Dissection of the Nisin Modification and Transport Machinery

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NisT, the transporter of the lantibiotic nisin, can transport fully modified, dehydrated and unmodified prenisin and fusions of the leader peptide with non-lantibiotic peptides

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Running title: Dissection of the Nisin Modification and Transport Machinery

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Lantibiotics are lanthionine-containing peptide antibiotics. Nisin, encoded by *nisA*, is a pentacyclic lantibiotic produced by some *Lactococcus lactis* strains. Its thioether rings are posttranslationally introduced by a membrane-bound enzyme complex. This complex is composed of three enzymes: NisB which dehydrates serines and threonines, NisC which couples these dehydrated residues to cysteines thus forming thioether rings and the transporter NisT. We followed the activity of various combinations of the nisin enzymes by measuring export of secreted peptides using antibodies against the leader peptide and mass spectroscopy for detection. *L. lactis* expressing the *nisABTC* genes efficiently produced fully posttranslationally modified prenisin. Strikingly, *L. lactis* expressing the *nisBT* genes could produce dehydrated prenisin without thioether rings and a dehydrated form of a non-lantibiotic peptide. In the absence of the biosynthetic NisBC enzymes, the NisT transporter was capable of excreting unmodified prenisin and fusions of the leader peptide with non-lantibiotic peptides. Our data show that NisT specifies a broad spectrum (poly)peptide transporter that can function either in conjunction with or independently from the biosynthetic genes. NisT secretes both unmodified- and partially or fully posttranslationally modified forms of prenisin and non-lantibiotic peptides. These results open the way for efficient production of a wide range of peptides with increased stability or novel bioactivities.
A wide spectrum of biological functions, such as hormone, growth factor, enzyme inhibitor, antigen, antibiotic and ionophore can be found among peptides. Cyclization of peptides has been shown to be a valuable method to obtain biostable analogs. Furthermore, by conformational constraints enhanced or modulated receptor interaction can be obtained (1, 2). Thioether rings can contribute to enhanced peptide stability, enhanced resistance against proteolytic degradation (3, 4) and modulation of receptor interaction (2).

Lantibiotics are bacterial peptides with intramolecular thioether bridges (5). They owe their name to their antibiotic activities and the presence of lanthionine residues. Lanthionines are thioether containing amino acids. A variety of activities has been demonstrated for lantibiotics e.g. autoinduction of lantibiotic synthesis (6), permeabilization of target membranes (7-11), inhibition of cell wall synthesis (12), lipid II binding (13), inhibition of phospholipase A$_2$ (14), modulation of autolytic enzymes (15) and of an angiotensin-converting enzyme (16). These activities all depend on the presence of thioether rings. By controlling the lanthionine-synthesizing enzyme complex, one might envisage the possibility to introduce thioether rings at any peptide position. However, at present only one new thioether ring has been synthesized in a lantibiotic (3). Most interestingly, in vitro activity of the lanthionine synthesizing enzyme LctM has recently been demonstrated (17).

The best known lantibiotic is nisin, which is produced by some Lactococcus lactis strains. Nisin is widely applied as a food preservative (18) because of its antimicrobial activity. It displays a variety of antibiotic activities against many Gram-positive bacteria (7, 19). Breukink et al. (13) found that nisin interacts with lipid II of the target cell, and already at very low concentrations this complex permeabilizes the membranes for small ions and solutes.

Nisin is composed of four methyllanthionines, one lanthionine, two dehydroalanines, one dehydrobutyrine and twenty six unmodified amino acids (20, 21). At position 33 mostly a
dehydroalanine is present but in some cases an unmodified serine can be found (22). The above-mentioned uncommon residues are posttranslationally produced by intracellular membrane-associated enzyme complexes (23, 24). The enzyme NisB dehydrates serines and threonines; NisC is responsible for thioether bridge formation by coupling the dehydroresidues to cysteines, and NisT exports fully modified prenisin. The extracellular serine protease NisP cleaves off the N-terminal 22-23 amino acid leader peptide (with or without initiating methionine), whereupon the active nisin is released (Fig. 1).

<FIGURE 1>

Here we show that the nisin-precursor transporter NisT, in different combinations with NisB and NisC, is able to transport a wide variety of modified and unmodified peptides. This now opens the way to the biotechnological production of modified peptides with novel bioactivities and improved stability. Moreover, we show that the processing enzyme, NisP, requires lanthionine formation of the propeptide for proper functioning.

