from pNZnisA-E3 and *nisBTC* was expressed from pIL3BTC, which includes the inverted repeat present in



Tn5276 between *nisA* and *nisB* (8, 20). This inverted repeat limits the expression of the *nisBTC* genes, affecting the stoichiometry of the NisA prepeptide and NisB, NisC, and NisT (9). Derivatives of the pIL3BTC plasmid were constructed by deleting *nisB*, *nisT*, *nisC*, or both *nisB* and *nisC* while leaving the inverted repeat intact. Cells containing pNZnisA-E3 and one of the five pIL3hp plasmids (Table 1) were grown in the presence of [<sup>35</sup>S]methionine. Prenisin production was induced by the addition of nisin to a concentration of 10 ng/ml. At this concentration, the maximum induction level is achieved (data not shown) (12). Labeled protein of both the cell and medium fractions was analyzed during 4 h of incubation (Fig. 2). The relative amounts of prenisin in the various medium fractions were determined using dilutions of the medium fraction of *nisABTC*-expressing cells as a standard (Table 2). Interestingly, in the absence of the transporter NisT, some dehydrated (and presumably cyclized) prenisin was detected in the medium but production was reduced by more than 99% relative to that in cells expressing the full system NisBTC (compare Fig. 2C with A and also see Table 2). Concomitantly, a peptide similar in size to fully modified prenisin (6 kDa) accumulated inside the *nisABC*-expressing cells (Fig. 2C). Western blot analysis using antibodies directed against the nisin leader sequence confirmed the identity of this peptide, showing a slightly higher level of accumulation of prenisin in *nisABC*-expressing cells than in cells expressing *nisABTC* (Fig. 3A, compare lanes 10 to 12 with lanes 4 to 6). Consistent with previous data, this finding indicates that nisin production is dependent on NisT. It should be noted, however, that the antibody does not recognize mature nisin or the NisA degradation products.

On the other hand, in the absence of the modification enzymes NisB and NisC but in the presence of NisT, the levels of production of unmodified prenisin were also very low (Fig. 2E and Table 2), albeit reproducibly slightly higher (2%) than those in the absence of NisT (Fig. 2C and Table 2). Likewise, cells expressing both NisT and NisC produced similarly small amounts of prenisin (Fig. 2B). In contrast, the expression of NisB together with the transporter NisT yielded dehydrated prenisin at a level of 30% relative to that of fully modified prenisin produced when the full system is present. Note that in both the cell and medium fractions from the strain expressing NisBC (Fig. 2C) or NisB alone in the presence of NisT (Fig. 2D), proteins with mobilities similar to that of nisin upon gel electrophoresis were observed (compare Fig. 2C and D with Fig. 1).

The observed levels of the various secreted prenisin forms were not due to differences in extracellular stability. Purified nisin, fully modified prenisin, dehydrated prenisin, and unmodified prenisin were found to be completely stable when incubated for up to 4 h in filter-sterilized spent medium from a culture of the *L. lactis* NZ9000 strain (Fig. 3B) or in buffer (data not shown). These data indicate that during the course of the production experiment, the secreted peptides were intrinsically stable and not lost by proteolytic degradation.

**Identities of prenisin forms produced by *L. lactis* cells in the absence and presence of NisB, NisC, and NisT.** Next, we established whether NisB and NisC expressed from the pILhp plasmids were fully functional. To confirm the dehydration of the produced prenisin, TCA-precipitated medium samples were analyzed by MALDI-TOF mass spectrometry (Table 2). For the

