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Investigation on translocation and proteolytic processing of the lanthipeptide nisin

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“The most exciting phrase to hear in science, the one that heralds new discoveries, is not ‘Eureka’, but ‘That’s funny …’ ”

Isaac Asimov

Abstract

Lanthipeptides are ribosomal synthesized and post-translational modified peptides containing unusual amino acids such as dehydroalanine, dehydrobutyrine and (methyl-)lanthionine. One of the best-studied lanthipeptides and used as a model system is nisin, produced by the Gram-positive bacterium *Lactococcus lactis*. It is synthesized as a precursor peptide (NisA) consisting of an N-terminal leader peptide and a C-terminal core peptide. Within the core peptide the dehydratase NisB and the cyclase NisC introduce the post-translational modifications, where the leader peptide acts as an enzyme recognition site. Furthermore, the leader peptide is a secretion signal for the ATP-binding cassette (ABC) transporter NisT. The exporter translocates NisA in a proposed channelling mechanism across the cytoplasmic membrane. Subsequently, the leader peptide is cleaved off by the extracellular located serine protease NisP and the mature nisin is released.

In the present thesis, the translocation of NisA is investigated by an *in vitro* and *in vivo* characterization of NisT. *In vitro* ATPase activity with and without substrate as well as pull-down assays with the interaction partner were performed with detergent-solubilised and purified NisT. It was found, that the basal ATPase rate was not stimulated by various substrates in a concentration-dependent manner. However, a conducted quantitative analysis of an *in vivo* secretion assay demonstrated the modulation by the modification machinery. Here, a strong enhancement of the apparent secretion rate was observed. Thus, the complex of NisT, NisB and NisC, which was observed by a pull-down assay, is prerequisite for an efficient NisA secretion. Furthermore, the recognition site of the leader peptide for NisT was investigated by mutational analysis in combination with an *in vivo* secretion assay. Similarly to the modification machinery, NisT recognises the FNLD-box motif and in addition charged residues as a secondary motif. Moreover, the analysis of leader hybrids allowed to conclude, how the leader peptide is influencing the substrate specificity of NisT and thereby the translocation process.

Finally, the thesis deals with the NisA maturation by characterising the leader peptidase NisP *in vitro*. Here, kinetic parameter of various substrates revealed, that NisP has the highest catalytic efficiency for modified NisA. Noteworthy, at least one lanthionine ring is necessary for efficient leader peptide cleavage. This reflects the preferred substrate specificity of NisP towards (methyl-)lanthionine-containing substrates.

Zusammenfassung

Lanthipeptide sind ribosomal synthetisierte und posttranslational modifizierte Peptide, die ungewöhnliche Aminosäuren wie Dehydroalanin, Didehydrobutyrin sowie (Methyl-) Lanthionin enthalten. Eines der am besten untersuchten und als Modelsystem genutzten Lanthipeptide, ist das vom Gram-positiven Bakterium *Lactococcus lactis* produzierte Nisin. Es wird als Vorläuferpeptid (NisA), bestehend aus einem N-terminalen Signalpeptid und einem C-terminalen Kernpeptid, synthetisiert. Innerhalb des Kernpeptids werden durch die Dehydratase NisB und die Zyklase NisC die posttranslationalen Modifikationen eingebaut, wobei das Signalpeptid als Enzymerkennungsstelle fungiert. Darüber hinaus wird das Signalpeptid vom „ATP-binding cassette“ (ABC) Transporter NisT erkannt, der NisA über die Zytoplasmamembran transportiert. Anschließend wird das Signalpeptid von einer extrazellulären Serinprotease NisP entfernt und das reife, antimikrobiell aktive Nisin freigesetzt.

In der vorliegenden Dissertation wird die Translokalisierung von NisA mittels der *in vivo* und *in vitro* Charakterisierung von NisT untersucht. Dabei wurden Experimente zur Bestimmung der *in vitro* ATPase Aktivität von Detergens solubilisierten und gereinigten NisT mit und ohne Substrat, sowie Interaktionsstudien mit den Interaktionspartnern NisB und NisC durchgeführt. Es wurde ermittelt, dass die basale ATPase Rate nicht durch die Zugabe von verschiedenen Substraten in einer konzentrationsabhängigen Weise stimuliert wurde. Jedoch zeigte die quantitative Analyse eines *in vivo* Sekretionsexperiments den modulierenden Effekt der Modifikationsmaschine. Dort wurde eine Erhöhung der apparenten Sekretionsrate durch die Modifikationsmaschine beobachtet. Folglich ist der Komplex bestehend aus NisT, NisB und NisC, der bei den Interaktionsstudien beobachtet wurde, unabdingbar für eine effiziente NisA Sekretion. Darüber hinaus wurde das Erkennungsmotiv für NisT im Signalpeptid mittels Mutationsanalyse in Kombination mit einem *in vivo* Sekretionsexperiment untersucht. Ähnlich zu der Modifikationsmaschine erkennt NisT das FNLD-Box Motiv und zusätzlich geladene Aminosäuren als zweites Erkennungsmotiv. Weiterhin erlaubt die Analyse von erstellten Signalpeptidhybriden eine Schlussfolgerung, wie das Signalpeptid die Substratspezifität von NisT und damit den Transport von NisA beeinflusst.

Abschließend behandelt die vorliegenden Dissertation die Reifung von NisA, indem die Signalpeptid Peptidase NisP *in vitro* charakterisiert wurde. Bei der Untersuchung der kinetischen Parameter von unterschiedlichen Substraten zeigte es sich, dass NisP die höchste katalytische Effizienz für modifiziertes NisA aufweist. Bemerkenswerterweise ist mindestens ein (Methyl-)Lanthioninring für eine effiziente Schneidereaktion des Signalpeptids notwendig. Dies reflektiert die präferierte Substratspezifität von NisP gegenüber (Methyl-)Lanthionin enthaltenden Substraten.

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Abbreviations

Å	Ångstrom
aa	amino acid
ABC	ATP-binding cassette
ADP	adenosine-5-diphospahte
AMP	antimicrobial peptide
ATP	adenosine-5-triphospahte
B. subtilis	*Bacillus subtilis*
C. difficile	*Clostridioides difficile*
cIEX	cation exchange chromatography
CP	core peptide
CS	cleavage site
Da	Dalton
DDM	n-dodecyl-β-D-maltopyranoside
Dha	(di)dehydroalanine
Dhb	(di)dehydrobutyric acid
DNA	deoxyribonucleic acid
dNisA	dehydrated NisA
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
e.g.	latin: exempli gratia
ERS	enzyme recognition site
et al.	latin: et alii
FC-16	fos-choline 16
FPLC	fast protein liquid chromatography
GTP	guanosine-5-triphospahte
His-tag	polyhistine-tag
HK	histidine kinase
HPLC	high-performance liquid chromatography
IC_{50}	inhibitory concentration, where growth is inhibited by 50%
IMAC	immobilised metal ion chromatography
i.a.	latin: inter alia
K	rate constant
K_m	Michaelis-Menten constant
Lan	lanthionine
L. lactis	*Lactococcus lactis*
LMNG	lauryl maltose neopentyl glycol
LP	leader peptide
M	molar
MeLan	methyl-lanthionine
mg	milligram
min	minute
ml	milliliter
NBD	nucleotide-binding domain

NisA	precursor peptide of nisin A
$NisA_{CP}$	nisin A core peptide
$NisA_{LP}$	nisin A leader peptide
nm	nanometer
nM	nanomolar
NRP	nonribosomal peptide
NRPS	nonribosomal peptide synthetase
NTA	nitrilotriacetic acid
PAGE	polyacrylamide gel electrophoresis
PDB	Protein Data Bank
pK_a	acid dissociation constant
PTM	post-translational modification
RiPP	ribosomally synthesized and post-translationally modified peptide
RP	reversed phase
S. agalactiae	*Streptococcus agalactiae*
S. aureus	*Staphylococcus aureus*
SBP	substrate-binding protein
sec	second
SEC	size exclusion chromatography
TBS	Tris-buffered saline
TBS-T	TBS with Tween 20
TCA	trichloroacetic acid
TEV	tabacco etch virus
TFE	trifluoroethanol
TM	transmembrane
TMH	transmembrane helix
TMD	transmembrane domain
uNisA	unmodified NisA
µl	microliter
µm	micromolar

amino acid	three letter code	one letter code
alanine	Ala	A
arginine	Arg	R
asparagine	Asn	N
aspartic acid	Asp	D
cysteine	Cys	C
glutamic acid	Glu	E
glutamine	Gln	Q
glycine	Gly	G
histidine	His	H
isoleucine	Ile	I
leucine	Leu	L
lysine	Lys	K
methionine	Met	M
phenylalanine	Phe	F
proline	Pro	P
serine	Ser	S
threonine	Thr	T
tryptophan	Trp	W
tyrosine	Tyr	Y
valine	Val	V

1 Introduction

1.1 Ribosomally synthesized and post-translationally modified peptides

1.1.1 The origin of peptides

In the 20th century great progress in the understanding of biological and especially biochemical process in nature provided us with tools to produce natural products. These natural products are substances that are produced by living organisms as part of their primary or secondary metabolism. Especially, the groups of the secondary metabolism like alkaloids (e.g. vinblastine and codeine), terpenoids (e.g. artemisinin) or polyketides (e.g. erythromycin) are interesting research targets to use them as lead compounds for pharmaceuticals (Newman and Cragg 2016). Peptides as an additional secondary metabolite are particularly interesting as lead compounds in biopharmaceutical industry since the discovery of antibiotics (Hudson and Mitchell 2018).

