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# Microbial production of thioether-stabilized peptides

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## Mechanistic dissection of the enzyme complexes involved in the biosynthesis of lacticin 3147 and nisin


### Abstract

The thioether rings in the lantibiotics lacticin 3147 and nisin are post-translationally introduced by dehydration of serines and threonines followed by coupling of these dehydrated residues to cysteines. The prepeptides of the two-component lantibiotic lacticin 3147, LtnA1 and LtnA2, are dehydrated and cyclized by two corresponding bifunctional enzymes, LtnM1 and LtnM2, and are subsequently processed and exported via one bifunctional enzyme LtnT. In the nisin synthetase comlex the enzymes NisB, NisC, NisT and NisP respectively dehydrate, cyclize, export and process prenisin. Here we demonstrate that the combination of LtnM2 and LtnT can modify, process and transport peptides entirely different from LtnA2 and that LtnT can process and transport unmodified LtnA2 and unrelated peptides. Furthermore we demonstrate a higher extent of NisB-mediated dehydration in the absence of thioether rings. Thioether rings apparently inhibited dehydration, which implies alternating action of NisB and NisC. Furthermore, specific –but not all- NisC-cyclized peptides were exported with higher efficiency as a result of their conformation. Taken together, these data provide further insight into the applicability of *Lactococcus lactis* containing lantibiotic enzymes for the design and production of modified peptides.



Appl Environ Microbiol. 2008 ;**74**:6591-7

## Introduction

The lantibiotics nisin and lacticin 3147 are produced by some distinct *Lactococcus lactis* species and inhibit a broad range of Gram-positive bacteria. Lantibiotics are peptide antibiotics that contain thioether-bridged amino acids: lanthionines and methyllanthionines (23, 118, 171). By binding to lipid II, the essential precursor of bacterial cell wall peptidoglycan, they inhibit cell wall synthesis and form hybrid pores in the membrane of the target cell (13, 15, 16, 56, 222, 223).

Two main classes of lantibiotics are discerned. In one class (class I) , comprising nisin (90), dehydration and cyclization are catalyzed by separate LanB and LanC enzymes. The nisin dehydratase NisB dehydrates serines and threonines of the prepeptide NisA. Subsequently, NisC couples the formed dehydroalanine and dehydrobutyrine to cysteines to form lanthionine or methyllanthionine, respectively. The ABC transporter NisT exports modified prenisin out of the cell (83, 153).

In the second class, comprising amongst others lacticin 481 (156), and the two-component lantibiotics and lacticin 3147 (168), dehydration and cyclization are performed by bifunctional LanM enzymes (23). During synthesis of lacticin 3147 each of the prepeptides LtnA1 and LtnA2 is modified by a separate modification enzyme, respectively LtnM1 and LtnM2 (119). Lacticin 3147 also contains other modifications resulting from conversion of dehydroalanines to D-alanines catalyzed by the enzyme LtnJ (28, 169). The modified peptides Ltnα and Ltnβ are processed and transported by LtnT. Both components are essential for the activity of the lantibiotic. Ltnα resembles mersacidin in its globular shape, and Ltnβ has some structural similarity to nisin (Fig. 1) (116, 118).

The discovery and development of novel antibiotics, is urgent because of the increase in resistance to multiple antibiotics. Lantibiotic mutants with modulated activity have been described (159). Furthermore the application of lantibiotic enzymes can generate biostable thioether-bridged therapeutic peptides, which are resistant against proteolytic degradation (55, 78). Thioether bridged peptides may also have modulated receptor interaction and extended delivery possibilities.

Several studies indicated that lantibiotic-modifying and transporter enzymes are organized in multimeric complexes (75, 226, 134). It was demonstrated before that cells containing all lacticin 3147 biosynthesis enzymes, except LtnM1, still produced Ltnβ (119). Here we studied whether in *L. lactis* either the combination of LtnM2 and LtnT or LtnT alone is functional and whether peptides that are entirely different from LtnA2 are LtnM2 and LtnT substrates.