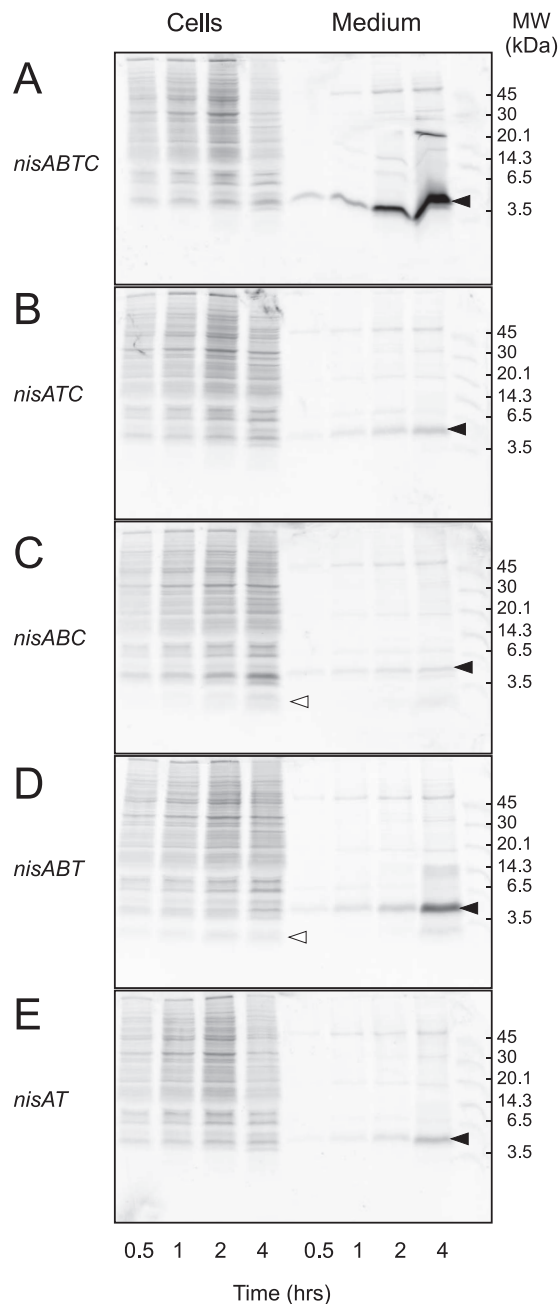


FIG. 2. Prenisin production by *L. lactis* NZ9000 cells expressing different combinations of the *nisB*, *nisT*, and *nisC* genes together with *nisA*. Cells expressing the indicated nisin genes in medium containing 0.1 mM unlabeled methionine were labeled with [<sup>35</sup>S]methionine, and at the indicated time points, cell (left) and medium (right) fractions were isolated as described in the legend to Fig. 1. Molecular sizes (MW) of marker proteins are indicated on the right. Prenisin is indicated by black arrowheads, and an intracellular prenisin degradation product is indicated by white arrowheads.

sample of *L. lactis* NZ9000 cells expressing *nisABTC*, peaks at a mass-over-charge ratio ( $m/z$ ) corresponding to prenisin forms containing or lacking the initiating methionine were observed, consistent with previous observations (8). Interestingly, for both species, peaks were present at masses 126 and 144 Da lower than the mass of the unmodified prenisin peptide. These mass losses

TABLE 2. Effects of NisB, NisC, and NisT on production of prenisin

| Relevant <i>L. lactis</i> NZ9000 genotype | Expected prenisin peptide <sup>a</sup> | Relative extracellular amt <sup>b</sup> | Growth inhibition after trypsin treatment <sup>c</sup> | Observed mass(es) (Da) in MALDI-TOF analysis (calculated mass) |   |
|---|--|---|--|--|---|
|   |  |   |  | With initiating methionine                                     | Without initiating methionine                 |
| <i>nisABTC</i>                            | Fully modified prenisin (D + T)        | 1                                       | +  | 5,838.8 (5,837), <sup>d</sup> 5,819.1 (5,819)                  | 5,705.7 (5,706), <sup>d</sup> 5,687.5 (5,688) |
| <i>nisATC</i>                             | Unmodified prenisin                    | 0.02                                    | -  | ND <sup>e</sup> (5,963)  | 5,832.3 (5,832)                               |
| <i>nisABC</i>                             | Fully modified prenisin (D + T)        | <0.01                                   | -  | ND (5,837), <sup>d</sup> ND (5,819)                            | 5,704.8 (5,706), <sup>d</sup> 5,687.9 (5,688) |
| <i>nisABT</i>                             | Dehydrated prenisin (D)                | 0.3                                     | -  | ND (5,837), <sup>d</sup> ND (5,819)                            | 5,706.1 (5,706), <sup>d</sup> 5,689.2 (5,688) |
| <i>nisAT</i>                              | Unmodified prenisin                    | 0.02                                    | -  | ND (5,963)   | 5,832.1 (5,832)                               |

<sup>a</sup> D, dehydrated serine (three) and threonine (five) residues; T, thioether bridges (five). The dehydration of one residue results in a mass loss of 18 Da, while thioether bridge formation does not result in mass change.