There are two major groups of peptides characterized by their synthesis. The ribosomally synthesized and post-translationally modified peptides (RiPP) and the non-ribosomally synthesized peptides (NRP). The biosynthesis pathway of the latter is employed by NRP synthetases (NRPS), which catalyse for example the biosynthesis of antibiotics and other macrocyclic peptides via thiol ester (e.g. vancomycin and bacitracin) (Konz, Klens et al. 1997, Hubbard and Walsh 2003). The modular assembly of the NRPS consists of five modules plus the peptidyl carrier proteins. The modules 1-4 catalyse the activation of amino acids, their linear condensation and optional their epimerization. In a final step a thioesterase-domain included in module 5 cleaves the mature peptide off from the NRPS (for more detailed see reviews (Challis and Naismith 2004, Marahiel 2009)). As NRP do not use mRNA as a template, they contain non-proteinogenic amino acids and fatty acids besides the standard post-translational modifications (PTM) of amino acids.

This is in contrast to RiPPs, where the peptide biosynthesis at ribosomes is restricted to an mRNA-based translation (Ingram 1970, Mazodier, Biville et al. 1988, Kelly, Pan et al. 2009, Wieland Brown, Acker et al. 2009, Condie, Nowak et al. 2011, Freeman, Gurgui et al. 2012, Terrat, Biass et al. 2012). Generally, are RiPPs ribosomally synthesized as precursor peptides (20-110 aa residues), which contain an N-terminal leader peptide (LP) and a C-terminal core peptide (CP) (Figure 1) (Arnison, Bibb et al. 2013). The LP is important for

recruiting the modification enzymes, keep the peptide inactive, for further processing and export (Garcia-Olmedo, Molina et al. 1998, Furgerson Ihnken, Chatterjee et al. 2008, Patton, Paul et al. 2008, Oman and van der Donk 2010, Donia and Schmidt 2011, Mavaro, Abts et al. 2011, Abts, Montalban-Lopez et al. 2013, Burkhart, Hudson et al. 2015). In some cases N-terminal of the leader peptide a signal sequence can be found to determine the pathway for the precursor peptide (in eukaryotes) (Jennings, West et al. 2001, Seronay, Fedosov et al. 2010). Within the core peptide the enzymes of the biosynthetic pathway catalyse the modification of certain amino acids (PTM site). There, a frequently observed PTM site are cysteine residues, where the thiol-group is converted for example to disulfides, thioether, sulfoxides or thiazol(in)es (Arnison, Bibb et al. 2013). Furthermore, common PTMs in RiPPs are dehydrations, acetylations, methylations, hydroxylations, O/N-glycosylations or head-to-tail cyclisations (Gross and Morell 1967, Onaka, Nakaho et al. 2005, Lee, Mitchell et al. 2008, Morris, Leeds et al. 2009, Walton, Hallen-Adams et al. 2010, Melby, Nard et al. 2011, van Belkum, Martin-Visscher et al. 2011).

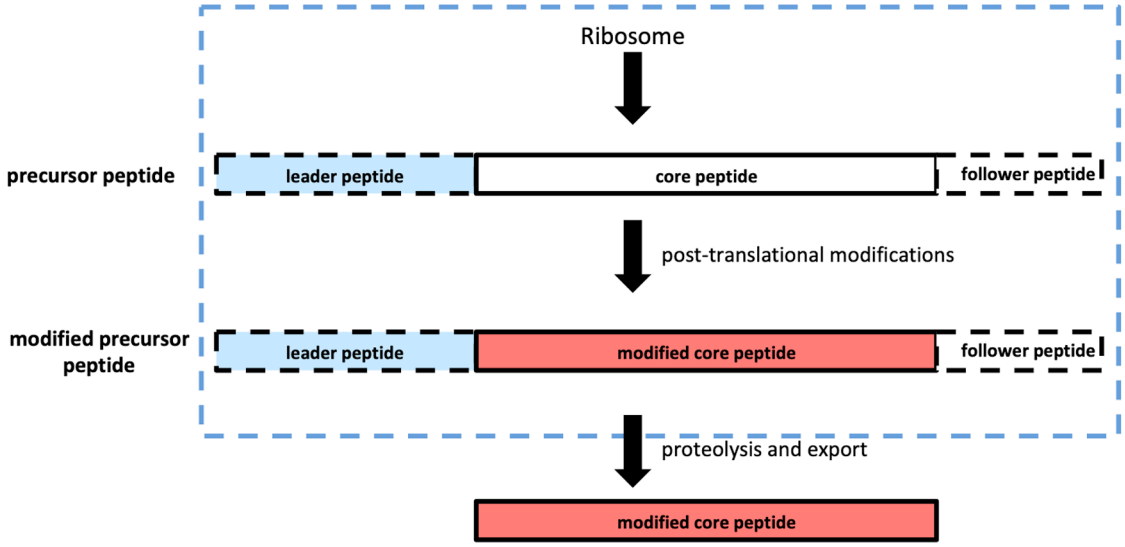


Figure 1: RiPP biosynthesis pathway.
Peptides are synthesized at the ribosomes as precursor peptide and are comprised of a leader peptide (blue) and a core peptides (white). In some cases a follower peptide (or an additional recognition site) is attached. Post-translational modifications are installed within the core peptide. After proteolysis and export the modified core peptide (red) is biological active. Scheme of biosynthesis pathway is based on a figure from (Repka, Chekan et al. 2017).

C-terminal of the CP an additional recognition sequence (follower peptide) for the modification enzyme can be attached, which lack the LP sequence (e.g. some bottromycins) (Hou, Tianero et al. 2012, Huo, Rachid et al. 2012). After modification the precursor peptide under goes further processing like proteolysis to remove the LP before or after export (van

der Meer, Polman et al. 1993, Allali, Afif et al. 2002, Lee, McIntosh et al. 2009, Luo, Hallen-Adams et al. 2009, Weiz, Ishida et al. 2011). The modified CP is the mature and active form of RiPPs and displays their bioactivities.

RiPPs are often located with their modification enzymes in gene cluster at one locus on the genome or on mobile gene elements (Arnison, Bibb et al. 2013, Bartholomae, Buivydas et al. 2017, Hudson and Mitchell 2018). The biosynthetic gene cluster (BGC) comprises of enzymes, which catalyse the PTMs (modification machinery, MM), a two-component system (TCS; optional), an exporter protein, a peptidase (optional) and enzymes for self-defence (immunity). Hence, a minimal system of MM, exporter and immunity proteins (theses proteins are dispensable depending on the properties of the RiPP) are sufficient for RiPP production.

1.1.2 Diversity of the RiPP superfamily

Since the end of the 20th century the constant analysis of metabolomes, transcriptomes and especially of bacterial genomes lead to the discovery of new RiPP BGCs. Therefore, bioinformatic tools (e.g. BAGEL4 (van Heel, de Jong et al. 2018), antiSMASH4.0 (Blin, Wolf et al. 2017), RiPPMiner (Agrawal, Khater et al. 2017), RiPPquest (Mohimani, Kersten et al. 2014), PRISM3 (Skinnider, Merwin et al. 2017)) are used for automatically analysis of these genomic data. In general, they can identify novel RiPPs based on comparative genome mining (e.g. common biosynthetic pathway), sequence motifs within the precursor peptides or open-reading frames (ORF), which are shorter than 100 aa (Ziemert, Alanjary et al. 2016, Hetrick and van der Donk 2017). For example, alone the analysis of 65421 prokaryotic genomes with the genome mining tool PRISM resulted in thousand potential BGCs (Skinnider, Johnston et al. 2016). This pool of potential new RiPPs demonstrate their high diversity in comparison to linear peptide or macrocyclic peptides and can therefore expanse the limits of the 20 canonical amino acids.

The superfamily of RiPPs consists of many subfamilies, which can be classified based on their taxonomic origin, biosynthesis, chemical structure and functional properties (Figure 2). RiPPs can be found in all kingdoms of life, so they are ubiquitously present in the domain of eukaryotes and prokaryotes. Examples of RiPPs in eukaryotes are cyclotides (plants) (Saether, Craik et al. 1995), amatoxins (fungi) (Walton, Hallen-Adams et al. 2010) or conopeptides (molluscs:cone snails) (Buczek, Bulaj et al. 2005, Halai and Craik 2009).

Especially, in bacteria a high diversity of RiPPs can be found. Some examples are the class of bottromycins (Huo, Rachid et al. 2012), cyanobactins (Donia and Schmidt 2011), lasso peptides (Hegemann, Zimmermann et al. 2015), lanthipeptides (Repka, Chekan et al. 2017), or microcins (e.g. microcin B17 or C7) from *Enterobacteria* (Duquesne, Destoumieux-Garzon et al. 2007).