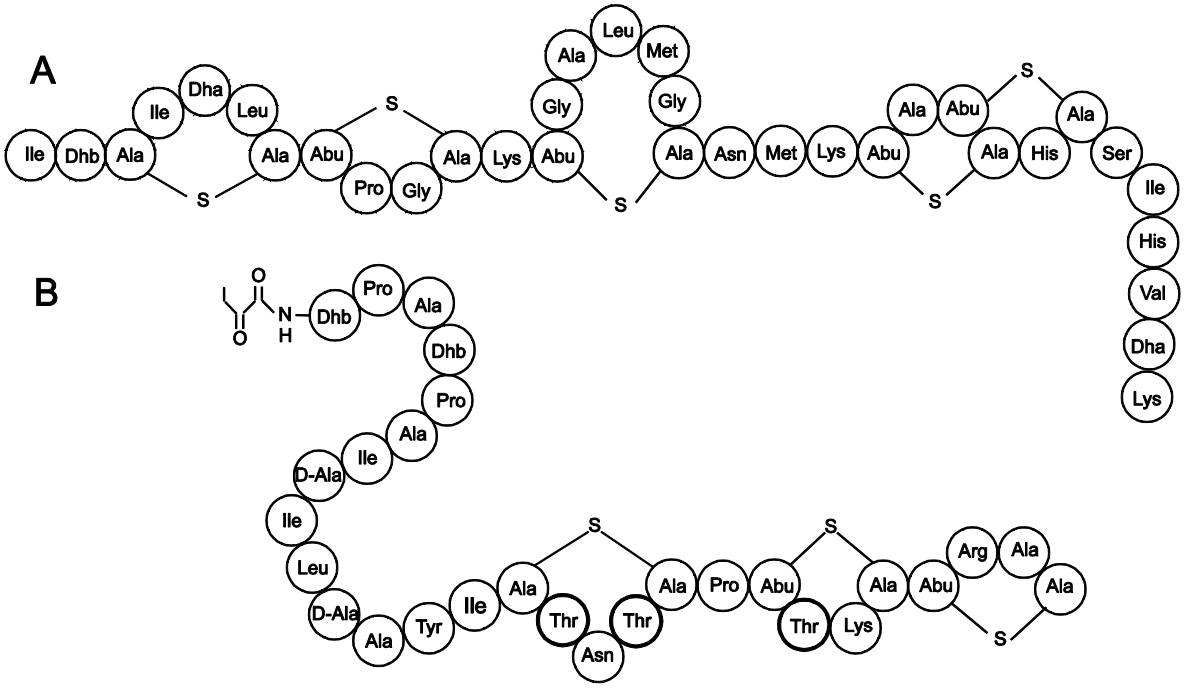


**Figure 1**. Nisin (A) and processed, fully modified Ltnβ peptide (B). In Ltnβ both threonines included in lanthionine ringA and the one threonine included in methyllanthionine ringB are not dehydrated.

Intriguingly, three threonines in the Ltnβ peptide (Fig. 1) that are included in the thioether rings are not dehydrated by LtnM2. In contrast NisB dehydrates threonines much better than serines and can also succesfully generate polydehydrobutyrine (157, 158). In addition we therefore studied the dehydration of lacticin A2-derived peptides corresponding to these ring-containing sequences by dissected enzyme complexes of the lacticin 3147 and nisin biosynthesis machineries.

## Materials and Methods

*Bacterial Strains and Plasmids***.** *L. lactis* NZ9000 was used for expression of the modification enzymes and peptides. The modification genes and transporter genes, *ltnM2T, nisBTC* were cloned into pIL253-derived plasmids (192) behind the nisin-inducible promoter. The encoding sequences for the N-terminal lacticin leader LtnA2 or nisin leader were fused to the sequence, encoding the substrate peptide, and placed under control of the nisin inducible promoter of pNZ8084-derived plasmids (93). Strains and plasmids are listed in Table 1.

*Molecular cloning***.** The *ltnA2* and *ltnTM2* genes were amplified from pBAC105, isolated from *L. lactis* IFPL105 and the *nisA* and *nisBTC* genes were amplified from chromosomal DNA of *L. lactis* NZ9700. PCR reactions were performed with Phusion DNA polymerase (Finnzymes, Finland). Restriction enzymes used for cloning strategies were purchased from New England Biolabs Inc.. Ligation

was carried out with T4 DNA ligase (Roche, Mannheim, Germany). Sequences encoding peptides different from the original propeptides were genetically fused to the leader or propeptide by means of PCR (87). Electrotransformation of *L. lactis* was carried out as previously described (61) using a Biorad gene pulser (Richmond, CA). Nucleotide sequence analysis was performed by BaseClear (Leiden, NL).