<Table 1>

MATERIALS AND METHODS

**Leader peptide.** Synthetic leader peptide without initiating methionine was purchased from Synpep, Dublin, CA, US. Bacterially produced leader peptide was purified by binding to teflon beads, a C18 column and elution with an acetonitrile gradient. Cleavage of leader peptide from fully modified prenisin resulted from a 15 min incubation at 37 °C with 1 mg/mL trypsin.

**Anti-leader peptide antibodies.** Polyclonal antibodies were raised in rabbits against the peptide H$_2$N-STKDFNLDLVSVSKKDC-CONH$_2$ coupled via the cysteine to keyhole limpet haemocyanin. Samples for Western blotting were prepared as follows. Ten mL (20 mL
for samples from cells with pLP1vp and pLP1ang) of bacterial culture supernatant was precipitated with 10% trichloroacetic acid (TCA), kept on ice for 2 h, pelleted by centrifugation at 18514 g during 30 min at 4 ºC, washed with 10% TCA and with acetone and vacuum dried. Pellets were dissolved in 50 μL (for TP9703, NZ9700 and PA1001 containing pBMDL5) or 20 μL sample buffer and applied on a gel.

**Bacterial strains and plasmids.** Strains and plasmids are listed in Table 1. L. lactis strains PA1001 and TP9703 were prepared from NZ9000 and NZ9700 respectively using the pOri gene replacement system (29). In TP9703, the nisP start codon of NZ9700 was replaced by a NotI restriction site.

**Molecular cloning.** Nisin gene(s) (combinations) were amplified from chromosomal DNA of L. lactis NZ9700. DNA amplification was carried out using Expand High Fidelity Polymerase (Roche, Mannheim, Germany) or Pfu polymerase (Invitrogen, CA) in case of insertion or deletion via round-PCR. Plasmid DNA was isolated using the Roche kit. DNA was restricted using restriction-PCR. Plasmid DNA was isolated using the Roche kit. DNA was restricted using restriction enzymes from New England BioLabs Inc. Ligation was carried out with T4 DNA ligase (Roche). DNA fragments were isolated from agarose gel using the Zymoclean gel DNA recovery kit (Orange, CA) or from a PCR mix by using the Roche PCR purification kit. For intermediate cloning steps pGEM-T (Promega) was used. Transformation of Escherichia coli (E. coli DB3.1 (ccdB mutant); E. coli DH5alpha and E. coli TOP10, all obtained from Invitrogen) was carried out using established procedures (30). Electrotransformation of L. lactis was carried out as previously described (31) using a Bio-Rad gene pulser (Biorad, Richmond, CA). Nucleotide sequence analyses were performed by BaseClear (Leiden, NL).

**Culturing.** L. lactis was grown in M17 broth (32) supplemented with 0.5% glucose (MG17) with or without chloramphenicol (5 μg/mL) and/or erythromycin (5 μg/mL). E. coli was cultured in TY medium with or without ampicillin (100 μg/mL) or erythromycin (100
μg/mL). Preceding mass spectrometry, cells were cultured and samples were prepared as follows. Overnight cultures of *L. lactis* grown in MG17 broth were diluted 1/100. At optical density at 660 nm of 0.4, cells were centrifuged and the medium was replaced by minimal medium (33) with or without 1/1000 volume of filtered (0.4 μm) overnight *L. lactis* NZ9700 culture medium containing nisin. Incubation was continued for 4 h or overnight after which mass spectrometry samples were prepared. In the case of cells containing pNGnisBT, 50 mL medium was subjected to TCA precipitation prior to further analysis. Growth inhibition experiments were performed as described previously (34), but in the absence of Tween.

Mass spectrometry. Samples were obtained by ziptip purification (C18 ziptip, Millipore). Ziptips were wetted and equilibrated with 50% acetonitrile followed by demineralized water. Then peptides were bound and washed with demineralized water, eluted with a solution of 0.1% trifluoroacetic acid (TFA) with 30- or 50% acetonitrile, vacuum dried and stored at –20 °C until analysis. The dried ziptip eluent was resuspended in 50% acetonitrile containing 0.1% (v/v) TFA and 1 μL was applied to the target. Subsequently, 1 μL of matrix (10 mg/mL alpha-cyano-4-hydroxycinnamic acid completely dissolved by mildly heating and vortexing in 50% acetonitrile containing 0.1% (v/v) TFA) was added to the target and allowed to dry. Mass spectra were recorded with a Bruker Biflex III MALDI-time-of-flight mass spectrometer. In order to maintain high sensitivity, an external calibration was applied. Ethanethiol treatment (35) was applied to confirm posttranslational modification.