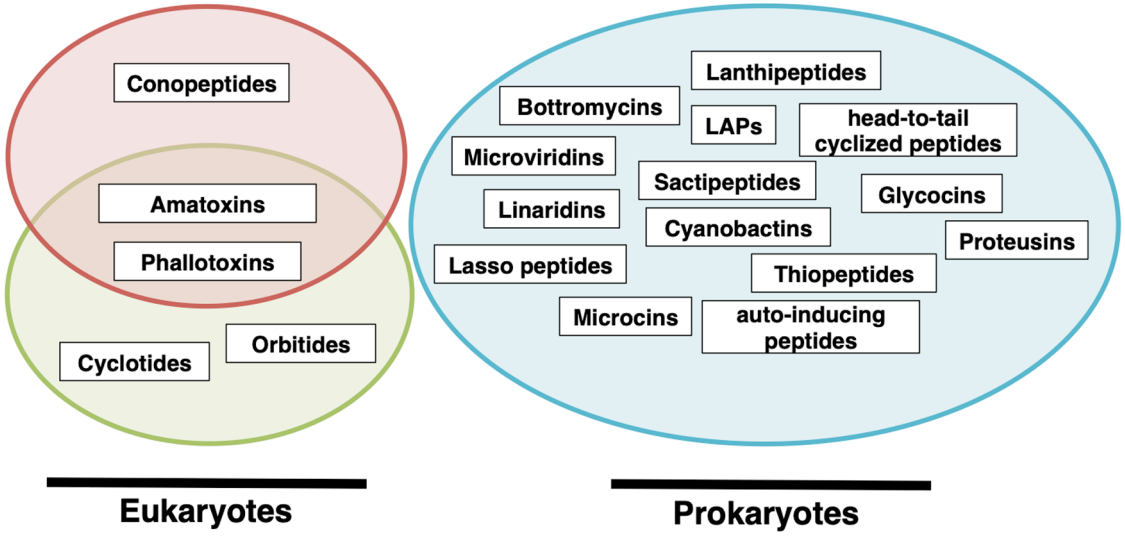


Figure 2: Classification of RiPPs.
The superfamily of RiPPs is found in prokaryotes (blue circle) and in eukaryotes (red circle: animals; green circle: plants), where they can be dissect in many classes based on their properties and origin.
LAPs: linear azol(in)e-containing peptides

The high diversity of RiPPs based on the divers PTMs and biosynthetic pathways lead to divers properties, such as antimicrobial, antiviral, antifungal, anticancer, antinociceptive and antiallodynic properties (Lin, Samanta et al. 1996, Gustafson, McKee et al. 2004, Bhat, Halasi et al. 2009, Houssen and Jaspars 2010, Meindl, Schmiederer et al. 2010, Fuchs, Jaskolla et al. 2011, Pandit and Gartel 2011, Ferir, Petrova et al. 2013, Iftime, Jasyk et al. 2015, Mohr, Volz et al. 2015).

Since the number of bacteria becoming resistant to standard antibiotics is increasing, an important property of RiPPs is their antimicrobial activity. Any peptide, which has an antimicrobial activity, is called antimicrobial peptide (AMP). In general, AMPs are low molecular weight peptides 2-10 kDa, have a cationic overall charge and an amphipathic structure (Tossi, Sandri et al. 2000). Additionally, if the AMPs have a bacterial origin they are called bacteriocins (Jacob, Lwoff et al. 1953, Tagg, Dajani et al. 1976, Jack, Tagg et al. 1995). AMPs show various mode of action such as membrane perturbation, membrane pore formation, binding to specific receptors, inhibition of peptidoglycan biosynthesis and inhibition of sporulation (Willey and van der Donk 2007, Malanovic and Lohner 2016,

Oppedijk, Martin et al. 2016). Within the expanding classes of RiPPs, lanthipeptides represent a large reservoir of possible AMPs (Goto, Li et al. 2010). They are mainly produced by Gram-positive bacteria from the phylum of Firmicutes and Actinobacteria. Moreover, lanthipeptide BGCs are found *inter alia* in Bacteroidetes, Chlamydiae, Cyanobacteria and Proteobacteria (Li, Sher et al. 2010, Marsh, O'Sullivan et al. 2010, Tracanna, de Jong et al. 2017). There, lanthipeptides with an antimicrobial activity are called lantibiotics (lanthionine-containing antibiotics) (Kellner, Jung et al. 1988). Lanthipeptide and especially lantibiotic discovery and characterization are important to find new lead compounds for the pharmaceutical industry. Thus, the knowledge in understanding the biosynthesis is requisite to find new ways to synthesis peptides with novel properties.

1.2 The superfamily of lanthipeptides

1.2.1 Overview and classification of lanthipeptides

The RiPP class of lanthipeptides is defined by two particular amino acids modifications. These peptides contain the bis-amino-bis acids lanthionine (Lan) or 3-methyllanthionine (MeLan) (Newton, Abraham et al. 1953, Ingram 1969). The PTM is catalysed by one or two enzyme in two steps. First, serine and threonine residues are dehydrated and result in the α, β unsaturated amino acids 2,3-didehydroalanine (Dha) and 2,3-didehydrobutyrine (Dhb) (Gross and Morell 1967, Gross and Morell 1968). A following Michael-type condensation of these amino acids to cysteine residues yields thioether crosslinks (Lan or MeLan rings). Lanthipeptides have polycyclic structures depending on the number of Ser/Thr and Cys residues. Besides these modifications lanthipeptides harbour other, unusually PTMs, which will be described in the next section (1.2.2).

The BGC of lanthipeptides are found especially in Gram-positive bacteria, but an occurrence is not restricted to this genera. They are also found in Gram-negative bacteria and cyanobacteria (Li, Sher et al. 2010, Tracanna, de Jong et al. 2017). The nomenclature of the lanthipeptide BGC is based on the generic locus symbol *lan* and Lan for the proteins. Each individual lanthipeptide BGC is named after the specific member (e.g. *nis* / Nis for nisin). A capital letter refers to the protein function within the biosynthetic cluster (e.g. A stands for the lanthipeptide precursor peptide, B for the dehydratase etc.).

In the past, lanthipeptides have either been classified based on their properties (e.g. their antimicrobial activity) or their structural diversity (Jung 1991, Sahl and Bierbaum 1998,

McAuliffe, Ross et al. 2001, Chatterjee, Paul et al. 2005). The general classification was based on their antimicrobial activity and they were classified as bacteriocins into class I, II, III and IV (Klaenhammer 1993, Heng and Tagg 2006). To class I belong small (< 10 kDa), heat- stable peptides that contain Lan, MeLan, dehydrated aa and other special PTMs. The class II is dedicated to bacteriocins, which are also small, heat-stable but lack Lan and MeLan. In contrast to class I and II are bacteriocins from class III. They are larger proteins (> 30 kDa) and heat-lable with or without bacteriolytic activity. Additionally, the class IV represents cyclic peptides. Their structural differences and functional properties further dissect class I bacteriocins into class A (with subclasses I/II) and class B (Figure 3), as it was proposed by Jung (Jung 1991).

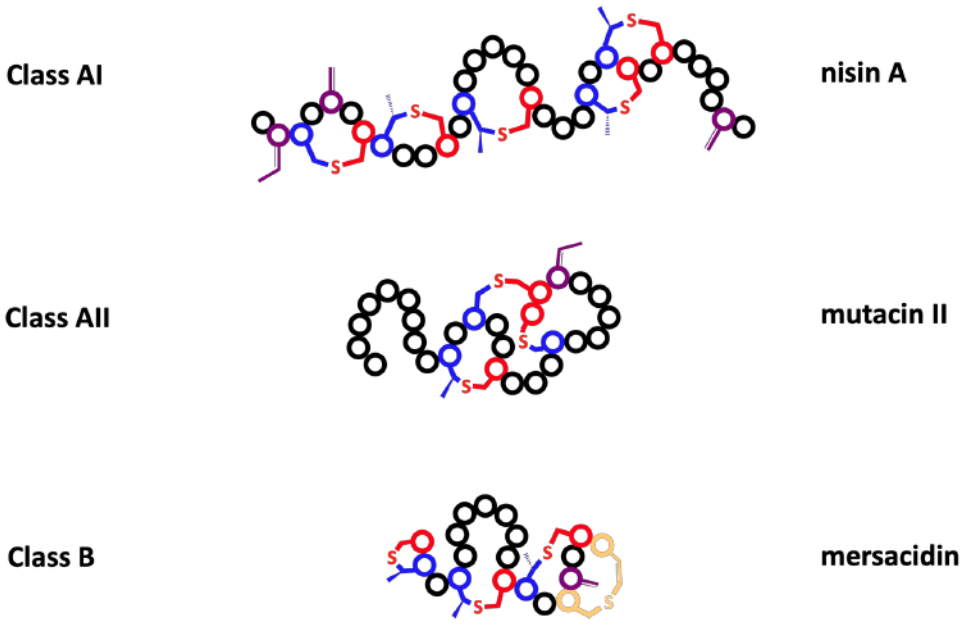


Figure 3: Examples of structural diversity of lanthipeptides.
Three examples of different classes of lanthipeptides structures are shown. The lanthipeptides are classified in classes A1 (nisin), A2 (mutacin II) and B (mersacidin) based on their structure. Colour code for the circles: former cysteine residue red; former serine or threonine residue blue; dehydrated amino acid (Dha or Dhb) violet; tailoring PTM (aminovinyl-cysteine) gold.

Class AI peptides are linear, cationic peptides with a similar ring structure. Members are for example nisin (Gross and Morell 1971), subtilin (Gross and Kiltz 1973), epidermin (Allgaier, Jung et al. 1986), gallidermin (Kellner, Jung et al. 1988) or Pep5 (Kaletta, Entian et al. 1989). However, peptides like mutacin II (Chikindas, Novak et al. 1995), lacticin 481 (Piard, M. et al. 1992) or nukacin ISK-1 (Sashihara, Kimura et al. 2000) have less elongated shape and belong to class AII. Both classes display antimicrobial activities such as pore formation or inhibition of peptidoglycan biosynthesis. Mersacidin (Chatterjee, Chatterjee et al. 1992) and cinnamycin (Kaletta, Entian et al. 1991) are members of class B. They are

globularly structured with less conformational freedom and a bacteriolytic property is uncommon within this group. These peptides interact with specific receptors (e.g. enzymes) to alter their action or inhibiting cell growth (Marki, Hanni et al. 1991, Brötz, Bierbaum et al. 1995). In 2006, a new sub-classification for class I bacteriocins (lantibiotics) was proposed, which divides the class into group Ia (linear), Ib (globular) and Ic (multi-component) (Heng and Tagg 2006).