| Strain or plasmid | Characteristic(s) | Source or reference |
|---|---|---|
| **Strains** | | |
| NZ9700 | *nisABTCIPRKFEG* | (90) |
| NZ9000 | MG1363 derivative; *pepN*::*nisRK*$^+$ | (91) |
| IFPL105 | pBAC105 | (117) |
| **Plasmids** | | |
| pIL253-derived | | (192) |
| pIL3BTC | *nisBTC* cloned behind the *Pnis* promoter; Cm$^r$ | (157) |
| pIL3BT | *nisBT* behind the *Pnis* promoter; Cm$^r$ | (15) |
| pIL31BTdC | Cyclase activity knocked out by mutation C326A in NisC | This study |
| pILPTM2 | *ltnT-ltnM2* cloned behind the *Pnis* promoter; Em$^r$ | This study |
| pILPltnT-2 | Disruption of *ltnM2* by BglII digestion; Em$^r$ | This study |
| pILPTM2-C818A | Substitution by means of PCR; Em$^r$ | This study |
| pNZ8048-derived | | (93) |
| pNZang1-7.2 | *Pnis* + encoding leader peptide NisA::NisA(1-17) :: IEGR::DKTYICP; Em$^r$ | This study |
| pNZang1-7.3 | *Pnis* + encoding leader peptide NisA::NisA(1-17):: IEGR::DATYICP; Em$^r$ | This study |
| pNZang1-7.8 | *Pnis* + encoding leader peptide NisA::NisA(1-17):: IEGR::DTVYCHP; Em$^r$ | This study |
| pA25-7.2 | *Pnis* + encoding leader peptide LtnA2::NisA(1-17):: IEGR::DKTYICP; Cm$^r$ | This study |
| pA25-7.3 | *Pnis* + encoding leader peptide LtnA2::NisA(1-17):: IEGR::DATYICP; Cm$^r$ | This study |
| pA25-7.8 | *Pnis* + encoding leader peptide LtnA2::NisA(1-17):: IEGR::DTVYCHP; Cm$^r$ | This study |
| pA24 | *Pnis* + encoding prepeptide LtnA2; Cm$^r$ | This study |
| pA27 | *Pnis* + encoding leader peptide LtnA2 ::TTPATPAISILSAYI; Cm$^r$ | This study |
| pA28 | *Pnis* + encoding prepeptide LtnA1 plus leader peptide LtnA2::ILSAYISTNTCPTTKCTRAC; Cm$^r$ | This study |
| pNZ29 | *Pnis* + encoding leader peptide NisA::ILSAYISTNTCPK; Em$^r$ | This study |
| pNZ30 | *Pnis* + encoding leader peptide NisA::ILPTTKCTRAC; Em$^r$ | This study |
| pNZ31 | *Pnis* + encoding leader peptide NisA::ILPTTKCARAA; Em$^r$ | This study |
| pNZ32 | *Pnis* + encoding leader peptide NisA::ILPTTKATRAA; Em$^r$ | This study |
| pTP-DSRWARVALID SQKAAVDKAITDIAEKL | *Pnis* + encoding leader peptide NisA::DSRWARVALIDSQKAAVDKAITDIAEKL; Em$^r$ | (157) |
| pTPtppii | *Pnis* + encoding leader peptide NisA::ITSISRASVA; Em$^r$ | (77) |

*Mass spectrometry***.** Samples were purified from the medium fraction by ziptip purification (C18 ziptip, Millipore) or directly spotted by putting 1 µl of the supernatant on the target. After drying, the spots were washed once with 4 µl MQ to

remove the salts. Subsequently, 1 µl of matrix (10 mg/ml α-cyano-4-hydroxycinnamic acid in 50% acetonitrile containing 0.1% (v/v) TFA) was added to the target and allowed to dry. CDAP (1-cyano-4-dimethylaminopyridinium tetrafluoroborate) was used to react with free cysteine residues. The vacuum-dried sample was resuspended in 9 µl 25 mM citrate buffer, pH 3.0, and reduced with 1 µl Tris[2-carboxyethyl]phosphine (TCEP) (10 mg/ml in MQ). After a 10 min incubation at room temperature 2 µl of CDAP (10 mg/ml in 100 % acetonitril) was added, followed by 15 min. of incubation at room temperature. Mass spectra were recorded with a Voyager DE PRO Maldi-TOF mass spectrometer in the linear mode (Applied Biosystems). In order to maintain high sensitivity, an external calibration was applied. All measurements were performed in at least three independent experiments with identical results.

*Gel electrophoresis***.** *L. lactis* cells were induced with nisin and grown further in minimal medium. Peptides were isolated from the supernatant using disposable SPE Bond Elut C18 cartridges from Varian. After applying the supernatant, the column was washed with MQ containing 0.1 % TFA and peptides were eluted with 100 % methanol containing 0.01 % TFA. Peptides out of 1 ml supernatant were applied on tricine gel (181). Analysis was performed using the Silver Staining Kit of Invitrogen or by Coomassie staining.

## Results

*Functionality of the enzymes LtnM2T***.** In order to analyze the functionality of the enzymes LtnM2 and LtnT in *L. lactis*, pILPTM2 was co-expressed with pA24, encoding the natural substrate LtnA2. Maldi-TOF analysis of the supernatant revealed a mass of 2846 Da identical to the expected mass of the 8-fold dehydrated Ltnβ peptide, lacking conversions of two dehydroalanines to D-alanines due to the absence of the enzyme LtnJ (169, 28) and which has undergone an N-terminal deamination yielding an α-keto amide (+1 Da) (25) (Fig. S1). Clearly the enzymes LtnM2 and LtnT, in the absence of any other lacticin 3147 enzyme, succesfully modified, processed and translocated the prepeptide LtnA2.

