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Mutagenesis of nisin's leader peptide proline strongly modulates export of precursor nisin

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Abstract The lantibiotic nisin is produced by *Lactococcus lactis* as a precursor peptide comprising a 23 amino acid leader peptide and a 34 amino acid post-translationally modifiable core peptide. We previously demonstrated that the conserved FNLD part of the leader is essential for intracellular enzyme-catalyzed introduction of lanthionines in the core peptide and also for transporter-mediated export, whereas other positions are subject to large mutational freedom. We here demonstrate that, in the absence of the extracellular leader peptidase, NisP, export of precursor nisin via the modification and transporter enzymes, NisBTC, is strongly affected by multiple substitutions of the leader residue at position -2, but not by substitution of positions in the vicinity of this site. Export levels of precursor nisin increased by more than 70% for position -2 mutants Asp, Thr, Ser, Trp, Lys, Val and decreased more than 70% for Cys, His, Met. In a strain with leader peptidase, the Pro-2Lys and Pro-2Asp precursor nisins were less efficiently cleaved by NisP than wild type precursor nisin. Taken together, the wild type precursor nisin with a proline at position -2 allows balanced export and cleavage efficiencies by precursor nisin's transporter and leader peptidase.

Keywords *Lactococcus lactis* · Lanthionine · Lanthipeptide · Leader peptide · Nisin · Transporter

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Introduction

Nisin A is a peptide antibiotic produced by some *Lactococcus lactis* strains. It is ribosomally produced as a precursor peptide of 57 amino acids, consisting of an N-terminal leader peptide and a C-terminal core peptide (Lubelski et al. 2008). The core peptide is post-translationally modified by the nisin modification enzymes, NisB (Grag et al. 2013; Koponen et al. 2002) and NisC (Koponen et al. 2002; Li et al. 2006), before it is exported as precursor nisin by the ABC-

transporter NisT. The leader peptide guides the interactions of the precursor peptide with the modification enzymes and transporter (Oman and van der Donk 2010). Outside the cell, the leader peptidase, NisP, cleaves off the leader peptide thus liberating active nisin.

Nisin is a pentacyclic peptide consisting of 21 unmodified amino acids, 2 dehydroalanines, 1 dehydrobutyrine, 1 lanthionine and 4 methyllanthionines (Gross and Morell 1971). Serines and threonines in the core peptide are dehydrated by NisB, resulting in dehydroalanines and dehydrobutyrines respectively. Subsequently, NisC catalyzes the coupling of the double bond in the dehydroamino acids to the thiol group of cysteines, forming a lanthionine (dAla-S-Ala) or methyllanthionine (dAbu-S-Ala). Nisin is a lanthionine-containing antibiotic, so-called called lantibiotic (Schnell et al. 1988). Lantibiotics are a subgroup of so-called lanthipeptides, which also comprise peptides without antibiotic activity (Arnison et al. 2013; Knerr and van der Donk 2012; Kodani et al. 2004; Willey and van der Donk 2007).

The nisin modification enzymes and transporter have a relaxed substrate specificity. This allows the use of *L. lactis* containing the nisin enzymes for the discovery of lanthionine-stabilized therapeutic peptides (Kluskens et al. 2005; Kuipers et al. 2004; Rink et al. 2010). Lanthionine-stabilized angiotensin-(1–7) has enhanced receptor specificity, enhanced intrinsic activity, strongly enhanced bioavailability, and potential for oral and pulmonary delivery (Kluskens et al. 2009; de Vries et al. 2010). It has multiple effective therapeutic activities for instance against acute respiratory distress syndrome (Wösten-van Asperen et al. 2011) and in the case of heart failure (Durik et al. 2012). Furthermore lanthionine-stabilized angiotensin-(1–7) and a lanthionine-containing agonist of the angiotensin type 2 receptor significantly reduce cardiopulmonary disease in hyperoxia treated neonatal rats (Wagenaar et al. 2013).