As the bacteriocin classification includes peptides without Lan and MeLan rings, but excludes lanthipeptides without antimicrobial activity, another classification system had to be established. Moreover, the increasing number of peptides from genome mining approaches made it difficult to classify lanthipeptides in a satisfactorily way. Therefore, these peptides are now classified based on their biosynthetic machinery, as genome mining approaches use the BGC to find lanthipeptides (Willey and van der Donk 2007).

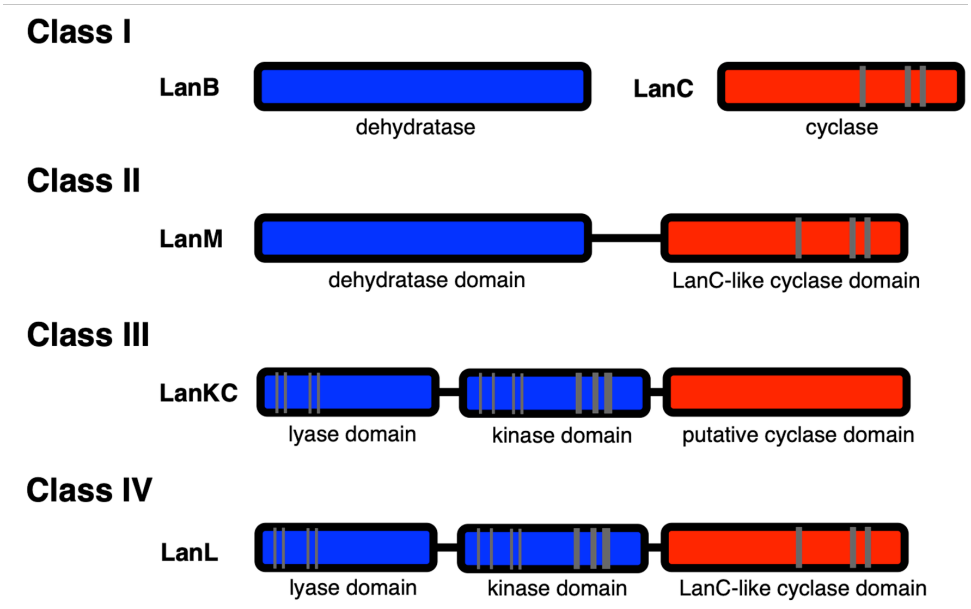


Figure 4: Classification of lanthipeptides based on biosynthetic enzymes.
The modification enzymes of lanthipeptides are dissected into a dehydratase (blue) and cyclase (red) activity. The grey areas define conserved regions for enzyme activity. A black line indicates a fusion of the putative domains. The classification is based on a figure from (Yu, Zhang et al. 2013).

Lanthipeptides are grouped into four classes based on their modification enzymes (Figure 4). In class I the dehydration is performed by a LanB enzyme and the cyclisation by a LanC enzyme, whereas in class II a bifunctional enzyme, termed LanM catalyses these steps. Likewise in the lanthipeptide class III and class IV the bifunctional enzymes LanKC and LanL dehydrate the PTM sites and subsequently the cyclase activity forms Lan and MeLan rings.

1.2.2 Lanthipeptide regulation, biosynthesis and modification

Lanthipeptides are organized in biosynthetic gene cluster (Figure 5), where the precursor peptide (LanA) is located with the enzymes for modification (LanB and LanC or LanM/LanKC/LanL), transport (LanT), procession (LanP), regulation (LanR and LanK), immunity (LanI, LanFEG) and accessory proteins (e.g. LanH) on one locus.

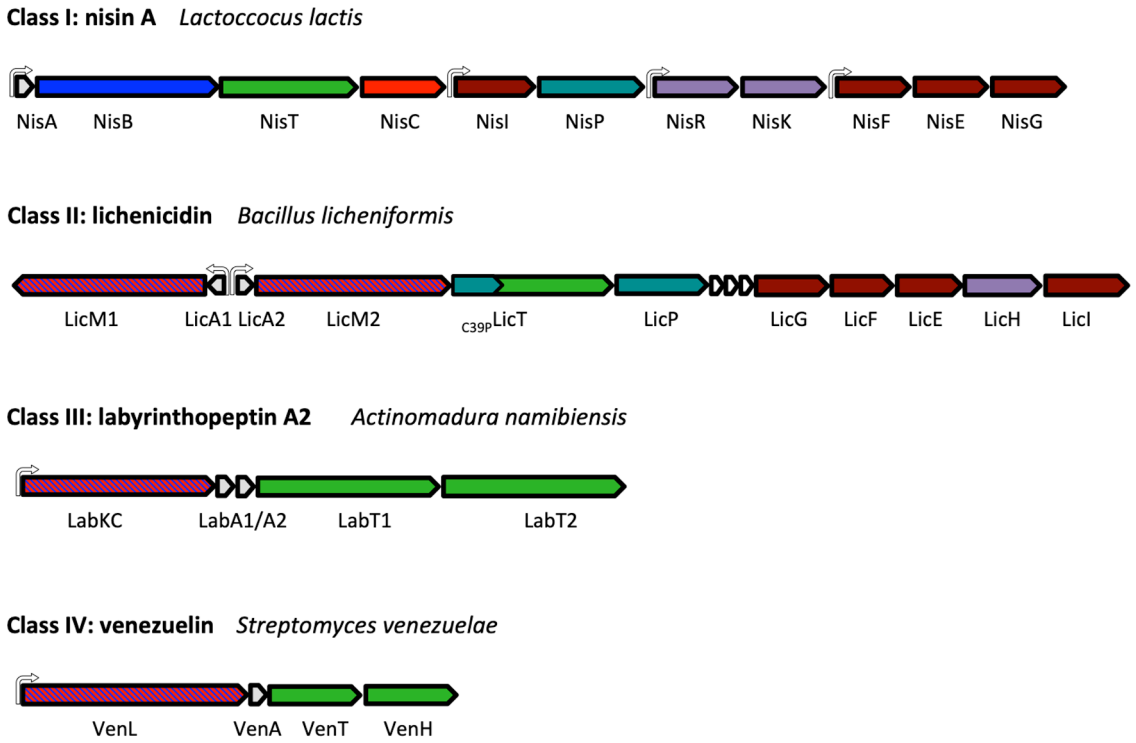


Figure 5: Biosynthetic gene cluster of lanthipeptides class I-IV.
Colour code: Precursor peptide (grey); dehydratase (blue) and bifunctional dehydratase/cyclase (blue, red stripped); cyclase (red); exporter (green); bifunctional exporter/protease (light blue/green); immunity proteins (dark red); two-component system (violet and light violet). The white arrows indicate known promotor. The protein names are under the genes

Some gene cluster are located on chromosomes (e.g. subtilin (Banerjee and Hansen 1988)), on mobile gene elements like conjugative transposons (e.g. nisin (Dodd, Horn et al. 1990, Rauch and de Vos 1992)) or plasmids (e.g. epidermin (Augustin, Rosenstein et al. 1992) or Pep5 (Ersfeld-Dressen, Sahl et al. 1984). The mobile gene elements point to be relicts of the horizontal gene transfer during evolution of the lanthipeptide BGCs between species of Gram-positive bacteria (Dias, Caetano et al. 2015, Johnson and Grossman 2015).

The biosynthesis of lanthipeptides starts at the ribosome, where the mRNA is translated to the aa sequence of the precursor peptide (Ingram 1970). From the ribosome the unmodified precursor peptide gets to the modification enzymes (LanB, LanM, LanKC or LanL). Here, the modifications within the CP are installed in two steps often in a LP-dependent modality. First, Ser and Thr residues are dehydrated to Dha and Dhb. As

mentioned above the reaction is catalysed by one enzyme (LanB), which is restricted to the class I lanthipeptides, or by a bifunctional enzyme (LanM class II, LanKC, or LanL for class III and IV). Many studies investigated the dehydration mechanisms, the reaction kinetics or the interaction of the LanB enzymes with its conjugated substrate (Koponen, Tolonen et al. 2002, Xie, Chatterjee et al. 2002, Lubelski, Overkamp et al. 2008, Lubelski, Khusainov et al. 2009, Mavaro, Abts et al. 2011, Garg, Salazar-Ocampo et al. 2013, Ortega, Hao et al. 2015, Ortega, Hao et al. 2016, Reiners, Abts et al. 2017, Repka, Hetrick et al. 2018). The mechanism of dehydration in LanB is glutamyl-tRNA^{Glu} dependent as shown for NisB and MibB (Ortega, Hao et al. 2015, Ortega, Hao et al. 2016). The hydroxyl-group of Ser and Thr residues is activated by the addition of a glutamyl-group originated from the glutamyl-tRNA^{Glu}. Next, a proton is subtracted from the α-carbon atom of the Ser/Thr residue by a base aa within LanB. Consequently, the β-elimination of the glutamate results in Dha and Dhb, respectively (Ortega, Hao et al. 2015, Ortega, Hao et al. 2016). It was shown that the directionality of the dehydration reaction within the CP (and also cyclisation) is from N- to C-terminus for some class I and class II LanBs/LanMs (e.g. NisB and HalM2) (Lubelski, Khusainov et al. 2009, Thibodeaux, Ha et al. 2014). In contrast to MibB and some bifunctional PTM enzymes (e.g. ProcM and LabKC), where the directionally is revers or follows a random order (Krawczyk, Voller et al. 2013, Mukherjee and van der Donk 2014, Ortega, Hao et al. 2016). During dehydration, the relative position of Ser and Thr residues to the LP does not influence the reaction as shown by alanine site-directed mutagenesis scanning (Lubelski, Overkamp et al. 2008). But the distance of the PTM sites form the enzyme recognition site (ERS) in the LP defines which Ser and Thr residues get dehydrated (Rink, Wierenga et al. 2007, Escano, Stauffer et al. 2015). In addition, the LP dependence of the dehydration reaction was shown, after which the LP increases the efficiency of the reaction (Khusainov and Kuipers 2012).