*LtnM2T dehydrate, process and export peptides comprising angiotensin-(1-7) variants.* We assessed the versatility of LtnM2T to modify and produce angiotensin-(1-7) variants and compared the results with an existing angiotensin-producing NisBTC system. Sequences coding for peptides comprising nisin(1-17) and angiotensin-(1-7) variants DKTYICP, DATYICP and DTVYCHP were placed behind the lacticin A2 leader or nisin leader. The encoding plasmids were each co-expressed in *L. lactis* with respectively pILPTM2 and pIL3BTC. The extent of LtnM2T- or NisBTC-mediated dehydration and translocation of the peptides in the supernatant of induced cultures was analyzed by Maldi-TOF (Table 2, Fig. 2AB, Fig. S2AB) and silver-stained tricine gels (Fig. 3). The amount of peptide produced via the NisBTC

system (Fig. 3, lanes 4-6) was higher than via the LtnM2T system (Fig. 3, lanes 1-3). LtnM2T-exported, dehydrated and processed peptides were clearly detectable by Maldi-TOF (Fig. 2B and Fig. S2AB). All three constructs were evidently dehydrated up to 5-fold by NisB (Table 2, Fig. 2A), whereas LtnM2-mediated dehydration was in two cases up to five-fold and for the peptide encoded by pA25-7.8 even up to 6-fold (fully) (Table 2, Fig. S2AB and Fig. 2B). In all cases 5-fold dehydration was the major product, implying that the effciency of LtnM2- and NisB-mediated dehydration of the peptides did not differ to a large extent.

TABLE 2. Comparison of peptide sequences fused to the nisin leader, modified and secreted by NisBTC with the same peptide sequences fused to the LtnA2 leader, modified, processed and secreted by LtnM2T.

| NisBTC | | LtnM2T | |
|---|---|---|---|
| **plasmid** | **max. dehydrations** | **plasmid** | **max. dehydrations** |
| pNZang1-7.2 | 5 x | pA25-7.2 | 5 x |
| pNZang1-7.3 | 5 x | pA25-7.3 | 5 x |
| pNZang1-7.8 | 5 x | pA25-7.8 | 6 x |

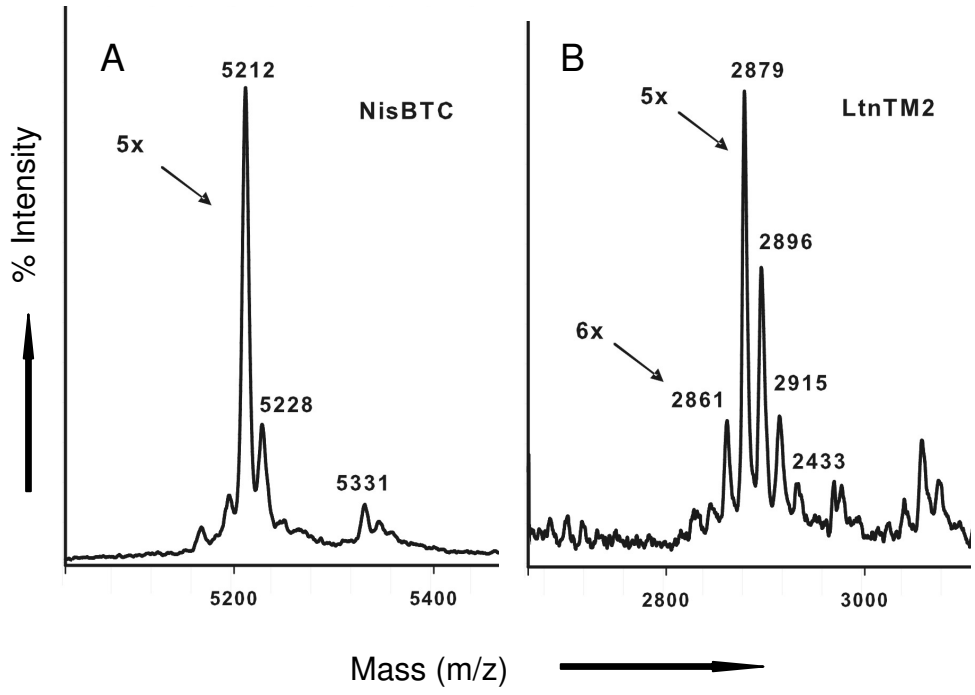


**Figure 2AB**. **Dehydration of angiotensin fusion peptides by NisB and LtnM2**. Culture supernatant was analyzed by Maldi-TOF as described in the methods section. (A) Supernatant of *L. lactis* NZ9000 pIL3BTC pNZang1-7.8. Expected mass of the protonated fully dehydrated fusion peptide (STKDFNLDLVSVSKKDSGASPRITSISLCTPGCKTGALMIEGRDTVYCHP) is 5194 Da. The main peak, 5212 Da, represents the 5-fold dehydrated peptide. (B) Supernatant of *L. lactis* NZ9000 pILPTM2 pA25-7.8. Expected mass of the processed, protonated and fully dehydrated peptide (ITSISLCTPGCKTGALMIEGRDTVYCHP) is 2861 Da.

The observed masses of the peptides in the supernatant indicated correct LtnT-mediated processing and export. In the absence of the transporter no peptide was detected in the supernatant excluding lysis or other translocation mechanisms. These data clearly prove that LtnM2 is capable of dehydrating peptides that are entirely different from its natural substrate. Importantly, these data also show that LtnT can subsequently remove the leader peptide and translocate the modified peptides *in vivo*.