<sup>b</sup> Amount of the peptide in the medium after 4 h of incubation, expressed as the ratio of the amount of the indicated peptide to the amount in the medium fraction of *nisABTC*-expressing cells.

<sup>c</sup> TCA-precipitated peptide was treated with trypsin to liberate nisin, and the inhibition of *L. lactis* LL108(pORI280) growth was assessed with a bioassay. +, present; -, absent.

<sup>d</sup> Result for the peptide with seven dehydrated residues.

<sup>e</sup> ND, not detected.

correspond, respectively, to seven and eight times the mass of water (18 Da), indicating efficient dehydration of the prenisin. In a previous study, a commercial nisin sample was shown to contain a form of nisin with an unmodified Ser33 residue ([Ser33]nisin), potentially due to incomplete dehydration (24). However, in the same study other (chemical) modifications were reported that resulted in the same molecular mass as that of [Ser33]nisin, namely, the addition of water to Dha5, resulting in [2-hydroxy-Ala5]nisin, which can be further converted to [Ile4-amide,pyruvyl-Leu6]des-Dha5-nisin. The detected peaks at masses of 5,838.8 and 5,705.7 Da (Table 2) therefore likely correspond to [Ser33]prenisin, [2-hydroxy-Ala5]prenisin, and [Ile4-amide,pyruvyl-Leu6]des-Dha5-prenisin.

Only in the case of NisBTC was the complete prenisin, including the initiating methionine, observed (Table 2), while under the other conditions tested (Table 2), only prenisin without the initiating methionine was observed. In all medium samples from cells expressing *nisB* (Table 2), molecules with masses of approximately 5,706 and 5,688 Da, corresponding to prenisin containing seven and eight dehydrated residues, respectively, were detected. In nisin, Ser29 is never dehydrated, which has been attributed to steric hindrance by the formation of the neighboring thioether ring E. In the case of *nisA* and *nisBT* coexpression, thioether rings are not formed since NisC is absent and, therefore, Ser29 may be available for dehydration. Nevertheless, prenisin with nine dehydrated residues

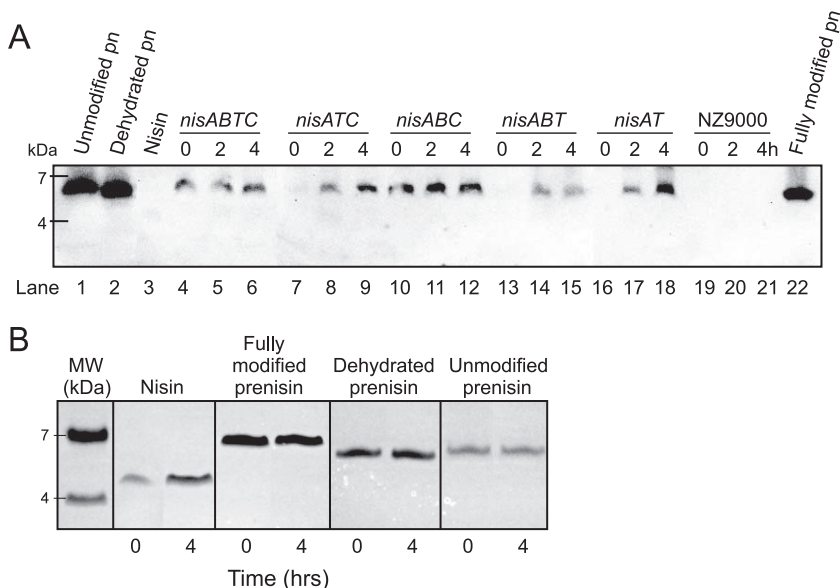


FIG. 3. Intracellular accumulation of prenisin and stability of secreted prenisin and derived peptides. (A) Intracellular prenisin accumulation in *L. lactis* NZ9000 cells lacking *nisABTC* or expressing different combinations of the *nisB*, *nisT*, and *nisC* genes together with *nisA* as indicated. Samples were taken prior to induction (0 h) and after 2 and 4 h of induction. Equal amounts of lysed cells were analyzed by Tricine-SDS-16% PAGE, followed by immunoblotting using antibodies directed against the leader sequence (lanes 4 to 21). Samples of about 2 μg each of purified unmodified prenisin (pn; lane 1), dehydrated prenisin (lane 2), nisin (lane 3), and fully modified prenisin (lane 22) were loaded as controls. Note that the antibody does not recognize the mature nisin. (B) Purified peptide at a concentration of ~0.3 mg/ml was incubated at 30°C in filter-sterilized spent medium from an *L. lactis* NZ9000 culture. At the time points indicated, samples were taken and analyzed by Tricine-SDS-16% PAGE and Coomassie brilliant blue staining. MW, molecular size markers.