Measurement of leader peptidase specificity. NisP-mediated cleavage of peptides was measured by MALDI-TOF MS. Log phase *L. lactis* strains were induced during 4 h or overnight. In indicated cases NisP-mediated cleavage was measured after pH-induced ring closure. Ring closure in dehydrated prenisin was achieved by 1 h incubation at pH 8.0, which was followed by readjustment of the pH to 4.3. Treatment of peptide containing supernatant
with cells of NZ9000 containing pNGnisTP was performed during 4 h, followed by MALDI-TOF MS analysis.

*Induction assay.* The presence of nisin was also tested using the sensitive GusA assay (6) that monitors the capacity of nisin to induce the nisin promoter.

RESULTS

*Detection of the nisin leader peptide.* We first investigated whether the nisin leader peptide would remain present in the culture medium of a nisin-producing *L. lactis* NZ9700 strain. Peptides isolated from the supernatant of this strain reacted with anti-leader peptide antibodies (Fig. 2, lane 3). A Coomassie-stained gel of a C18 column elution fraction showed the presence of peptide (Fig. 3A) with a mass of 2350.9 Da (MALDI-TOF MS). An identical peptide (Fig. 3B) was observed when ziptip-treated supernatant from a nisin-producing *L. lactis* NZ9700 strain, grown overnight in minimal medium (33), was analyzed via mass spectrometry. This mass corresponds to the nisin leader peptide without the initiating methionine. In addition, peaks with masses corresponding to the nisin leader peptide with the initiating methionine (2482.2 Da) and of nisin (3354.2 Da) were detected (Fig. 3B). In control incubations with *L. lactis* strain NZ9000, which does not produce nisin, no peaks were observed. Synthetic leader peptide of residue 2-23 gave a mass peak identical to the peak we assigned to the cleaved leader peptide without the initiating methionine. These data for the first time demonstrated the presence of intact nisin leader peptide in the culture medium following secretion and processing.

<FIGURE 2>

<FIGURE 3AB>
**Thioether ring formation by NisBTC.** In order to investigate whether NisBTC are sufficient for thioether ring formation we cloned the *nisABTC* genes. Both uninduced (Fig. 2, lane 13) and induced PA1001 (Fig 2, lane 14) containing pBMDL5 produced prenisin. Apparently, when no inducing nisin was added, some transcription still occurred; this has previously also been reported for wild-type nisin producers (6). Subsequently, we analyzed the prenisin peptides. The uninduced strain PA1001 containing pBMDL5 produced two peptides with masses corresponding to dehydrated prenisin or fully modified prenisin with and without the initiating methionine (5817.4 and 5686.5 Da) and a third peptide corresponding to unmodified prenisin (5833.9 Da). Similar peptide masses were observed after induction of PA1001 containing pBMDL5 (Table 2), but the mass peak of dehydrated or fully modified prenisin (5688.9 Da) was more pronounced.

<Table 2>

The trypsin-treated supernatant of induced PA1001 containing pBMDL5 showed a growth inhibiting activity comparable to that of NZ9700, indicating the presence of fully modified prenisin. Uninduced cells showed a much lower activity. In addition, trypsin-treated culture medium of uninduced and nisin-induced PA1001 cells containing pBMDL5 gained in induction capacity as measured with the GusA assay (6).

The production of fully modified prenisin by PA1001 containing pBMDL5 was confirmed by overlaying agar plate cells with NZ9000 (Fig. 4A) and with NZ9000 containing pNGnisTP (Fig. 4B). As expected, NZ9700 (position 2) produced clear halos with both overlays, whereas PA1001 (position 1) did not form a halo. By contrast, PA1001 containing pBMDL5 (position 3) and TP9703 (position 4) only produced halos when overlayed with NZ9000 containing pNGnisTP (Fig. 4B). Consistent with the lower activity of trypsin-treated TP9703 supernatant, the halos produced by TP9703 were much smaller than of PA1001 cells containing pBMDL5. These data demonstrate that both induced- and -to a lesser extent-
uninduced PA1001 cells containing pBMDL5 produce fully modified prenisin, thus showing that the NisBTC enzymes are sufficient for the thioether ring formation.