*LtnT stays active in the absence of LtnM2***.** We investigated whether LtnT itself, in the absence of the other lacticin 3147 synthetase enzymes, retains activity. When LtnT was co-expressed with LtnA2 or the DTVYCHP-comprising peptide preceded by the LtnA2 leader, processed peptides could be identified in the supernatant (Fig. S3AB). In the absence of the transporter no peptide was detected in the supernatant excluding lysis or other translocation mechanisms. These results indicate that functional dissection of LtnT from the other synthetase enzymes is possible. LtnT can process and translocate LtnA2 and unrelated substrate peptides provided that the LtnA2 leader is present.

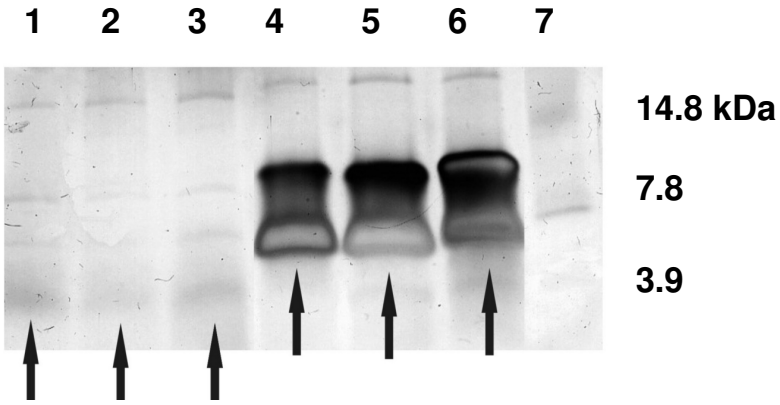


**Figure 3**. **Secretion of modified peptides by LtnT and by NisT**. *L. lactis* cells were induced with nisin and grown overnight in minimal medium. Peptides out of 1 ml supernatant were applied on a tricine gel. The gel was stained using the Silver Staining Kit of Invitrogen. *Lane 1*: NZ9000 pILPTM2 pA25-7.2; *Lane 2*: NZ9000 pILPTM2 pA25-7.3; *Lane 3*: NZ9000 pILPTM2 pA25-7.8; *Lane4*: NZ9000 pIL3BTC pNZang1-7.2; *Lane 5*: NZ9000 pIL3BTC pNZang1-7.3; *Lane 6*: NZ9000 pIL3BTC pNZang1-7.8; *Lane 7*: Kaleidoscopic marker of Biorad. In Lanes 6, 7 and 8, clearly dimers were detected likely resulting from high peptide concentrations.

*Do thioether rings prevent the dehydration of threonines in the peptide Ltnβ?* In the natural, post-translationally modified Ltnβ peptide two threonines included in the first lanthionine ring and one threonine included in the second methyllanthionine are not dehydrated by LtnM2 (Fig. 1). In order to determine whether dehydration is impaired by the thioether rings installed by LtnM2, an LtnM2 mutant was made in which the cyclase activity was knocked out by exchanging the conserved cysteine at position 818 in LtnM2 (107), resulting in plasmid pILPTM2-C818A. However, when

LtnA2-encoding pA24 was co-expressed with pILPTM2-C818A, secretion of the dehydrated natural substrate LtnA2 could not be detected in the supernatant by Maldi-TOF. In the absence of cyclase activity spontaneous formation of not-original large rings in the LtnA2 peptide can not be excluded and these might hamper the processing or transport by LtnT. The peptide variants comprising angiotensin-(1-7), encoded by the plasmids pA25-7.2 and pA25-7.8 and co-expressed with pILPTM2-C818A, were successfully dehydrated, processed and translocated, indicating that the dehydratase activity of C818A-LtnM2 and the processing and transport activity of LtnT were unaltered (data not shown).

Moreover, when truncations of the LtnA2 peptide, TTPATPAISILSAYI (pA27) and ILSAYISTNTCPTTKCTRAC (pA28) were preceded by the lacticin A2 leader-peptide and co-expressed with the enzymes LtnM2 and LtnT, no secreted peptides could be detected, neither processed nor unprocessed. Apparently processing and / or the transport by LtnM2T has substrate-specific limitations. For further studies on the possible influence of thioether rings on dehydration we therefore continued this study by applying the nisin biosynthesis NisBT(C) enzymes.

*Enhanced dehydration in the absence of NisC***.** When the truncated variants of the LtnA2 peptide, ILSAYISTNTCPK and ILPTTKCTRAC preceded by the nisin leader and encoded by the plasmids pNZ29 and pNZ30, were co-expressed with pIL3BT (Fig. 4AC) or pIL3BTC (Fig. 4BD) a higher extent of dehydration was observed in the absence of NisC. In this context it seems that the threonine residues that are not dehydrated by LtnM2 can be dehydrated by NisB. To verify whether unmodified threonines in the thioether rings were involved, the C-terminally adapted fragment of LtnA2, ILPTTKCARAA, was fused to the nisin leader (pNZ31). Co-expression of pNZ31 with pIL3BT (Fig. 4E) or pIL3BTC (Fig. 4F) showed again a higher extent of dehydration of the peptide in the absence of NisC. Incubations with TCEP and CDAP and the lack of CDAP modification, confirmed that the peptide ILPTTKCARAA was almost fully cyclized by NisC (Fig. S4AB).