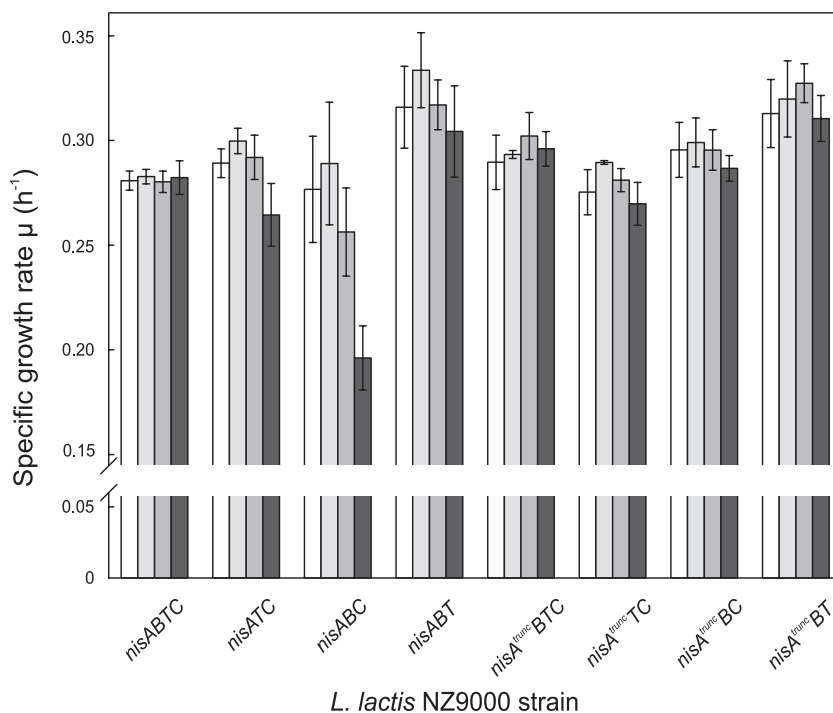


FIG. 4. In the absence of *nisT*, the expression of *nisABC* inhibits the growth of *L. lactis* NZ9000. *L. lactis* NZ9000 strains containing different combinations of *nisA* or truncated *nisA* [*nisA<sup>trunc</sup>*, encoding NisA( $\Delta$ 23–34)] and the *nisB*, *nisT*, and *nisC* genes (as indicated) under the control of the nisin-inducible promoter were grown in CDM containing nisin at a concentration of 0 (white bars), 0.1 (lightest gray bars), 2 (medium gray bars), or 10 (darkest gray bars) ng/ml. The specific growth rate ( $\mu$ ) was determined from the increase of the OD<sub>660</sub> in the exponential growth phase. Each bar indicates the average specific growth rate of three independent cultures. The standard deviations are indicated by error bars.

could not be detected, suggesting that one of the residues escapes dehydration. In a parallel experiment with cells expressing a truncated form of NisA lacking amino acids 23 to 34 [NisA( $\Delta$ 23–34)], similar dehydration efficiency was observed. Molecules with masses 72 and 90 Da lower than that of the unmodified truncated prenisin peptide (with and without initiating methionine, 4,695 and 4,563 Da, respectively) were detected when the gene encoding NisA( $\Delta$ 23–34) was coexpressed with *nisBTC* or *nisBT* (data not shown). The 90-Da mass reduction corresponds to the expected dehydration of Thr2, Ser3, Ser5, Thr8, and Thr13. The 72-Da reduction in mass may result from incomplete dehydration or complete dehydration followed by the addition of water to Dha5.

To assess the correct dehydration and cyclization of prenisin, a growth inhibition assay was used. A medium fraction of *nisABTC*-expressing cells treated with trypsin to remove the leader peptide inhibited the growth of *L. lactis* LL108(pOri280) (21), indicating the presence of active nisin and thus confirming the proper cyclization (Table 2). As expected, in the absence of NisB or NisC no active nisin was obtained (Table 2).