The nisin leader peptide contains a conserved FNLD sequence which is important for NisB activity (Plat et al. 2011; Khusainov et al. 2011; Mavaro et al. 2011), for NisC activity (Abts et al. 2013) and for NisT-mediated export (Plat et al. 2011). The exact mechanism(s) of lanthipeptide leader peptide-induced export out of the cell are unknown. Different mechanisms of LanT-mediated export have been discussed (Plat et al. 2013). Introduction of a negatively charged cleavage site for enterokinase, DDDDK, in the C-terminal part of the nisin leader appeared to hamper export of precursor nisin (Plat et al. 2011). We here investigated whether inserting positively charged residues in the C-terminal part of the leader peptide might modulate export via NisBTC. Position -2 in the wild type nisin leader is a helix-breaking proline. We found that substitution of this proline can have an important, amino acid-dependent impact on the efficiency of export of precursor nisin.

Materials and methods

Bacterial strains and plasmids

Lactococcus lactis NZ9000 was used for expression of the modification enzymes and precursor nisin constructs. *L. lactis* was grown in M17 broth (Terzaghi and Sandine 1975) supplemented with 0.5% glucose (GM17) or in minimal medium (Rink et al. 2005) with or without chloramphenicol (5 μg/ml) and/or erythromycin (5 μg/ml). Prior to analysis of peptides produced in the media, cells were cultured as follows. Overnight cultures of *L. lactis* NZ9000, grown in GM17 broth containing antibiotics, were diluted 1/100 in minimal medium. Production of mutated precursor nisin was induced by adding supernatant of *L. lactis* NZ9700 1:1000, containing approximately 1 mg/l wild type nisin. Strains and plasmids are listed in Table 1.

Molecular cloning

Standard genetic manipulations were performed using established procedures (Sambrook et al. 1989). Constructs coding for mutated precursor nisin were made by amplifying plasmid pNZnisA-E3 (Kuipers et al. 2004) via round-PCR using a downstream sense primer and an upstream antisense primer. Each primer pair contained one 5′ phosphorylation and a (non-annealing) peptide-encoding tail. The pORI280 system was used for integration of the P-2K mutation on the chromosome of an industrial stain (Leenhouts et al. 1996). This mutation was confirmed by sequencing of the mutation-containing PCR fragment obtained using chromosomal DNA as template. DNA amplification was performed with Phusion DNA polymerase (Finnzymes, Finland). Digestions were performed using restriction enzymes from New England BioLabs (Ipswich, MA). Ligation of the plasmids was carried out with T4 DNA ligase

Table 1 Bacterial strains and plasmids

Strain or plasmid	Characteristics	References
Strain		
L. lactis NZ9000	*nisRK* +	Kuipers et al. (1997)
L. lactis NZ9700	*nisABTCIPRKEFG*	Kuipers et al. (1997)
L. lactis LL108(pORI 280)	Em^r Cm^r	Kuipers et al. (1997), Leenhouts et al. (1998)
L. lactis pNGnisT pNGnisP	*nisTP*	Kuipers et al. (2004)
Plasmid		
pNZnisA-E3	*nisA*	Kuipers et al. (2004)
pIL3BTC	*nisBTC*	Rink et al. (2005)

(Roche, Mannheim, Germany). Electrotransformation of *L. lactis* was carried out as previously described using a Bio-Rad gene pulser (Richmond, CA) (Holo and Nes 1995). Nucleotide sequence analysis was performed by BaseClear (Leiden, Netherlands).

Purification

Precursor nisin was purified from culture supernatants. Minimal medium culture supernatant was diluted with an equal volume of 100 mM lactic acid (pH 2.5). The precursor peptides were subsequently purified by a single passage of the supernatant over a 5 ml HiTrap SP Sepharose cation-exchange column (GE Healthcare). Elution was performed at pH 4.0 with 1 M NaCl in 50 mM lactic acid. The fraction containing the precursor peptide was desalted on a PD10 column (GE Healthcare) and subsequently lyophilised or dried in a speed-vac. Nisin (mutants) were further purified and/or analysed via reversed-phase high-performance liquid chromatography (HPLC) on a C12 or C18 column with a gradient of 10–50% acetonitrile in 0.1% trifluoroacetic acid.

Cleavage of the leader peptide from precursor nisin

The leader peptide was cleaved off by incubating precursor nisin with 0.02 mg/ml trypsin for 30 min to 1 h at 37 °C. Alternatively, precursor nisin was incubated with NisP-expressing cells *L. lactis* NZ9000 pNGnisT pNGnisP cells at 30 °C.