<FIGURE 4>

Export of dehydrated prenisin via NisBT. Next we investigated the functionality of NisBT in the absence of NisC. Strikingly, NZ9000 cells containing pBMDL8B produced prenisin (Fig. 2, lane 12). Mass spectrometry analysis of the supernatant of these cells with plasmid-encoded NisABT, (the *nisA* gene of this construct having two leader peptide mutations: S –6 P and P –2 L) demonstrated the production of dehydrated prenisin (5711.2 Da) and unmodified prenisin (5857.0 Da) (Table 2). This shows that NisBT can act independently of NisC and also demonstrates that the NisT transport activity is not strictly coupled to full posttranslational modification of prenisin.

Modification and transport of a non-lantibiotic peptide. In order to investigate whether fusions of the leader with a non-lantibiotic peptide could be modified by NisB and transported by NisT, peptide production by NZ9000 containing pNGnisBT and pLP1ang, encoding a fusion of the leader peptide with NRSYICP, was investigated. Both unmodified (3317.9 and 3186.5 Da) and dehydrated fusions of leader peptide with angiotensin<sup>1-7</sup> (3302.9 and 3169.2 Da) with and without methionine<sub>1</sub>, were observed (Table 2). In order to confirm the observed dehydration, prior to analysis the peptide samples were treated with ethanethiol, which reacts with dehydroresidues. Indeed, after ethanethiol treatment, the peptide with the mass of dehydrated leader-angiotensin<sup>1-7</sup> had disappeared whereas a peptide corresponding to ethanethiol-modified dehydrated peptide appeared (3235.6 Da). As expected, ethanethiol treatment did not alter the mass of the non-dehydrated peptide (3188.6 Da). These data clearly prove that the fusion of leader peptide with angiotensin<sup>1-7</sup> was dehydrated and transported by NisBT.


**NisT has a broad substrate specificity.** We subsequently determined whether NisT in the absence of NisBC, is capable of transporting various unmodified fusion peptides containing the nisin leader peptide. Experiments were performed with NZ9000 cells containing two plasmids, one coding for NisT and the second for a leader peptide fusion. Export of unmodified prenisin (Fig. 2, lane 11), unmodified prenisin with a C-terminally fused enkephalin peptide (Fig. 2, lane 5), a fusion of the leader peptide with a vasopressin variant (Fig. 2, lane 7) and a fusion of the leader peptide with an angiotensin variant (Fig. 2, lane 9) was measured using anti-leader peptide antibodies. Mass spectra clearly demonstrated export of unmodified prenisin without the initiating methionine (5833.2 Da), of a fusion peptide of unmodified prenisin with a C-terminal enkephalin variant (6403.2 Da), of a fusion of nisin leader peptide with vasopressin (3405.2 Da) and of a fusion peptide of the nisin leader peptide with angiotensin-7 (3186.6 Da), (Table 2). Control experiments without pNGnisT and with strain NZ9743 (26) containing disrupted nisT showed no detectable levels of secreted peptide in the culture medium. Furthermore, in the cell fraction of induced NZ9743 cells an antibody-reactive peptide was detected (data not shown). Taken together the data clearly demonstrate that NisT can act independently of the other lantibiotic enzymes and further show that the substrate specificity of NisT is much wider than only the fully modified prenisin.

<Table 3>

**NisP is specific for thioether ring containing prenisin.** We measured which leader peptide-containing peptides could be cleaved by the leader peptidase. The leader peptide could neither be cleaved from the leader peptide-angiotensin fusion nor from the unmodified or dehydrated prenisin (Table 3). However, after keeping the dehydrated prenisin 1 h at pH 8.0, the leader peptide could be cleaved off. At pH 8.0 thioether rings can be closed spontaneously (36, 37) after which the peptide apparently had become substrate for the leader
peptidase. The pH 8.0- and NisP-treated dehydrated prenisin had however no antimicrobial activity (data not shown), which indicates that no fully modified prenisin had been formed. Fully modified prenisin itself was also cleaved by the leader peptidase, and a control experiment showed that, in the absence of leader peptidase, the pH 8.0 treatment alone did not liberate the leader peptide (Table 3).