Control experiments provided consistent data. The cysteines in the peptide ILPTTKCTRAC were exchanged by alanines leading to ILPTTKARAA encoded by pNZ32. This plasmid was co-expressed with pIL3BTC (Fig. S5A) or pIL3BT (Fig. S5B) which resulted in an identical extent of dehydration. Furthermore, knocking out the cyclase activity by the mutation C326A (107) resulting in the the plasmid pIL31BTdC did not alter the extent of peptide dehydration. Identical dehydration levels were observed when co-expressing with pIL31BTdC or pIL3BT (data not shown). Expression of the activity-deficient NisC mutant was confirmed by Western blotting (data not shown). All these data clearly demonstrate reduced dehydration in the presence of the cyclase NisC, consistent with reduced dehydration of threonines that are included in the thioether rings.

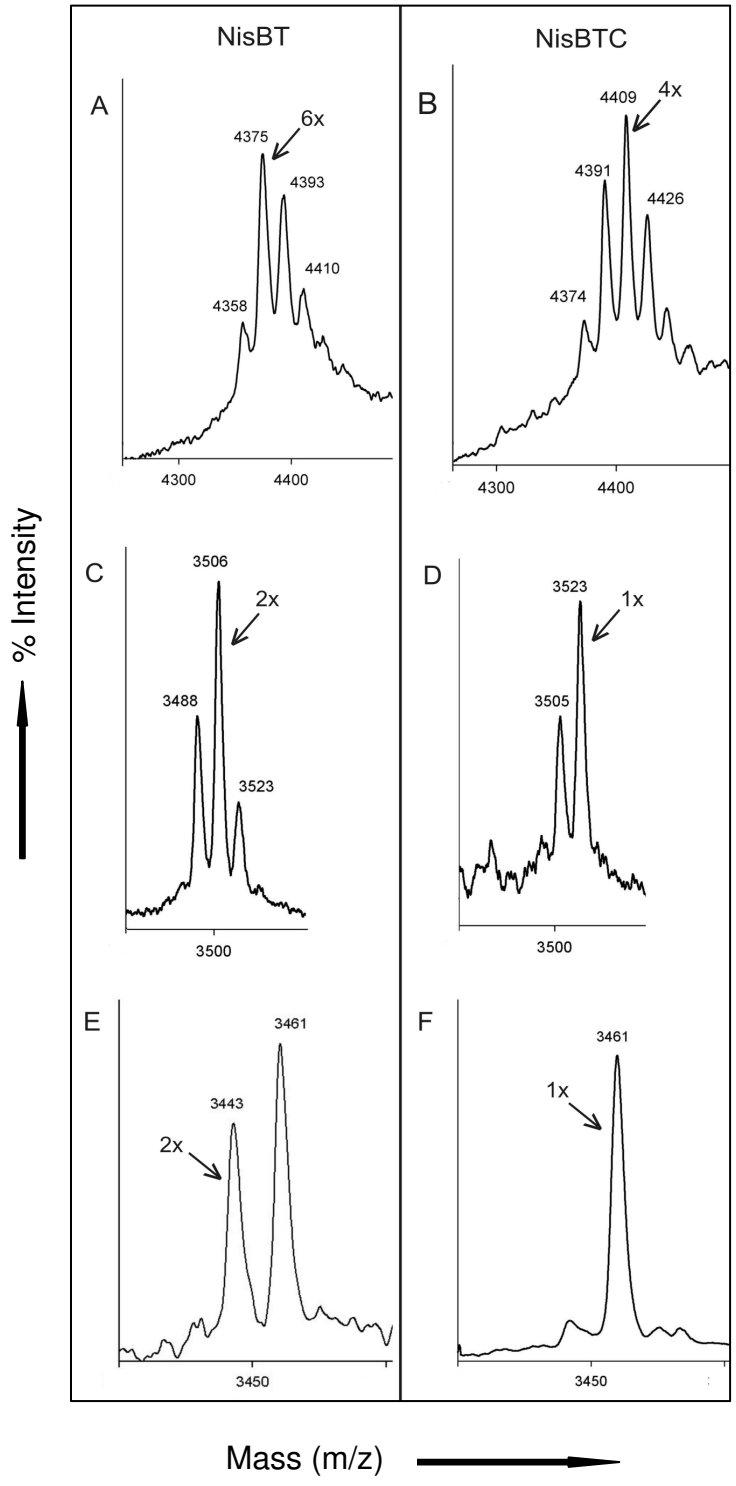


**Figure 4ABCDEF**. **Enhanced dehydration by NisBT compared to NisBTC**. Culture supernatant was analyzed by Maldi-TOF. Expected masses of the fully dehydrated and protonated fusion peptides encoded by: pNZ29 (nisin leader::ILSAYISTNTCPK): 3672 Da; pNZ30 (nisin leader::ILPTTKCTRAC): 3487 Da and pNZ31 (nisin leader-ILPTTKCARAA): 3443 Da. (A) pNZ29 co-expressed with pIL3BT, main peak is of peptide 4-fold dehydrated; (B) pNZ29 co-expressed with pIL3BTC, main peak is of peptide 2-fold dehydrated; (C) pNZ30 co-expressed with pIL3BT, main

peak is of peptide 2-fold dehydrated; (D) pNZ30 co-expressed with pIL3BTC, main peak is of peptide 1-fold dehydrated; (E) pNZ31 co-expressed with pIL3BT; (F) pNZ31 co-expressed with pIL3BTC.