To get more insight into the mechanism a high resolution structure (e.g. crystal structure) is prerequisite. Two crystal structures of LanB proteins are available, which are the dehydratases of nisin (NisB) and NAI-107 (MibB). Both are dimeric proteins with a two-domain organization consisting of an N-terminal glutamylation (~800 aa) and a C-terminal elimination domain (~350 aa) (Ortega, Hao et al. 2015, Ortega, Hao et al. 2016). While, NisB was co-crystallized with its precursor peptide NisA (electron density fits to the leader peptide Lys-20 to Lys-9), only the apo-structure of MibB was determined. By mapping the electrostatic potential of NisB a possible binding site of the glutamyl-tRNA^{Glu} was identified

in the N-terminal domain and mutational studies further identified residues, which are important for glutamylation and elimination (Khusainov, van Heel et al. 2015, Ortega, Hao et al. 2015).

Sequential after the dehydration reaction follows the cyclisation, catalysed by LanC enzymes, where the Lan and MeLan thioether cross-links are formed (Koponen, Tolonen et al. 2002). LanC enzymes catalyse the regio- and stereoselective addition of a nucleophilic thiol-group from a cysteine residue to the electrophilic dehydro aa (Dha or Dhb). Within the catalytic centre one zinc ion, coordinated by conserved residues activates the thiol-group by lowering its pK_a value (Okeley, Paul et al. 2003, Helfrich, Entian et al. 2007). This activation enables the nucleophilic attack onto the β-carbon atom of the Dha or Dhb, where the enolate protonation yields in the D- or L-configuration at the α-carbon atom. LanC enzymes share a few conserved motifs, which are mainly distributed in the active centre and are important for their activity. In the case of NisC these residues are Cys284, Cys330, His331 involved in coordinating the zinc ion (Li, Yu et al. 2006, Li and van der Donk 2007). Generally, the cyclisation reaction is irreversible and it is proposed, that the ring topology is defined by the substrate CP sequence as only one product (isomer) is facilitate by LanC enzymes (Yu, Zhang et al. 2013). Nevertheless, the reversibility of the reaction was demonstrated for NisC and HalM2, where in the case of HalM2 a His at position 791 is suggested to be the base for the retro-conjugate addition (Yang and van der Donk 2015).

For LanC enzymes one crystal structure is available, which elucidates the reaction mechanism. The crystal structure of NisC comprises of a α, α-toroid fold and an SH2-like domain, which forms a shallow grove. The active centre with the zinc ion is coaxial in the α, α-toroid fold, whereas the extension is suggested to be important in substrate binding (Li, Yu et al. 2006). The substrate is supposed to bind in the shallow grove, which enables the different rings sizes of the Lan and MeLan rings in NisA. The study by Li *et al.* also showed the first *in vitro* activity of a LanC enzyme, where the installation of Lan and MeLan rings was proven by MS analysis and the antimicrobial activity of the peptide after LP removal.

A bifunctional LanM enzyme, consisting of a dehydration domain and a LanC-like cyclase domain with homology to LanC proteins, catalyses the modification of class II lanthipeptides (Figure 4). In contrast to class I LanB enzymes the dehydration of the precursor peptide occur via phosphorylation and is ATP-dependent (Mukherjee and van der Donk 2014, Dong, Tang et al. 2015, Shimafuji, Noguchi et al. 2015, Wang, Ge et al. 2016). In

LanM enzymes Ser and Thr residues are phosphorylated by a kinase domain and yielding phosphoserine (pSer) and phosphothreonine (pThr) residues. Interestingly, the elimination of the phosphate takes place at the same active site and results in Dha and Dhb (Chatterjee, Miller et al. 2005, Thibodeaux, Ha et al. 2014). This mechanism was shown by *in vitro* experiments *inter alia* with the LanMs of lacticin 481 (Xie, Miller et al. 2004), haloduracin (McClerren, Cooper et al. 2006) and prochlorosins (Mukherjee and van der Donk 2014). A more detailed view on the mechanism is possible by evaluating the high-resolution crystal structure of a LanM protein (CylM) (Dong, Tang et al. 2015). The fold of the dehydration domain is similar to Ser/Thr kinases and lipid kinases (LK) or LK-like kinases, whereas the cyclase domain has a similarity to the NisC structure. In general, the dehydration domain is larger than the mentioned kinases and include additional subdomains (e.g. a kinase-activation domain and a activation loop). The binding site of the LP seems to be located in the N-terminal domain as well as in the cyclisation domain (Dong, Tang et al. 2015). Noteworthy, a direct interaction of dehydration domain and the cyclisation domain was not observed and therefrom each domain can function independently when expressed and isolated as single domains (Dong, Tang et al. 2015, Shimafuji, Noguchi et al. 2015, Yu, Mukherjee et al. 2015, Thibodeaux, Wagoner et al. 2016).

The structure of the LanC-like domain of LanM proteins is comparable to LanC proteins and displays a similar fold (Dong, Tang et al. 2015). The overall fold is a α, α-toroid fold but the SH2-like extension is missing. Instead an antiparallel β-sheet might adopt the substrate binding site in LanM proteins during cyclisation. The mechanism of cyclisation is based on the thiol activation by a coordinated zinc ion like in LanC proteins (Paul, Patton et al. 2007). Similar to LanC, LanM proteins catalyse the formation of only one isomer, thus a sequence based but enzyme assisted formation of the Lan and MeLan rings is suggested (Repka, Chekan et al. 2017). The reaction is under kinetic control, where the enzyme enables an increased reactivity of the nucleophile and electrophile by lowering transition state energies. Consequently, depending on the CP sequence only one ring topology is formed (Yu, Mukherjee et al. 2015). Interestingly, in contradiction to class I lanthipeptides (only DL-configuration) also a DL- and LL-stereochemistry has been observed for class II lanthipeptides (as shown for cytolysin and lichenicidin β) (Dischinger, Josten et al. 2009, Tang, Thibodeaux et al. 2016).

In class III lanthipeptides the bifunctional enzyme LanKC carries out the modification of precursor LanA to form mLanA. In the CP of these peptides an additional PTM besides Lan and MeLan is observed, which it is termed labionin (Meindl, Schmiederer et al. 2010). The formation of this PTM is originated from a second conjugate-addition instead of the protonation of the enolate (see class I lanthipeptides). Here, the enolate attacks another Dha and after protonation a labionin is formed. In general, LanKC proteins consist of an N-terminal lyase domain, a central kinase domain and a C-terminal cyclase domain (Muller, Ensle et al. 2011). Although, the cyclase domain exhibits a sequence homology to LanC and the LanC-like domain of LanM proteins, the conserved residues of the catalytic centre (e.g. for zinc ion binding) are absent (Kodani, Hudson et al. 2004, Muller, Schmiederer et al. 2010). The kinase domain presumably catalyses the phosphorylation of Ser and Thr residues in an NTP-dependent manner. Where, for example LabKC utilizes GTP, EryKC ATP and CurKC can use all NTPs to catalyses the phosphorylation (Muller, Schmiederer et al. 2010, Krawczyk, Voller et al. 2012, Voller, Krawczyk et al. 2012). Next, the β-elimination of phosphoserine/-threonine at the lyase domain forms Dha and Dhb, respectively. In contrast to LanM proteins, intermediates of the reaction were detected, which indicates that phosphorylation and elimination are not located at one active site but at their specific domains (Krawczyk, Ensle et al. 2012, Jungmann, Krawczyk et al. 2014). The directionally of the dehydration and the cyclisation in class III lanthipeptides was shown to be C- to N-terminal (Krawczyk, Ensle et al. 2012). Little is known about the exact mechanism of dehydration and the following cyclisation. A conserved sequence of SX_2SX_3C seems to define the characteristic for Lab formation. Although, alternate sequences have been described for other class III lanthipeptides (Meindl, Schmiederer et al. 2010, Wang and van der Donk 2012). Interestingly, in class III lanthipeptides either Lan/MeLan or labionin are found to be the main products (Voller, Krawczyk et al. 2012, Jungmann, van Herwerden et al. 2016). But how this enzymatic process is controlled on a molecular level is still unknown. Examples of class III lanthipeptides are SapB, labyrinthopeptin, catenulipetin, and curvopeptin, which all do not exhibit antimicrobial activities and originally this distinguished these peptides from lantibiotics (Kodani, Hudson et al. 2004, Krawczyk, Voller et al. 2012, Voller, Krawczyk et al. 2012, Wang and van der Donk 2012). Labyrinthopeptin A1 displays antiviral activity against the HIV (human immunodeficiency virus) and HSV (herpes simplex virus) (Ferir, Petrova et al. 2013). Interestingly, one class III lanthipeptide (NAI-112) shows not only low antimicrobial

activity against *Staphylococci* and *Streptococci* but also an antinociceptive activity (Iorio, Sasso et al. 2014).