Gel electrophoresis and quantification

Production levels of precursor nisin (mutants) were analysed via gel electrophoresis. Peptides were isolated from the supernatant of minimal medium cell cultures by TCA precipitation. Peptides were separated on tricine SDS gel (Schägger and von Jagow 1987). Analysis was performed by Coomassie staining (PageBlue^{TM}). Peptide bands were quantified by measuring the gel band density using ImageJ (Schneider et al. 2012). Cell free extract was obtained using a previously described washing procedure (Kleerebezem et al. 1997). As a control for the applied quantification, HPLC peak areas were compared for wild type precursor nisin and mutants in the medium. The chromosomal P-2K and wild type strains were compared in repeated small scale (10L) fermentations in parallel under conditions that essentially replicate commercial-scale production, including pH control during growth, inoculum ratios, and general media conditions. Samples were removed aseptically, hourly, as the culture approached stationary phase and thereafter until the end of fermentation. The samples were processed and analysed quantitatively for the presence of nisin using a validated reversed-phase analytical method using a HP HPLC System. Cleavage of wild type precursor nisin was compared with cleavage of the P-2K and P-2D mutants by incubating the precursor peptides with NisP-expressing cells. Samples were collected at different time points, and analyzed with HPLC to generate quantitative data of NisP cleavage.

Antimicrobial activity

Antimicrobial activity of the nisin mutants was measured against nisin-sensitive *L. lactis* strains. Indicator strain *L. lactis* LL108(pORI 280) (Kuipers et al. 1997; Leenhouts et al. 1998) or *L. lactis* NZ9000 pNGnisT pNGnisP (Kuipers et al. 2004) was grown in

GM17 containing chloramphenicol (5 μg/ml) and erythromycin (5 μg/ml) to an optical density of 0.1 at 600 nm. After 3–6 h of incubation in microwell plates with a series of two-fold dilutions of the nisin mutant, growth inhibition was measured at 600 nm. The 50% inhibitory concentration (IC_{50}) was obtained from the midpoint of the sigmoidal growth curve. Halo-forming capacities of (mutant) nisin-producing *L. lactis* NZ9000 colonies were measured using an overlay with the NisP-expressing *L. lactis* NZ9000 pNGnisT pNGnisP strain.

Mass spectrometry

Mass spectrometry was performed to confirm that the correct (precursor) nisin mutants were produced and, in case of leader cleavage, that the intended mutant leader peptide was produced. In case of (precursor) nisin mutants samples were pre-incubated with 1 mg/ml tris[2-carboxyethyl]phosphine (TCEP) to prevent cysteinylation of free cysteines. For all samples, and also when just the leader peptide was measured, either 1 μl of culture supernatant or purified peptide was applied to a target and allowed to dry. Spots from culture supernatants were washed once with 5 μl of Millipore water to remove the salts. Spots were subsequently overlaid with 1 μl of matrix (5 mg/ml α-cyano-4-hydroxycinnamic acid in 50% acetonitrile containing 0.1% [vol/vol] trifluoroacetic acid). Mass spectra were recorded with a Voyager-DE PRO matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) mass spectrometer (Applied Biosystems). In order to maintain high sensitivity, an external calibration was applied.

Results

Introduction of C-terminal lysines in the nisin leader peptide

To investigate the influence of positively charged residues in the C-terminal part of the leader peptide, we introduced lysines at position P-2, D-7 and S-3/P-2 (Fig. 1). Analysis of the production on a tricine SDS gel showed a nearly two-fold increased production for the P-2K mutant (Fig. 2a). Nisin mutant I+4K/L+6I ("KSI", a positive control, (Rink et al. 2007b)) was also produced better than wild type nisin. The D-7K mutant and the S-3K/P-2K ("KKR") double mutant showed decreased production. These data demonstrate that lysines in positions -2 or +4, which are both close to the leader peptide to core peptide border, enhance production.

Shifted trypsin-mediated cleavage of precursor nisin mutant P-2K

The antimicrobial activity of the mutants was tested by overlaying a duplicate gel with a nisin-sensitive strain. In the case of I+4K/L+6I and S-3K/P-2K precursor nisin the size of the halo correlated roughly to the amount of precursor produced (Fig. 2b).

In contrast, the relatively small halo surrounding the P-2K mutant clearly showed low activity of the P-2K mutant. In the activity assay, the leader peptide was removed from the (mutant) precursor nisin by adding trypsin in the overlay. Trypsin usually cleaves most efficiently after an Arg but it will also cleave after a Lys. We therefore investigated whether this comparatively low activity of P-2K nisin might result from incomplete removal of the leader peptide.