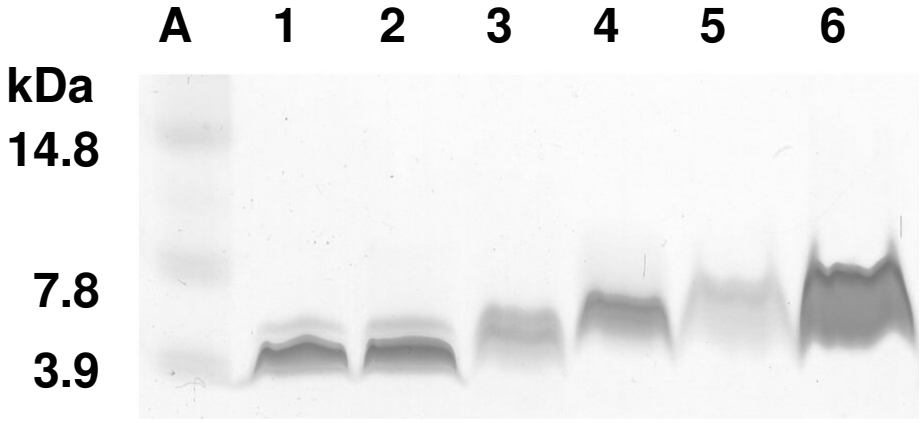


**Figure 5**. **Secretion of peptides via NisBT and NisBTC**. *L. lactis* cells were induced at OD600 = 0.4 with nisin and grown further in minimal medium for 5 hours. Peptides out of 3 ml supernatant were applied on a tricine gel and analyzed by Coomassie staining. *Lane* A: Kaleidoscopic marker of Biorad. *Lane 1*: NZ9000 pIL3BT pNZ29 (nisin leader:: ILSAYISTNTCPK); *Lane 2*: NZ9000 pIL3BTC pNZ29; *Lane 3*: NZ9000 pIL3BT pNZ30 (nisin leader::ILPTTKCTRAC); *Lane4*: NZ9000 pIL3BTC pNZ30; *Lane 5*: NZ9000 pIL3BT pNZ31 (nisin leader:: ILPTTKCARAA); *Lane 6*: NZ9000 pIL3BTC pNZ31.

*NisC-mediated cyclization enhances the production level of some peptides but not of others***.** We investigated whether or not the presence of NisC might enhance export. The amount of secreted peptide produced via NisBT or NisBTC was compared and analyzed on a Coomassie-stained tricine gel (Fig. 5). No noteworthy difference in secretion level was observed between NisBT- and NisBTC-transported peptides when peptide-encoding plasmid pNZ29 was co-expressed with pIL3BT or pIL3BTC. The secretion level of the peptide encoded by pNZ30 was somewhat higher when co-expressed with pIL3BTC instead of pIL3BT. However, when pNZ31 was co-expressed with pIL3BTC, the secretion level was clearly higher than when co-expressed with pIL3BT.

Subsequently we wanted to distinguish a channeling effect of NisC itself from enhanced export of peptides due to the cyclized peptide conformation. When peptides without cysteines hence without potential for thioether ring formation, such as the ones encoded by pNZ31, pTP-DSRWARVALIDSQKAAVDKAITDIAEKL and pTPtppii (Fig. S6), were co-expressed with either pIL3BT or pIL3BTC, no differences in transport could be detected. Nor did the presence of the inactive NisC mutant C326A improve the transport efficiency (data not shown). These observations indicate that in these experiments no channeling effect of NisC for transport of peptides by NisT occurs. Hence differences in export via NisBT compared to NisBTC, observed for the peptides encoded by the plasmids pNZ29 and pNZ30, are likely caused by differences

in conformation between (methyl)lanthionine-containing peptides versus linear peptides with free cysteines.

## Discussion

We here studied post-translational modifications, processing and transport by (sub)complexes of class I and class II lantibiotic enzymes. Since neither the activity of lacticin 3147 M1 and M2 enzymes, nor the nisin dehydratase activity by NisB, has been reconstituted *in vitro*, we here performed *in vivo* studies on the dissection of the enzyme complexes of these two lantibiotics, lacticin 3147 and nisin.