The class IV lanthipeptides are modified by a lanthipeptide synthetase LanL. This enzyme comprises also of a putative N-terminal pSer/pThr lyase, a central serine/threonine kinase domain and a LanC-like domain located at the C-terminus (Goto, Li et al. 2010). Similar to class III lanthipeptides, their dehydration follows the mechanism of phosphorylation of Ser and Thr residues with a phosphate elimination yielding Dha and Dhb (Goto, Okesli et al. 2011). Subsequently, the LanC-like domain catalyses the intramolecular addition of cysteine residue onto Dha or Dhb resulting in Lan and MeLan ring formation. But in difference to LanKC enzyme the cyclase domain of LanL has a zinc binding site comprising of three cysteine residues (Zhang, Yu et al. 2012). The first characterized member of class IV lanthipeptides was venezuelin (VenA), which is produced by *Streptomyces venezuelae* (Goto, Li et al. 2010). Based on the BGC, this lanthipeptide was assigned to another class (IV) as the modification enzyme LanL differs from the class III modification enzyme LanKC. Other members like streptocollin show a similar ring topology (Iftime, Jasyk et al. 2015, Hegemann and van der Donk 2018). In 2018, a study identified the directionally of the dehydration reaction for globisporin (SgbA) to be from N- to C-terminus (Hegemann and van der Donk 2018). Additionally, they identified the central kinase domain of SgbA to be important to bind the LP of SgbA, where the N-terminal part of the LP was necessary for full modification of the CP. Nothing is known about the activity of the mature peptide or the crystal structure of the modification enzymes so far.

Besides the hallmark modification of lanthipeptides (Lan and MeLan) and the dehydrated aa additional unusual PTMs (e.g. halogenation of Trp residues) are found in some lanthipeptides. These additional PTMs are either catalysed by tailoring enzymes and targeting the N or C-termini of the CP or are installed within the CP by specific enzymes.

The N-terminal modifications are described for the lanthipeptides Pep5, epilancin K7 and epicidin 280. There, the dehydrated aa at the N-terminus (position +1) undergo a spontaneous deamination. For example in Pep5, where the Dha forms a 2-oxobutyryl residue or the deamination of Dhb in lactocin S, which results in an S-2-oxopropionyl residue (Skaugen, Nissen-Meyer et al. 1994). In epilancin K7 and epicidin 280 the 2-oxopropionyl residues are reduced by an oxidoreductase (LanO) to a 2-hydroxylpropionyl residue (lactyl) (Kamp, Hooven et al. 1995, Heidrich, Pag et al. 1998).

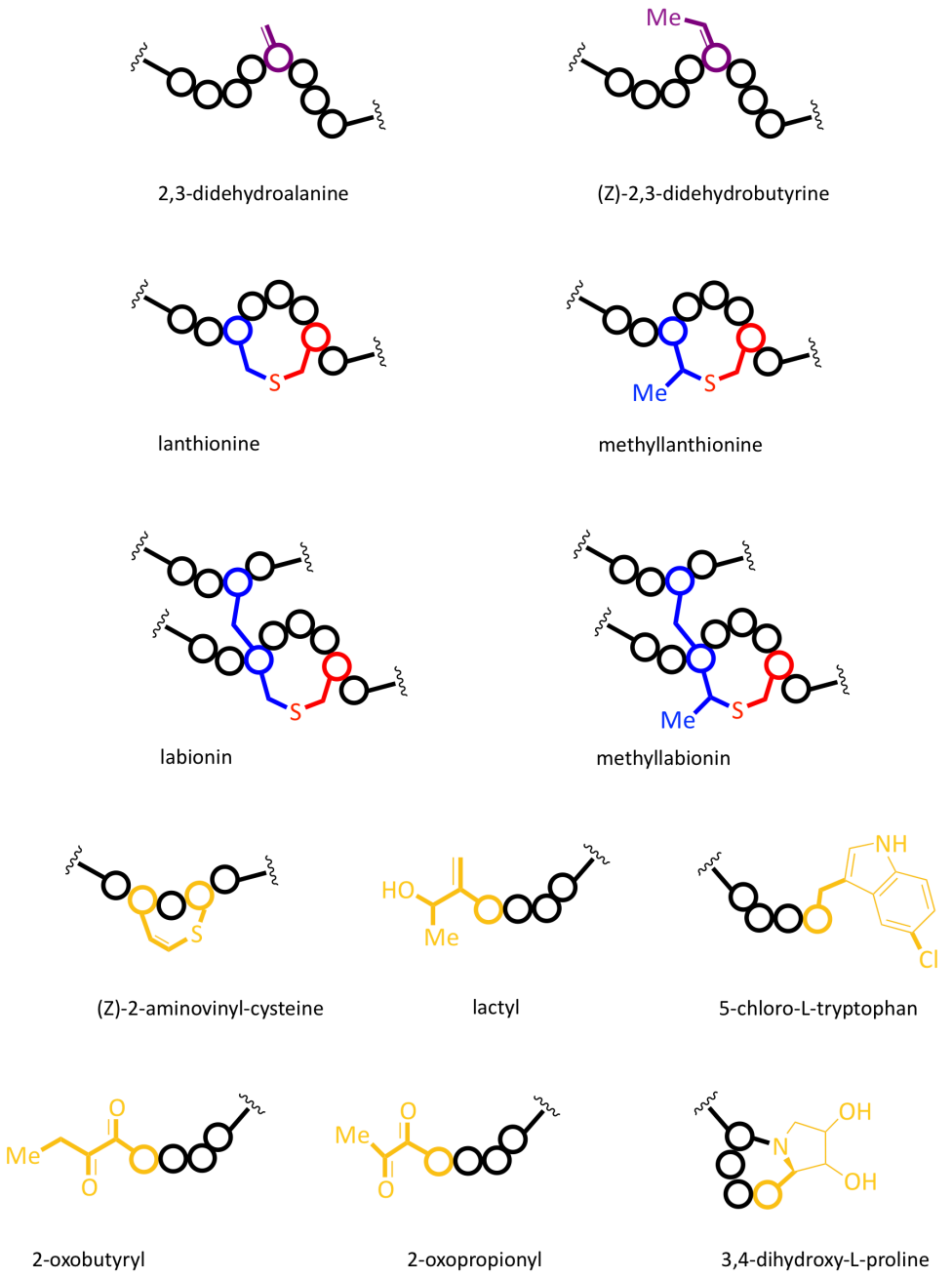


Figure 6: Common post-translational modifications of lanthipeptides.
Colour code: black any amino acid, red former cysteine residue; blue former serine or threonine residue; violet: dehydrated amino acids (Dha or Dhb); gold: tailoring PTM. Scheme of lanthipeptide modifications is based on a figure taken from (Repka, Chekan et al. 2017). It is modified and created with BioDraw Ultra 12.

The C-terminal modifications are found for example in the lanthipeptides epidermin, gallidermin, mersacidin, NAI-107, duramycin A and cinnamycin. In the latter cases the addition of a C-terminal Lys to Dha forms a lysinoalanine bridge (LysN-Ala, or Lal), which is catalysed by a LanN enzyme (Kaletta, Entian et al. 1991, An, Cogan et al. 2018). Another special reaction is catalysed by a flavin-dependent enzyme LanD. First, it catalyses the oxidative decarboxylation of a Cys residue located at the C-terminus. After decarboxylation the resulting (Z)-enethiol reacts with a Dha to form a S-[(Z)-2-aminovinyl]-D-cysteine

(AviCys), which is described for epidermin, gallidermin and NAI-107 (Kellner, Jung et al. 1988, Kupke, Stevanovic et al. 1992, Kupke, Kempter et al. 1994, Castiglione, Lazzarini et al. 2008). A similar modification was found in mersacidin, where the enethiol attacks a Dhb residue and forms a 2-aminovinyl-3-methyl-D-cysteine (AviMeCys) (Jung 1991).

The PTMs in the CP are installed by specific enzymes, which are found in some lanthipeptide BGC. In the CP of the lanthipeptide lactocin S and the two-component lanthipeptide lacticin 3147 a hydrogenation reaction was observed, where a LanJ (LasN, and LtnJ) enzyme catalyses the stereospecific hydrogenation of a Dha residue to a D-alanine (Skaugen, Nissen-Meyer et al. 1994, Cotter, O'Connor et al. 2005). Another special modification is the halogenation of a tryptophan residue described for the lanthipeptide NAI-107. Here, MibH catalyses the chlorination of Trp residue and MibS provides the reduced cofactor FAD (Ortega, Cogan et al. 2017). Additionally, a hydroxylation of a proline residue to dihydroxyproline, which is presumably catalysed by MibO, is found in NAI-107 (Foulston and Bibb 2010). In the class II lanthipeptide actagardine the oxidation of MeLan was observed, which yields in a sulfoxide, where a flavin-dependent monooxygenase GarO catalyses the selective oxidation in the D ring (Shi, Bueno et al. 2012). In contrast to class I and II, only two PTMs from tailoring enzymes are observed in class III and IV lanthipeptides. The formation of a disulfide bond between two cysteine residues was found in the labyrinthopeptin A1-A3, but a specific enzyme for this reaction was not found in the BGC. A *N*-glycosalytion of a Trp residue with a 6-deoxyhexose was described for NAI-112, where a putative glycosyltransferase is located near the corresponding *lanA* locus.