MALDI-TOF MS of intact P-2K precursor nisin (Fig. 3a), trypsin-cleaved P-2K precursor nisin (Fig. 3b) and NisP-cleaved P-2K precursor nisin (Fig. 3c) showed that trypsin cleaves primarily between K-2 and R-1, resulting in R-nisin (Fig. 3b). This shifted cleavage likely results from sterical hindrance from ring A. Incubating the P-2K precursor nisin with NisP-producing *L. lactis* cells (Kuipers et al. 2004) resulted in complete removal of the leader peptide (Fig. 3c). After incubation with the NisP-producing cells, the antimicrobial activity of the peptides was measured in a dilution assay. The P-2K-precursor-nisin-derived nisin showed a two-fold higher activity than wild type nisin (data not shown) which is consistent with its two-fold higher production level. These results show that, in this particular case of P-2K precursor nisin, trypsin has a higher affinity for the -2K position than for -1R, and that the presence of an additional N-terminal Arg decreases the antimicrobial activity of nisin. Moreover, the results demonstrate that the P-2K substitution does not hamper interaction with the modification enzymes and allows cleavage by NisP-producing *L. lactis* (Kuipers et al. 2004).

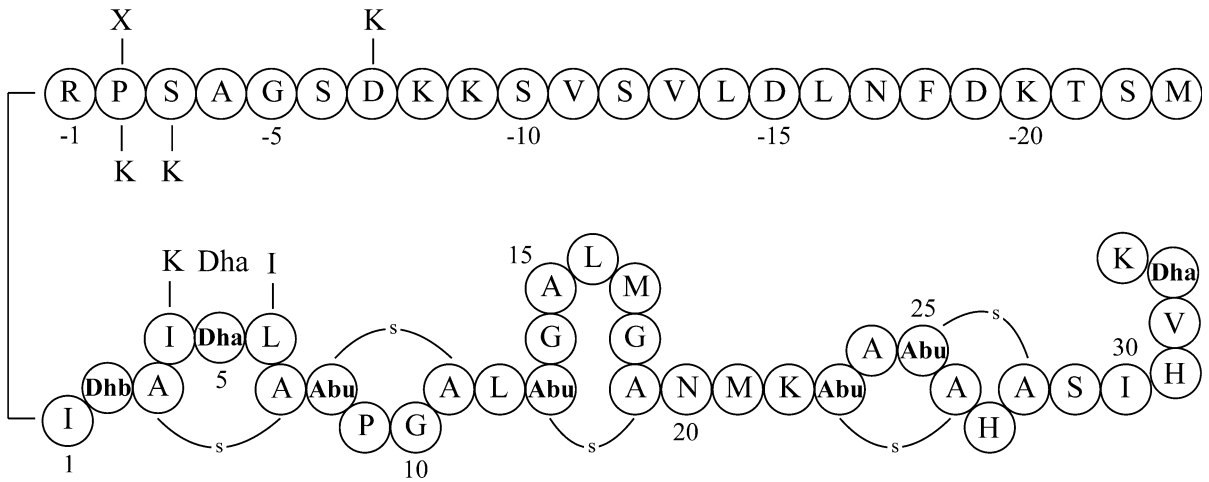

Fig. 1 An overview of the amino acid substitutions in precursor nisin used in this study."*X*" indicates randomization

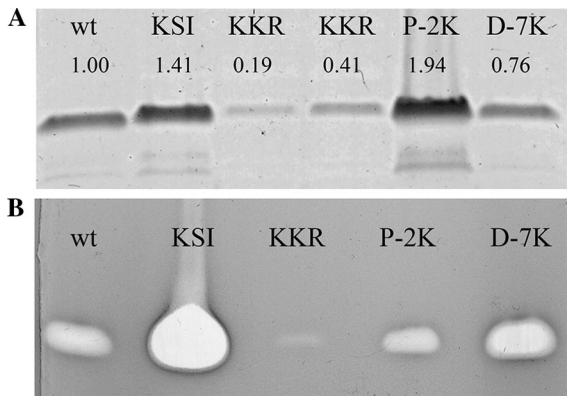

Fig. 2 Production and antimicrobial activity of nisin mutants. **a** A comparison of the production in the supernatant of wild type precursor nisin (wt) with precursor nisin mutants I+4K/L+6I (KSI), S-3K/P-2K (KKR, two transformants), P-2K, and D-7K. Production was measured by TCA-precipitating 1 ml of supernatant, running it over a tricine SDS gel, and subsequent staining with PageBlue. Band intensities were quantified using ImageJ and relative values are given below the peptide names. **b** Overlay of a duplicate gel with nisin-sensitive strain LL108pORI280. Precursor nisin peptides were cleaved by adding trypsin