In order to discriminate between NisT-dependent- and -independent NisP activity, a plasmid containing the nisP gene was constructed. An antimicrobial activity assay involving fully modified prenisin and mass spectrometry analysis confirmed that the NZ9000 cells containing pNGnisP expressed active NisP as they were able to cleave the leader peptide from externally added fully modified prenisin (data not shown). Hence, NisP can act independently of other lantibiotic enzymes.

**DISCUSSION**

The leader peptide of nisin may fulfill several functions. First, prior to export it may have a role in the posttranslational modification and recognition events (17, 38). Secondly, it is needed for recognition by the transport system, and third, it keeps the lantibiotic in an inactive state until maturation has taken place (4). Here, we demonstrated that the nisin leader peptide accumulates in the bacterial culture medium of nisin producing *L. lactis* cells.

The subtilin leader peptide has been shown to act as a translocation signal in *B. subtilis* (39) and in *E. coli* (40). Export of alkaline phosphatase of *E. coli* when fused with the subtilin leader peptide seemed to be enhanced in the presence of a transporter that is encoded within the subtilin operon (39). When the leader peptidase of subtilin is inhibited by
phenylmethyl-sulfonyl fluoride, accumulation of fully modified presubtilin, subtilin and a series of degradation products in the medium has been observed (41). Mutagenesis studies of the leader peptide of various lantibiotics revealed that some of the leader peptide residues are essential for export and possibly for interaction with the modifying enzymes (42-44). In contrast to the present work, it has also been suggested that fully modified prenisin is the only form of nisin recognized by the transporter (45).

A membrane-associated enzyme complex of NisBTC has been reported to be responsible for dehydration of the serine and threonine residues of prenisin, the thioether ring formation by cross-linking of dehydroresidues to cysteines, and the final export step (23). In nisin, Ser29 is not dehydrated whereas Ser33 sometimes escapes dehydration. Overexpression of NisB results in a more frequent dehydration of Ser33 (22). Here we report that the genes \textit{nisABTC} are sufficient for production and export of fully modified prenisin. Strikingly, we also demonstrate that NisB and NisT suffice to export dehydroresidue-containing peptides. This implies that ring formation is not a prerequisite for export. Dehydrated prenisin is, however, not a substrate for NisP which indicates that further modification is needed before NisP recognizes the prenisin as substrate. Incubation of the dehydrated form of prenisin at pH 8.0 results in spontaneous ring closure (36, 37). Under those conditions, the peptide becomes a substrate of NisP and the leader peptide can be cleaved off. \textit{L. lactis} NZ9000 with NisABT but without NisC indeed produced the dehydrated prenisin with the dehydroalanines and dehydrobutyrines present and without the ring closure. The serine and threonine residues in the leader peptide are never dehydrated as confirmed by mass spectrometry (Fig. 3B). NisBT-expressing cells produced the dehydrated prenisin with eight dehydrated serine and threonine residues; they also produced a fusion of leader peptide with dehydrated angiotensin$^{1-7}$ (Table 2). This implies that bacteria that only contain LanBT (homologs of NisBT) or LanT and the equivalent LanM part (the N-terminal
dehydration domain of LanM) can produce peptides with dehydroresidues. Therefore, two more amino acids are in principle available as building blocks for the synthesis of novel (poly)peptides with desired properties. Dehydroresidues have been reported to be essential for the activity of some bio-active (poly)peptides (46-51). Although the mechanism of NisB action is not known, statistical studies on the variability of the flanking regions of the eight dehydratable serine and threonine residues in nisin and related lantibiotics suggest that NisB, in contrast to the specific NisP, is equipped with a broad substrate specificity. Engineering of dehydroresidues in nisin and Pep5 has been demonstrated (3, 52). Therefore, a wide variety of peptides with dehydroresidues might be produced and exported via NisBT. In this context it is interesting to note that prePep5 fragments with dehydrated residues of Pep5 are exported (53) when the pepC gene is largely deleted. However, those studies all concern original lantibiotics and it would be of interest to introduce such residues in peptides that are normally not modified. Lantibiotic transporters are generally considered to export only specific lantibiotics synthesized by the gene products encoded in the same operon structure. Also some nisin-subtilin chimeras are exported (54) while nisin Z export can be directed by the subtilin leader peptide (55). Those studies, however, all pointed at a rather narrow substrate specificity. Here we demonstrate that the specificity of the NisT transporter is much wider than originally anticipated. Various unrelated peptides, in either a modified form (in the presence of NisB) or an unmodified form (in the absence of NisB) can be secreted provided that they are fused to the leader peptide.