**Mature nisin accumulates inside the cell in the absence of NisT.** In the nisin producer strain *L. lactis* N8, a deletion in the *nisT* gene leads to the accumulation of mature nisin in the cytoplasm of the cells (17). Apparently, fully modified prenisin is produced but remains in the cytoplasm due to the absence of the transporter. The accumulation of mature nisin likely results from the conversion of prenisin into nisin by intracellular proteases. The expression of *nisABC* significantly reduced the specific growth rate of the host strain by approximately 30% at the highest

induction level (10 ng of nisin/ml), whereas no significant reduction of the growth rate was observed for the other strains that lacked one or both of the modification enzymes (Fig. 4). Indeed, in the cellular fractions of *nisABC*-expressing cells, peptides similar in size to fully modified prenisin (6 kDa) (Fig. 2C and 3A) and nisin (3.4 kDa) accumulated (Fig. 2C).

A truncated form of nisin lacking amino acids 23 to 34 has been shown previously to display reduced growth-inhibiting activity against a nisin-sensitive *L. lactis* strain (21). The residual antimicrobial activity of this peptide was attributed to its retained ability to bind to lipid II while the pore-forming activity was lost. When the same truncated form of NisA was expressed together with NisBC, no significant reduction of the growth rate of the host strain was observed (Fig. 4).

## DISCUSSION

Nisin production depends on the concerted activities of the modifying and membrane-associated enzymes NisB and NisC, the transporter NisT, and the extracellular protease NisP. By means of the yeast two-hybrid system and coimmunoprecipitation experiments, NisB, NisT, and NisC have been shown previously to be interacting proteins (28). However, the functional significance of this interaction has remained elusive, as all three proteins have been shown to function in the absence of the other two (8, 9, 13). Here we have presented a quantitative assessment of the role of the individual Nis proteins in prenisin production. Plasmids carrying the *nisB*, *nisC*, and *nisT* genes separately or in combination were employed, and their

expression was induced by the addition of nisin. In the absence of any one of the three components of the nisin synthetase complex, a profound reduction in prenisin production was observed. In line with previous observations (17), NisT was essential for the secretion of the completely modified prenisin from the cell. Remarkably, in the absence of the dehydratase NisB, a very low level of unmodified prenisin (only 2% of the level observed for *nisABTC*-expressing cells) was found in the medium, irrespective of the presence (in *nisATC*-expressing cells) or absence (in *nisAT*-expressing cells) of NisC. Mass spectrometry showed that the small amounts of secreted prenisin corresponded to the unmodified form. On the other hand, in cells expressing both *nisB* and *nisT*, a substantial level of dehydrated prenisin (about 30% of that in the control cells) was detected in the medium fraction. The loss of production was not due to the proteolysis of the secreted peptides in the external medium, as the long-term incubation of the fully modified prenisin and prenisin and the dehydrated or unmodified prenisin with spent medium did not reveal any major differences in stability. Rather, our results suggest that compromised translocation is the main reason for the reduced production of dehydrated and unmodified prenisin. Consistent with this hypothesis is the observation that a prenisin-derived peptide (~3 kDa) accumulated in the *nisABT*-expressing cells (Fig. 2D). The same fragment was observed in *nisABC*-expressing cells (Fig. 2C) but could not be detected in *nisAT*- or *nisATC*-expressing cells (Fig. 2A and B). The possibility that the deletion of *nisB* somehow affects the ribosomal synthesis of prenisin (NisA) was investigated by Western blot analysis of the cellular fractions with nisin leader antibodies (Fig. 3A). Cells expressing *nisABT*, *nisATC*, or *nisAT*, however, accumulated comparable levels of prenisin following 4 h of production. Therefore, we hypothesize that the strongly reduced production of unmodified and dehydrated prenisin was due to reduced export activity and that the prenisin peptides, among others, were partially proteolyzed in the cell to release nisin, which is growth inhibitory (Fig. 4). In line with this suggestion was the intracellular presence of small peptides in the *nisABC* strain, which were likely nisin and further degradation products (Fig. 2C; also see below).

In the absence of NisT, the production of prenisin in the medium was reduced more than 100-fold, with a concomitant accumulation of nisin in the cells, which likely originates from the nonspecific removal of the leader sequence from the fully modified prenisin by cytoplasmic proteases (Fig. 2C). Thioether rings have been shown previously to stabilize and protect (poly)peptides from proteolytic cleavage (7, 30) and may allow for the fully modified prenisin and nisin to accumulate inside the cell. In support of this possibility, cells expressing *nisABC* showed a significantly reduced growth rate under strong inducing conditions. Under identical conditions, the expression of a truncated form of prenisin lacking the 12 C-terminal amino acids (23 to 34) and unable to form pores (21) did not result in a reduction of the growth rate. Therefore, we conclude that in the absence of NisT, *nisABC*-expressing cells accumulate toxic levels of nisin in the cytosol, leading to pore formation and the inhibition of cell wall synthesis.