An important aspect of lanthipeptide biosynthesis is the regulation of the BGC, where the transcription is under the control of promotor region upstream and within the gene clusters. In many bacteria the regulation of a BGC is dependent on a two-component system (TCS) in a quorum-sensing manner (Parkinson and Kofoid 1992, Kleerebezem, Quadri et al. 1997). The TCS of some lanthipeptides (e.g. nisin, mersacidin, bovicin HJ50) comprises of LanK and LanR (Kuipers, Beerthuyzen et al. 1995, Altena, Guder et al. 2000, Liu, Zhong et al. 2009). LanK is a on the cell surface located receptor histidine kinase (sensor). This receptor is sensing extracellular changes and transfers the signal via autophosphorylated histidines to the receiver. The receiver is a response regulator LanR and the signal from LanK is transferred via the phosphylation of aspartate residues (van Kraaij, de Vos et al. 1999, Willey and van der Donk 2007, Spiess, Korn et al. 2015). The regulator binds to the promotor region

and the transcription of the operon begins. Besides the autoinduction of lanthipeptide expression via the TCS, other mechanisms to regulate the transcription of the biosynthetic operons are described. In the subtilin operon the expression of SpaRK is depended on the sigma factor SigH and also under the negative control of AbrB expression (a general transition state regulator) (Stein, Borchert et al. 2002). The lanthipeptide NAI-107 produced by the actinobacteria *Microbispora coralline* also need an extracellular sigma factor (MibX) and a starvation signal from limited amounts of nitrogen to trigger transcription (Fernandez-Martinez, Gomez-Escribano et al. 2015). In the case of epidermin two systems control the expression of the structural genes from the BGC. The *epiA* promotor is activated by EpiQ (response regulator) but the system lacks the corresponding histidine kinase (Peschel, Augustin et al. 1993). Additionally, an accessory gene regulator from the *agr* locus seems to control the expression of the epidermin protease but is not involved in any promotor regulation of the BGC (Kies, Vuong et al. 2003).

Especially, the regulation systems for lanthipeptide expression of other class II-IV lanthipeptides show a high diversity and often lack LanK/R proteins. For example the expression of the lacticin 481 BGC is depended on pH acidification and under the control of P1 and P3 promotor (Hindre, Le Pennec et al. 2004). The regulation of the class II lanthipeptide lacticin 3147 is depended on the specific regulator protein LtnR. This protein is a repressor from the PBSX-family of transcriptional repressors and is down-regulating the lanthipeptide production (McAuliffe, Hill et al. 2000, McAuliffe, O'Keeffe et al. 2001). The operon of lactocin S is regulated by the Rgg-like regulator LasX, which binds to the two promotor ($Plas_{A-W}$, $Plas_{XY}$) of the BGC. There, LasX represses its own transcription but increases the transcriptional activity of the $Plas_{A-W}$ promotor (Rawlinson, Nes et al. 2002). Similar to lactocin S, the BGC of mutacin II lacks a LanK protein. Its regulator protein is homologous to the Rgg-family regulators and regulates the transcriptional activity by repression (Merritt and Qi 2012). In the class III lanthipeptide avermipeptin the transcription of the BGC is depended on an A-factor regulatory cascade (Voller, Krawczyk et al. 2012). Another example for the regulation of class III lanthipeptide biosynthesis is the regulatory mechanism for SapB. There, the expression is regulated by specific activators with coregulators (e.g. RamR, BldD and CprA) (O'Connor, Kanellis et al. 2002, Kodani, Hudson et al. 2004). Nothing is known about the regulation of class IV lanthipeptides until now.

1.2.3 The function of the leader peptide

Unlike the general secretion signal (*sec* signal peptide) for unfolded polypeptides (*Sec* pathway) (Natale, Bruser et al. 2008) or the twin-arginine signal for folded polypeptides (Tat pathway) (Palmer, Sargent et al. 2010) in prokaryotes, the LP of lanthipeptides is not only a secretion signal for its dedicated exporter but has many functions.

Leader peptides from lanthipeptides have an average length between 20 and 70 aa (Li, Sher et al. 2010). Generally, the LPs show no sequence homology to *sec* signal peptides or other known signal peptides. One exception seem to be cinnamycin, which has a AXA-cleavage motif for the signal peptidase I (Widdick, Dodd et al. 2003). The LP facilitates many functions such as the recognition site for the modification enzymes (ERS) and it defines the PTM sites in the core peptide (Figure 7) (Chatterjee, Patton et al. 2006, Li, Yu et al. 2006, Mavaro, Abts et al. 2011, Muller, Ensle et al. 2011, Abts, Montalban-Lopez et al. 2013, Ortega, Hao et al. 2015). Additionally, it keeps the mLanA inactive until the secretion and maturation process is finished (van der Meer, Rollema et al. 1994). The LP is also important for the secretion process (Kuipers, de Boef et al. 2004). Furthermore, the LP harbours the cleavage site (CS) for the dedicated LP peptidases (van der Meer, Rollema et al. 1994, Ortega, Velasquez et al. 2014).

In general, LPs have hydrophilic properties with a negative net charge at pH 7. But the net charge is ranging from positive/neutral (e.g. +1 nisin and 0 subtilin) to negatively charge (-4 gallidermin) or strong negatively charged (-9 NAI-107). It remains unclear, if this property represent an important aspect for the interaction with the biosynthetic enzymes or it is just an inherent sequence-dependent effect. A widely discussed property of lanthipeptide LPs is their secondary structure, which is observed with some class I and II lanthipeptides. An amphiphilic, α-helical structure of LPs are described for epidermin, Pep5, nukacin ISK-1 and lacticin 481 (Schnell, Entian et al. 1988, Weil, Beck-Sickinger et al. 1990, Furgerson Ihnken, Chatterjee et al. 2008, Nagao, Morinaga et al. 2009). There, an important function for the modification enzymes is suggested. Moreover, the peptidase domain of the exporter LctT of lacticin 481 recognizes the α-helical property within the LP, where the introduction of the α-helix breaker proline reduced the proteolysis of the precursor peptide (Furgerson Ihnken, Chatterjee et al. 2008). Therefore, even proteolytic enzymes/the exporter seems to need the secondary structure elements for proper recognition of $LanA_{LP}$.

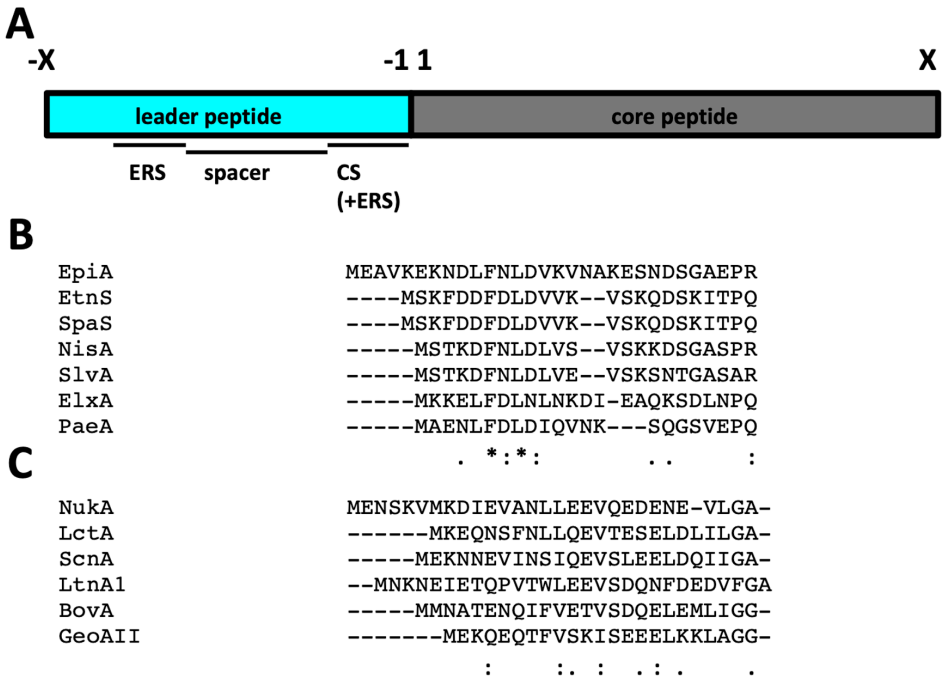


Figure 7: Scheme of lanthipeptide and sequence alignment of leader peptides.
A) The leader peptide sequence of lanthipeptides comprises of an enzyme recognition site (ERS), a spacer region and a cleavage site (CS). The -X defines the possible length of the leader peptide and X the length of the core peptide. A multiple sequence alignment with Clustal Omega of some leader peptides (Madeira, Park et al. 2019). **B)** LPs from class I and **C)** class II show conserved sequence motifs within the leader peptide sequence.
Similar amino acids (.); conserved amino acids (:); highly conserved amino acids (*)

In contrast to these observations, is the random coil NMR structure of the nisin LP in aqueous solution (van den Hooven, Rollema et al. 1997). In addition, no α-helical but a β-strand property of the LP was observed in the co-crystal structure of NisB and NisA (Ortega, Hao et al. 2015). Presumably, the interaction with certain enzymes alters the secondary structure and it is dependent on the LP sequence as well as on the interacting enzyme. Here, the interaction of the LP with its modification enzymes is the key element of lanthipeptide biosynthesis. Therefore, it has to ensure the specificity to PTM sites, while it is recognized by at least two different proteins /domains. The enzyme interaction is achieved in most cases via the LP, where conserved aa motifs are recognized (e.g. FN/DLD-box in class I lanthipeptides). The modification at the PTM site is separated from the recognition site and no modifications were found in the LP sequence as shown for nisin and Pep5 (Weil, Beck-Sickinger et al. 1990, Kuipers, Rollema et al. 1993, van der Meer, Polman et al. 1993). It is assumed, that during LP binding at the modification enzymes the equilibrium is shifted to an active form of these enzymes. Thus, the LP probably induces conformational changes in the modification enzymes, which increases the PTM activity within the CP (Khusainov and Kuipers 2012).