Previous attempts to demonstrate functionally active NisP upon overproduction in L. lactis have not been conclusive (4). Here we show that, in the absence of other lantibiotic enzymes, NisP can be functionally expressed in L. lactis. The enzyme shows a clear leader peptidase activity on fully modified prenisin. These data furthermore demonstrate that the NisP activity is not coupled to the transport step by NisT. This agrees with observations that
extracellularly added fully modified prenisin is processed by *L. lactis* NZ9800 (4). Remarkably, neither the dehydrated prenisin nor the leader peptide fusions were cleaved by NisP. This strongly suggests that the leader peptidase is specific for thioether ring-containing prenisin. In this respect, the dehydrated prenisin became a substrate for NisP after pH-induced ring closure which is very suggestive of region- and stereospecific closure of one or more rings. Using model peptides non-enzymatic, stereospecific ring closure has been shown for ring B (36, 37) and ring E (37) of nisin, whereas region specificity and a three to one stereo preference was shown for ring A of subtilin (37). These data indicate that production of dehydroresidue-containing peptides may be followed by extracellular specific ring closure, e.g. at pH 8.0.

Thioether rings are essential for most lantibiotic activities. Opening of ring A or C (9) or replacement of a thioether ring by a disulfide bridge and reducing it (56) causes a severe loss of activity. In addition, the rings can protect (poly)peptides against proteolytic degradation (3, 4) and their presence may modulate the activity of peptides (2). Active lanthionine analogs of somatostatin and enkephalin have been synthesized chemically (2, 57), but this involved elaborate methods that more easily could be performed by a fermentative route. The transport by NisT of medically relevant therapeutic peptides like enkephalin, vasopressin and dehydrated angiotensin (variants) compel further research on modifying such peptides by the lantibiotic enzymes.

Summarizing, we have shown that NisBT is sufficient to dehydrate and export the dehydrated non-lantibiotic angiotensin\(^1-7\), the dehydrated prenisin, and that the NisT transporter is equipped with a wide substrate specificity, transporting various peptides provided they are fused to the nisin leader peptide. Production of peptides via NisBT provides an adequate system to study the substrate specificity of NisB, and may enable the synthesis and export of a wide variety of peptides with dehydroresidues. This process can
then be followed by extracellular stereospecific ring closure to avoid possible export incompatibilities of bulky thioether ring containing peptides.

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REFERENCES


Fig. 1. Posttranslational modifications of prenisin.

1: Serines and threonines of unmodified prenisin are dehydrated by NisB. 2: The resulting dehydrated prenisin contains dehydroalanines (Dha) and dehydrobutyrines (Dhb). 3: NisC forms thioether rings between dehydrated residues and cysteines resulting in fully modified prenisin. 4: NisT transports the fully modified prenisin and NisP cleaves off the leader peptide liberating nisin.
Fig. 2. Extracellular peptide detection by anti-leader peptide antibodies.

*L. lactis* cells were induced with nisin (+) or not induced (-), incubated for 4 h (NZ9000 containing pNGnisT and pLP1vp) or overnight, and subjected to TCA precipitation and Western blotting. Lanes 1, 2: strain TP9703; lane 3 strain NZ9700; lanes 4, 5: strain NZ9000 containing pNGnisT and pNGenkT; lanes 6, 7: strain NZ9000 containing pNGnisT and pLP1vp; lanes 8, 9: strain NZ9000 containing pNGnisT and pLP1ang; lanes 10, 11: strain NZ9000 containing pNGnisT and pNZnisA-E3; lane 12: strain NZ9000 containing pBMDL8b; and lanes 13, 14: strain PA1001 containing pBMDL5. Each experiment was repeated at least three times with similar results.
Fig. 3. Isolation of nisin leader peptide from culture medium.

Fig. 3A: Leader peptide isolated from *L. lactis* NZ9700 culture medium by binding to teflon beads and a C18 column was subjected to gel electrophoresis and stained with Coomassie.