Randomization of position -2

Is the increased production of the P-2K precursor nisin mutant positive charge-dependent or could other factors be involved? We investigated this by substituting the Pro on position -2 with other amino acids and subsequently measured production (Table 2). Interestingly, large differences were observed between production levels of the different mutant types ranging from a two-fold increase to a ten-fold decrease. Besides precursor nisin mutant P-2K, also the precursor nisin mutants P-2D, P-2T, P-2S, P-2W, and P-2V show strongly enhanced production. Very low production was measured for the P-2C, P-2H, and P-2M. Especially surprising is the high production of the P-2D mutant and the low production of the P-2H mutant. These results clearly demonstrate that the enhanced production of P-2K precursor nisin is not determined by positive charge alone.

Production stability

As an additional control, the transformation of each mutant was repeated and the precursor nisin production of the resulting mutants was measured. For ten mutation types production was satisfactorily reproducible. Only three type of mutations displayed variation in production: P-2A, P-2Q and P-2R. Strikingly P-2R mutants led to precursor nisin production which varied from very high for some transformants to very low for others. Apparently by unknown cause(s) these three specific mutations led to variable results, whereas the majority of the mutations caused stable production.

Chromosomal P-2K leader substitution

The P-2K mutation was inserted on the chromosome of a commercial nisin production strain. This strain, in contrast to the studies above, also naturally expresses NisP. Incorporation of the P-2K mutation on the chromosome of this strain was confirmed by mass

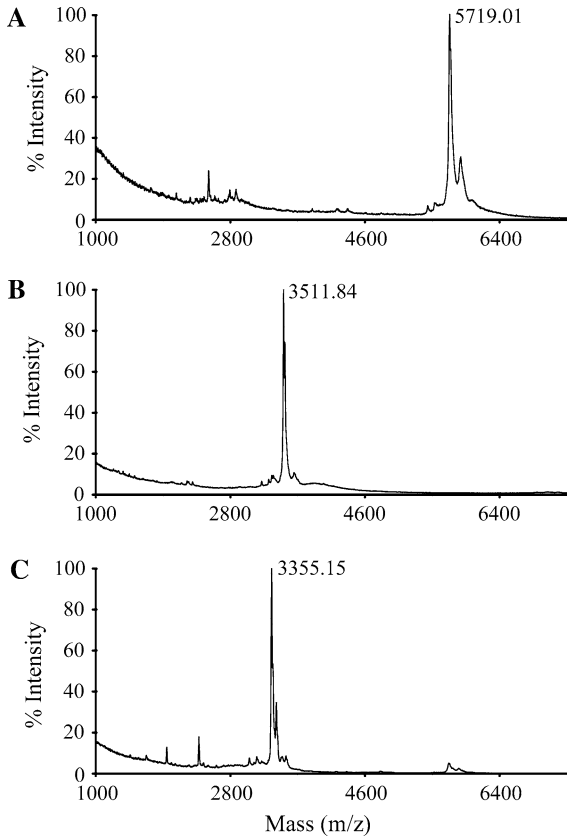

Fig. 3 Removal of leader peptide from precursor nisin P-2K. **a** Mass spectrometry analysis of precursor nisin containing the P-2K mutation. The [MH+] peak of 5719.01 Da corresponds to precursor nisin with the P-2K substitution; theoretical value without Met1: 5719.86 Da. **b** Mass spectrometry analysis after incubation with trypsin. The [MH+] peak of 3511.84 Da corresponds to R-nisin, theoretical value: 3511.38 Da. **c** Mass spectrometry analysis after incubation with NisP-expressing cells (Kuipers et al. 2004). The [MH+] peak of 3355.15 corresponds to nisin; theoretical value 3355.20 Da