It has been reported that mutagenized LtnA1 and LtnA2 are each still modified, processed, and transported and retain antimicrobial activity (29, 30, 48). In all cases, the mutagenized substrates were still highly homologous to their natural counterparts. In the study by Cotter (30), not all of the alanine-substituted peptides could be detected. It is not clear whether the lack of detection of some of the mutagenized peptides is due to impaired processing and/or transport.

LtnT is an ABC transporter with a dual function. First, the ABC transporter is a maturation protease; its intracellular proteolytic domain resides in the N-terminal part of the protein. Second, after removal of the leader peptide LtnT translocates the substrate across the membrane (57). In the nisin biosynthesis machinery these processes are carried out by two distinct enzymes. NisT transports prenisin across the membrane, NisP extracellularly removes the leader-peptide (212, 230). Hence, removal of the nisin leader is not a prerequisite for transport of peptides fused to the leader (86).

As can be seen in Table S1 there is peptide-dependent variation in processing and export. Modification of the peptide substrates does not seem to be a qualification for processing and transport by LtnT. Clearly more research is needed to establish a clear picture of the substrate specificity of the lacticin 3147 synthetase enzymes and transporter involved.

In this study we demonstrated for the first time that LtnM2 can dehydrate peptides unrelated to LtnA2 and that LtnT surprisingly was able to process and translocate these modified peptides. Moreover, LtnT was shown to process and transport substrate peptides in the absence of all other lacticin 3147 synthetase enzymes. Van der Donk and co-workers demonstrated *in vitro* activity of lacticin 481, whose modification enzyme LctM has homology with LtnM2, independent of LctT (226). An *in vivo* study showed that in the absence of LctT a fully modified but truncated variant of the lacticin 481 was detected in the supernatant by mass spectrometry (208). This truncated, modified variant seems to be translocated via another transporter. In addition, NukM can modify its substrate in the absence of NukT (134). The latter study also proposed a membrane-located multimeric enzyme complex in which NukT and NukM are associated like the enzymes of the proposed

membrane-associated multimeric lantibiotic-synthesis complex (NisBTC / SpaBTC) (189, 75). We here demonstrate that, like the enzymes NisB, NisC, NisT and NisP which can function independently (86, 87, 106), the combination of LtnM with LtnT and LtnT alone can also function independent of other lacticin 3147 synthetase enzymes.

We hypothesized that dehydration and cyclization by the enzymes LanM, LanB and LanC are alternating activities and that thioether rings may inhibit dehydration of residues that are included in the thioether ring. In a previous study by Van der Donk the possibility that cyclization prevented dehydration of specific residues of the lacticin 481 peptide was not excluded (126). For the lacticin 3147 LtnM2 enzyme we have no conclusive data as a result of apparent substrate limitations of LtnT. We therefore compared modification by partially dissected enzyme complexes: NisBT versus NisBTC. All the tested truncations of the LtnA2 peptide had a higher extent of dehydration in the absence of NisC. In the case of export via NisBT, fully dehydrated peptides were observed. The apparent impaired dehydration of residues included in the ring is fully consistent with the possibility that the rings themselves interfere with accessibility of NisB. This points at the occurrence of alternating dehydrating and cyclization activities. For alternating activities it is likely that association between NisB and NisC is needed, consistent with the existence of a multimeric enzyme complex (189). It is tempting to speculate on a model in which the nisin leader might bind close to an interface between NisB and NisC allowing the modifiable propeptide substrate to flip from NisB to NisC and back again during alternating activities. Although no data are available yet, such a model is even easier to envisage for LctM enzymes in which the dehydratase and cyclase catalytic sites are present within one and the same enzyme.

Lantibiotic cyclases regio-specifically cyclize thioether rings (160, 235). Because of their high reactivity, dehydroalanines may spontaneously react with cysteines. As a result, regio-specific cyclase activity might be required before unwanted thioether bridges are formed. Therefore alternating activity of dehydratase and cyclase might in some peptides be crucial to ascertain the correct bridging pattern, essential for activity. To provide a complete picture it should be emphasized that alternating dehydratase and cyclase activity may occur, but is not an absolute necessity. For instance, *in vitro* reconstitution demonstrated that NisC can cyclize fully dehydrated prenisin in the absence of NisB and NisT (106). In these studies dehydration and cylization were uncoupled processes and could therefore not be alternating processes. Many lantibiotics possess dehydrated residues in a thioether ring, like Dha5 and Abu25 in nisin. Clearly, while we do not know whether dehydration of these particular residues takes place before or after ring formation, they are dehydrated. Future studies might investigate the possibility that the conformation

of the peptide substrate itself affects whether, when and where the dehydration and cyclization activities alternate.

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*Footnotes*

The abbreviations used in this paper are defined as follows:Dha, dehydroalanine; MALDI-TOF, matrix-assisted laser desorption ionization–time of flight; TFA, trifluoroacetic acid; TCEP, Tris(2-carboxyethyl)phosphine; CDAP, 1-cyano-4-dimethylamino-pyridinium tetrafluoroborate; Cmr, chloramphenicol-resistant; and Emr, erythromycin resistant.

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