Fig. 3B: Detection of the nisin leader peptide by MALDI-TOF MS. The supernatant of overnight *L. lactis* NZ9700 grown on minimal medium was ziptip-treated followed by MALDI-TOF MS analysis. Expected average masses (M+H<sup>+</sup>) are for the nisin leader peptide residues 2-23: 2352.6 Da, nisin leader peptide residues 1-23: 2483.8 Da, nisin: 3355.2 Da. The experiment shown is a typical result that was repeated more than ten times with identical results.
Fig. 4. Leader peptidase activity in NisP producing cells.

PA1001 (position 1), NZ9700 (2), PA1001 containing pBMDL5 (3) and TP9703 (4) cells were grown overnight on agar plates that contained no antibiotic next to inducing amounts of nisin. Subsequently, the cells were overlayed with log phase-grown sensitive strain NZ9000 (A) and NZ9000 containing pNGnisTP (B) cells and further grown for one more night. The size of the halo indicates the presence of active nisin processed by NisP. The experiment was repeated three times in duplicate with similar result.
Table 1. *Lactococcus lactis* strains and plasmids

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<td>Derived from NZ9700 by disruption of <em>nisA</em></td>
<td>(21)</td>
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<td>NZ9743</td>
<td>Derived from NZ9700 by disruption of <em>nisT</em></td>
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<td>NZ9000</td>
<td><em>nisRK</em></td>
<td>(25)</td>
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<td>PA1001</td>
<td>ΔacmA (27), ΔhtrA (28), derived from NZ9000</td>
<td>This study</td>
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<tr>
<td>TP9703</td>
<td>Δ<em>nisP</em>, derived from NZ9700</td>
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**pNZ8048 derived plasmids**

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<th>Plasmid</th>
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<tr>
<td>pBMDL8b</td>
<td><em>nisABT</em> (S-6P, P-2L <em>nisA</em>) in a gateway plasmid. An inverted repeat is present between <em>nisA</em> and <em>nisB</em> as on the chromosome of NZ9700, Em-r</td>
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<td>pNGnisBT</td>
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</table>

*Em-r:* erythromycin resistance gene  
*Cm-r:* chloramphenicol resistance gene
**Table 2.** Modification and export of peptides in *L. lactis*. Cells were induced and grown 4 h (NZ9000 containing pNGnisT and pNGang, NZ9000 containing pNGnisT and pLP1vp), or overnight in minimal medium followed by TCA precipitation and/or direct ziptip treatment of the supernatant and MALDI-TOF MS. Experiments were repeated at least three times with similar result. Theoretical values are average masses in Da (M+H^+).

<table>
<thead>
<tr>
<th><em>L. lactis</em> strain</th>
<th>plasmids</th>
<th>Peptide</th>
<th>Observed mass (Da) (M+H^+)</th>
<th>Theoretical mass with Methionine</th>
<th>Theoretical mass w/o Methionine</th>
</tr>
</thead>
<tbody>
<tr>
<td>NZ9000</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PA1001</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PA1001</td>
<td>pBMDL5 (nisABTC)</td>
<td>Fully modified preisin</td>
<td>5688.9 5820.3</td>
<td>5820.0 5688.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Unmodified preisin</td>
<td>5836.3</td>
<td>5964.0 5832.8</td>
</tr>
<tr>
<td>NZ9000</td>
<td>pBMDL8b (nisABT)</td>
<td>Fully dehydrated S-6P, P-2L preisin</td>
<td>5711.2 5846.1</td>
<td>5714.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Unmodified preisin</td>
<td>5857.0</td>
<td>5990.1 5858.9</td>
</tr>
<tr>
<td>NZ9000</td>
<td>pNGnisBT pLP1ang</td>
<td>Leader peptide fused to angiotensin:</td>
<td>3186.5 3317.9</td>
<td>3317.8 3186.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Dehydrated</td>
<td>3169.2 3302.9</td>
<td>3299.8 3168.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Dehydrated, ethanethiol addition</td>
<td>3235.6</td>
<td>3362.8 3231.6</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Unmodified, (ethanethiol)</td>
<td>3186.6</td>
<td>3317.8 3186.6</td>
</tr>
<tr>
<td>NZ9000</td>
<td>pNGnisT pNZnisA-E3</td>
<td>Unmodified preisin</td>
<td>5833.2 5964.0</td>
<td>5832.8</td>
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<tr>
<td>NZ9000</td>
<td>pNGnisT pNGenkT</td>
<td>Unmodified preisin C-terminally fused to enkephalin</td>
<td>6403.2 6535.6</td>
<td>6404.4</td>
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<tr>
<td>NZ9000</td>
<td>pNGnisT pLP1vp</td>
<td>Leader peptide fused to vasopressin</td>
<td>3405.2 3537.0</td>
<td>3405.8</td>
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<tr>
<td>NZ9000</td>
<td>pNGnisT pLP1ang</td>
<td>Leader peptide fused to angiotensin</td>
<td>3186.6 3317.8</td>
<td>3186.6</td>
<td></td>
</tr>
</tbody>
</table>
Table 3. The leader peptidase is specific for thioether ring containing prenisin. Cleavage of peptides by NisP was measured by MALDI-TOF MS. NisP was expressed by the peptide producing cells or cells expressing NisP were added or –in control experiments—no NisP was present. By incubating one hour at pH 8.0 thioether ring closure in dehydrated prenisin was induced. Theoretical values are average masses in Da (M+H+).