spectrometry of the produced leader peptide and DNA sequence analysis. Production was compared at various time points using HPLC. At no time point did mature nisin production of P-2K mutant strain exceed that of the wild type strain. Mature nisin production by the P-2K mutant strain reached at 24 h maximally 93.4% of that of the wild type strain. Hence, we observed enhanced P-2K and P-2D *precursor* nisin production in cells without NisP, whereas production of *mature* nisin by a production strain carrying the chromosomal P-2K mutation was not increased. To investigate whether this could be caused by less efficient cleavage of P-2K precursor nisin by NisP we investigated whether or not NisP cleaves the P-2 mutant as readily as wild type precursor nisin. Preliminary data indicated that both P-2 K and P-2D precursor nisin are less efficiently cleaved by NisP than the wild type leader. Taken together, the data indicate that the wild type nisin precursor with a proline at position -2 allows balanced export- and cleavage efficiencies by precursor nisin's transporter and leader peptidase.

Discussion

Lanthipeptide leader peptides are very interesting since each single leader peptide functionally interacts with proteins as different as a serine/threonine dehydratase, a cyclase, a transporter and –if present- a leader peptidase (Plat et al. 2013). Here we studied the role of C-terminal mutations in the nisin leader peptide on export. Previous observations (Plat et al. 2011) showed decreased production when substituting C-terminal residues in the leader peptide of nisin with negatively charged residues. Therefore we aimed in this study at enhancing production levels by mutating C-terminal amino acids of the leader peptide into lysines. A strong increase in production was measured for the nisin P-2K mutant. By contrast the D-7K did not cause increased production nor did the simultaneous introduction of -2K and -3K, indicating the relevance of the selective mutagenesis of the -2

Table 2 Relative production by *L. lactis* of P-2X precursor nisin mutants

X	D	T	S	W	K	V	N	G
Relative production	1.97 ± 0.28	1.88 ± 0.20	1.75 ± 0.21	1.73 ± 0.28	1,72 ± 0.10	1.70 ± 0.24	1.40 ± 0.08	1.36 ± 0.12
I	P	L	E	F	Y	C	H	M
1.27 ± 0.11	1.00 ± 0.00	0.96 ± 0.22	0.62 ± 0.11	0.62 ± 0.12	0.58 ± 0.08	0.25 ± 0.03	0.13 ± 0.04	0.09 ± 0.04

position. Substituting the Pro at position -2 with other amino acids showed a more complicated picture: P-2D causes enhanced production; P-2H causes reduced production. Interestingly, also P-2T caused enhanced production and precursor nisin Q also contains a Thr at position -2. In an old pioneer study P-2V and P-2G did not have any detectable effect on *mature* nisin production (van der Meer et al. 1994), whereas in the present study both these mutations caused increased production of *precursor* nisin, respectively 1.70 ± 0.24 and 1.36 ± 0.12 (Table 2). In addition to the inherent differences between studying production of mature nisin and precursor nisin, the observed production differences may result from the here used improved two-plasmid expression system. The modulation of export depends on the -2 leader site and is amino acid dependent. This precludes that modulated production is caused by more optimal or less optimal codons. Furthermore the reproducibility of the data for plasmid-containing cells that result from different transformations also precludes that modulated production is caused by heterogeneity of lactococcal cells. Our data indicate that production of wild type precursor nisin, containing a Pro at position -2, is average compared to production of P-2X precursor nisin mutants. Taken together the data indicate that the helix breaking proline-2 might be relevant for optimal NisP-mediated cleavage but possibly not optimal for export of precursor nisin.

Lanthipeptide leader peptides are much more hydrophilic than the lanthipeptides themselves. Most class I leader peptides end up in the extracellular medium after cleavage from the core peptide by LanP or other proteases (Kuipers et al. 2004; Stein and Entian 2002). Outside the FNLD box a large mutational freedom has been demonstrated without affecting the capacity to modify substrate. Even a 6His tag could be introduced (Plat et al. 2011). Residues between the FNLD box and the core peptide might function as a spacer (Plat et al. 2011). The same hypothesis has been suggested for a class III lanthipeptide labyrinthopeptin (Müller et al. 2011). A large part of the diverse leaders of class II ProcM substrates is dispensable (Zhang et al. 2014). Replacement of the conserved FNLD sequence in the nisin leader peptide by four alanines eliminates the capacity to induce NisB activity (Plat et al. 2011) by eliminating binding to NisB (Khusainov et al. 2011; Mavaro et al. 2011). Furthermore, this leader peptide with the FNLD box replaced by four alanines, hardly induced any export. Deleting a sequence in NisB itself, which resembles the FNLD sequence, also eliminated NisB activity (Khusainov et al. 2011). The co-crystal structure of NisB and precursor nisin together with exciting mechanistic information provided an explanation of the importance of the leader peptide FNLD box for interaction with NisB (Ortega et al. 2014).