Siegers et al. (28) have shown that NisB, NisC, and NisT are contained in a membrane-bound nisin synthetase complex. The possible benefit of complex formation may be that preni-

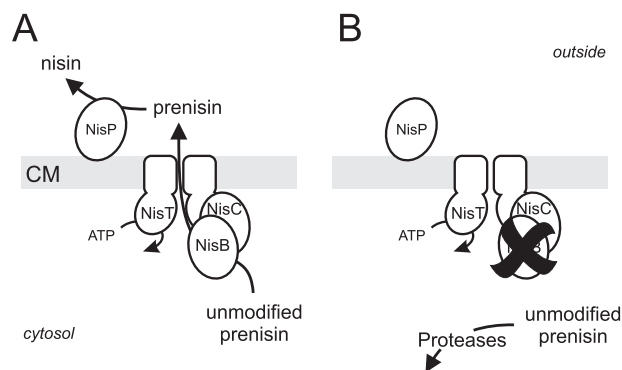


FIG. 5. Proposed channeling mechanism of prenisin transfer between the nisin modification enzymes NisB and NisC and the transporter NisT. (A) Prenisin is targeted to the NisBTC complex, dehydrated by NisB, cyclized by NisC, and subsequently secreted by NisT. Outside the cell, the mature prenisin is processed by NisP into the active nisin. CM, cell membrane. (B) In the absence of NisB, the production of prenisin is almost completely abolished, suggesting a role of NisB in membrane targeting and channeling of unmodified prenisin in the cell. Without this targeting, the unmodified prenisin is proteolyzed in the cell.

sin modification and secretion are well-coupled processes. The extremely fast appearance of fully modified prenisin in the medium (Fig. 1) is consistent with such a channeling mechanism. In the presence of NisB but in the absence of NisC, significant albeit reduced production was observed, whereas the absence of NisB resulted in an almost complete loss of production under all conditions tested. Therefore, we propose that NisB is a central component of the NisBTC complex that is essential for the transfer of the modified prenisin to NisT (Fig. 5). An alternative explanation for our results is that NisT is highly specific for the fully modified prenisin only. However, we consider this hypothesis less likely, as Rink et al. (20) reported the efficient production of a series of unmodified peptides in a NisT strain provided that these peptides contained the nisin leader sequence.

The results of the pulse-labeling experiments (Fig. 1) with [ $^{35}$ S]methionine and *L. lactis* strain NZ9700, which contains a single copy of the nisin-sucrose transposon Tn5276 (11), show that the secretion of fully modified prenisin and subsequent processing into nisin are independent processes. Fully modified prenisin was already detected in the medium after 1 min of incubation, indicating that synthesis, modification, and secretion are relatively fast processes. The secreted fully modified prenisin was subsequently slowly processed into nisin by NisP. Processing occurs either at the membrane surface or, alternatively, in the culture medium, as some NisP is released into the medium. Overall, the data demonstrate that the removal of the leader peptide is not coupled strictly to the export event and that unprocessed prenisin is first released into the medium (Fig. 5). Importantly, secretion thus appears to be a faster event than processing. This view is further supported by findings that the disruption of the *nisP* gene allows the secretion of the unprocessed fully modified prenisin (8), which demonstrates that these processes are not mechanistically coupled.

Employing the enzymes NisB and NisC and the transporter NisT of the nisin biosynthesis complex for the biological production of peptides containing dehydroalanine and dehydro-



butyryne residues and thioether bridges may open a window to a multitude of improvements on antimicrobial and therapeutic peptides known today. Recent studies have shown that these proteins are capable of modifying and transporting a range of peptides that are not related to the natural substrate prenisin (7, 8, 22). This finding further demonstrates that the substrate specificity of NisT is relatively broad and supports the notion that the lack of production in the absence of NisB (and NisC) is due to the poor targeting and channeling of the prenisin from the nisin modification enzymes to the transporter.

#### ACKNOWLEDGMENT

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