<table>
<thead>
<tr>
<th>L. lactis strain</th>
<th>Plasmid</th>
<th>Post-treatment</th>
<th>Peptide</th>
<th>Observed mass (Da) (M+H+)</th>
<th>Theoretical mass after cleavage</th>
<th>Theoretical mass w/o cleavage</th>
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</thead>
<tbody>
<tr>
<td>NZ9000</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>PA1001</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
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</tr>
<tr>
<td>NZ9000</td>
<td>pLP1vp + pNGnisTP</td>
<td>-</td>
<td>leader peptide-vasopressin</td>
<td>-</td>
<td>2352.6</td>
<td>1072.2</td>
</tr>
<tr>
<td>NZ9000</td>
<td>pLP1ang + pNGnisTP</td>
<td>-</td>
<td>leader peptide-angiotensin</td>
<td>3187.1</td>
<td>2352.6</td>
<td>853.0</td>
</tr>
<tr>
<td>NZ9000</td>
<td>pNZnisA-E3 + pNGnisTP</td>
<td>-</td>
<td>unmodified prenisin</td>
<td>5836.3</td>
<td>2352.6</td>
<td>3499.2</td>
</tr>
<tr>
<td>NZ9000</td>
<td>pBMDL8b (nisABT) NZ9000/pNGnisTP cells</td>
<td>dehydrated S-6P, P-2L prenisin</td>
<td>5714.9</td>
<td>2378.7</td>
<td>3355.2</td>
<td>5714.9</td>
</tr>
<tr>
<td>NZ9000</td>
<td>pBMDL8b (nisABT) pH 8.0 and NZ9000/pNGnisTP cells</td>
<td>dehydrated S-6P, P-2L prenisin with 1 or more thioether rings</td>
<td>2377.1</td>
<td>3349.8</td>
<td>2378.7</td>
<td>3355.2</td>
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<tr>
<td>PA1001</td>
<td>pBMDL5 (nisABTC) pH 8.0</td>
<td>fully modified prenisin</td>
<td>5686.3</td>
<td>2352.6</td>
<td>3355.2</td>
<td>5688.8</td>
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<tr>
<td>PA1001</td>
<td>pBMDL5 (nisABTC) NZ9000/pNGnisTP cells</td>
<td>leader peptide and fully modified nisin</td>
<td>2351.9</td>
<td>3353.9</td>
<td>2352.6</td>
<td>3355.2</td>
</tr>
</tbody>
</table>
Figure 1
Figure 2
Figure 3

Dissection of the Nisin Modification and Transport Machinery

2200 2400 2600 2800 3000 3200 3400 3600

M/Z

Relative intensity

2350.9 2482.2

3354.2

2482.2

3354.2

2350.9

2200 2400 2600 2800 3000 3200 3400 3600

M/Z

Relative intensity

2350.9 2482.2

3354.2

2482.2

3354.2

2350.9

KDa

20 25

10 15
Figure 4
NisT, the transporter of the lantibiotic nisin, can transport fully modified, dehydrated and unmodified prenisin and fusions of the leader peptide with non-lantibiotic peptides
Anneke Kuipers, Esther de Boef, Rick Rink, Susan Fekken, Leon D. Kluskens, Arnold J.M. Driessen, Kees Leenhouts, Oscar P. Kuipers and Gert N. Moll

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