Experiments using Isothermal Titration Calorimetry (ITC) and a combination of Size Exclusion Chromatography (SEC) with Multi-Angle Light Scattering (MALS) analysis have demonstrated that NisC binds the FxLx motif of the nisin leader peptide (Abts et al. 2013). Replacement with all alanines of six regions of 2–4 leader peptide amino acids followed by co-purification with NisB and NisC yielded a more complex and detailed picture on binding and modification and indicated that leader regions LVSV(-14-11), STKD(-22-19) and especially FNLD(-18-15) contributed to interaction with NisB and NisC (Khusainov et al. 2013). In addition replacement of PR(-2-1) with alanines strongly reduced co-purification of NisB and NisC (Khusainov et al. 2013). The latter implies that it can not be excluded that replacement of P-2 might indirectly affect export by modulated interaction of the leader with the modification enzymes.

A propensity to form an alpha helical structure has been demonstrated for the leader peptides of nisin, lacticin 481 (Patton et al. 2008) and nukacin (Nagao et al. 2009) and predicted for other leader peptides (Oman and van der Donk 2010). The nisin leader peptide showed a random coil in aqueous solution (van den Hooven et al. 1997), but adopts a helical form in a mixture of trifluoroethanol and water (Beck-Sickinger and Jung 1993; Lian et al. 1992). However, the nisin leader peptide binds to NisB as an antiparallel-strand (Ortega et al. 2014). Introduction of a proline in position -8 or -6 or -4 in lacticin 481 abolished LctM synthetase activity, while introduction of a proline in position -5 reduced LctM synthetase activity (Patton et al. 2008). Introduction of a proline in position -8 or -9 in the nukacin leader, abolished, respectively reduced antimicrobial activity in the supernatant (Nagao et al. 2009). Mutational freedom, except for the introduction of proline, appeared to be high for any position. Proline has a turn propensity and thereby disturbs alpha helical structure (Piela et al. 1987). On the contrary, here we eliminated a helix-breaking

proline residue at position -2 of the nisin leader peptide by substituting it for other amino acids. Replacement of proline for Asp, Thr, Ser, Trp, Lys and Val enhanced export without compromising the extent of NisB- and NisC-mediated modification.

With respect to removal of the leader peptide, different cases occur within lanthipeptides. Most Class I leader peptides are cleaved off extracellularly by a dedicated peptidase LanP (Plat et al. 2013) or in the case of subtilin (Stein and Entian 2002) by non-dedicated extracellular peptidases. Also transport of the nisin leader peptide itself, if it is produced without any attached core peptide, has been demonstrated (Rink et al. 2007a). In contrast the leader peptide of Class II lanthipeptides is cleaved off by an intracellular peptidase domain within the transporter. Hence, most Class I transporters export the leader peptide, whereas class II transporters do not.

NisT has been reported to be present as a dimer (Ortega et al. 2014; Siegers et al. 1996). We previously presented three models of the NisT-mediated export (Siegers et al. 1996). A tentative model involves binding of the FNLD box to NisT, which induces ATP-driven segregation of the two NisT molecules and opening of a NisT-pore, through which the N-terminal site of the core peptide first diffuses to the outside of the cell. Alternatively, the C-terminus of the peptide moves first out of the cell, after the "knock on the door" effectuated by the binding of the FNLD box to NisT. A third possibility might be that the FNLD box binds to NisT after which an ATP-driven pumping of the whole peptide with N- to C-terminal directionality moves out of the cell.

In conclusion, the present study demonstrates that mutagenesis of position -2 of the nisin leader peptide strongly modulates export of precursor nisin. Future research will establish the mechanistic role of this leader site in export, the order of the export of the leader and the core peptide and the overall mechanism of leader peptide-induced transport via NisT.

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Conflict of interest The authors confirm that this article content has no conflict of interest.

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