In the class I lanthipeptides the FN/DLD-box motif within LP is highly conserved and it is the recognition site for the LanB and LanC proteins (Mavaro, Abts et al. 2011, Plat, Kluskens et al. 2011, Abts, Montalban-Lopez et al. 2013, Khusainov, Moll et al. 2013). There, the interaction of LP with the LanB is mediated via hydrophobic interactions with the FN/DLD motif, which was observed in the crystal structure of NisB with NisA (Ortega, Hao et al. 2015). Similarly the LP binds LanC via hydrophobic interactions with the Phe and Leu residues of the FNLD-motif (Abts, Montalban-Lopez et al. 2013). The lanthipeptide mutacin 1140 has beside the FN/DLD-box (FDVQ) an addition important four aa motif (EDLF), that is important for the PTM enzymes (Escano, Stauffer et al. 2014). The hydrophobic aa Leu and Phe are suggested to be critical for PTM enzymes interaction. A construction of a minimal LP investigated in the nisin system supports the importance of the FNLD-box. Plat *et al.* could show that a sequence of MSTKDFNLDLR fused to $NisA_{CP}$ is sufficient for recognition by NisB and allows partial dehydration (Plat, Kluskens et al. 2011).

The LPs of other lanthipeptide classes have other motifs. In class II a higher number of negatively charge aa and a C-terminal located motif consisting of a ELXXφX (X is any aa and φ a hydrophobic aa) motif can be found (Xie, Miller et al. 2004, Nagao, Morinaga et al. 2009, Zhang, Yang et al. 2014). Interestingly, the α-helical property is connected to the latter mentioned motif and clearly shows that the interaction of the LP with the modification machinery of class II lanthipeptides follows a different mechanism as class I lanthipeptide modification. Similarly to class I the LP defines the length and thereby the distance to the PTM sites (e.g. shown for LctA, HalA, LicA and LabA1/3) (Chatterjee, Patton et al. 2006, Muller, Ensle et al. 2011, Bindman and van der Donk 2013). In class III lanthipeptides an φL/FE/DLQXφ motif is described, where the hydrophobic aa at position 2 and 5 seem to be important for the modification enzyme (Muller, Ensle et al. 2011). The *in trans* addition of the LP of StaA increases the activity of StaKC (Jungmann, van Herwerden et al. 2016). Thus, the LP seems to be an allosteric activator, which enhances the activity of the modification enzymes like observed for class I-II modification enzymes. The LP importance was also shown for class IV lanthipeptides by binding experiments of the fluorescence-labelled $SgbA_{LP}$ with SgbL (Hegemann and van der Donk 2018). There, especially a α-helical region at the N-terminus of the LP is crucial for binding and maturation by SgbL.

The secretion of the lanthipeptides are LP-dependent and even fusions of non-lanthipeptides to the LP are translocated by the exporter (Kuipers, de Boef et al. 2004,

Kluskens, Kuipers et al. 2005). But so far nothing is known about the recognition site of the exporter proteins within the LP. But it is assumed, that also the FNLD-box might be important for the secretion process (Plat, Kluskens et al. 2011). Additional to the ERS at the N-terminal part of the LP, a second recognition site is located at the C-terminus. These amino acids are the recognition and cleavage site for the leader peptidases. In class I lanthipeptides of the nisin-group the common motif is $GA(X)_nR$ (n: number of residues 1-8). However, depending on the sub-group other motifs like $D/EL/V(X)_nQ$, GAXTP or GAXPQ are found (Pep5-group and epidermin group) (van der Meer, Rollema et al. 1994, Ortega, Velasquez et al. 2014). Some lanthipeptides produced by *Bacilli* do not even have a specific lanthipeptide peptidase (e.g. subtilin or ericin A) (Stein, Borchert et al. 2002, Corvey, Stein et al. 2003). In class II lanthipeptides the consensus motif at the very C-terminus is different and comprises of an $ELX_n\varphi XGG/A/S$ motif (Chen, Qi et al. 2001, Furgerson Ihnken, Chatterjee et al. 2008, Caetano, Barbosa et al. 2014). The recognition motifs of class III and IV lanthipeptide processing are nearly unknown but some known examples will be describe in the following sections.

1.2.4 Lanthipeptide secretion and maturation

The lanthipeptide precursor peptides are primed after modification for direct secretion or for cytoplasmic maturation prior to the translocation process. Therefore, the secretion and maturation process can be dissect into different variants, which are depended on the classification of the lanthipeptide, but it is not necessary the case (Figure 8). First, a general overview of the different variants will be given, before a more detailed description of the secretion (1.3) and maturation (1.4) process follows.

The class I lanthipeptides are mainly secreted by a LanT-type (or NisT-type) ATP-binding cassette (ABC) transporter consisting of 580 - 620 aa and comprises of two domains located on one polypeptide chain. Examples of these homodimeric transporters are NisT (Quiao and Saris 1996), SpaT, (Izaguirre and Hansen 1997) and PepT (Meyer, Bierbaum et al. 1995), which export their substrate without further procession. In these operons (except subtilin-like lanthipeptides) are specific peptidases located, which remove the LP in a final maturation step and activate the precursor peptide. This leader peptidases (LanP) are either locate in the cytoplasm (e.g. PepP (Meyer, Bierbaum et al. 1995), EciP (Heidrich, Pag et al. 1998), ElxP (Ortega, Velasquez et al. 2014)) or extracellularly (e.g. EpiP (Geissler, Götz et al.

1996) and NisP (van der Meer, Polman et al. 1993)). In the latter case, the cleavage of the LP takes place after secretion.

In contrast, many class II lanthipeptides are secreted by AMS (ABC transporter of maturation and secretion) transporter, which are SunT-type ABC transporters (Gebhard 2012). These exporters consists of 680-720 aa and have a three domain organisation (Repka, Chekan et al. 2017). They are bifunctional, homodimeric transporters, where proteolytic activity and translocation of the precursor peptide are tightly coupled processes (Nishie, Shioya et al. 2009, Zheng, Nagao et al. 2017). Examples are the exporter LctT (Uguen, Hindre et al. 2005), MrsT (Altena, Guder et al. 2000), BovT (Liu, Zhong et al. 2009), LicT (Begley, Cotter et al. 2009) and NukT (Nagao, Aso et al. 2005). Interestingly, in the operon of LicT is an additional peptidase LicP located, which further trims the LP of the precursor peptide yielding mature peptide after translocation (Tang, Dong et al. 2015).

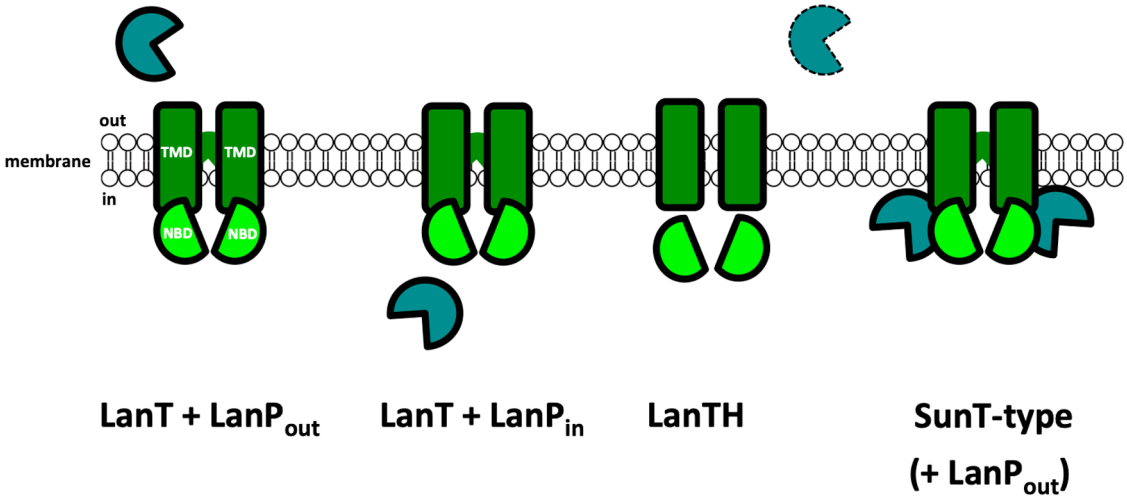


Figure 8: Diversity and localization of lanthipeptide transporters and peptidases.
Colour code: Transmembrane domain (TMD) of transporter in dark green and ATPase domain (NBD) in light green. Peptidase or peptidase domain (C39P) is indicated in light blue.

The class III lanthipeptides are secreted by LanT-type ABC transporters after modification and presumably after their finial maturation step. There, it was first suggested, that the proteolytic cleavage of the LP is performed by prolyl oligopeptidase (POP) (Voller, Krawczyk et al. 2013). But they are regularly not found in the BGCs of lanthipeptides and a general allocation to the BGC is not possible. Recently, a Zn-dependent protease AplP was found to be responsible for LP cleavage of NAI-112 and thereby represents a class of previously unknown leader peptidases (Chen, Xu et al. 2019).

In class IV lanthipeptides little is known about the transporter and to date no protease is described (Goto, Li et al. 2010, Hegemann and van der Donk 2018). In the BGC

two proteins responsible for the translocation process can be identified. There, one protein consists of 330 - 380 aa and is the ATP-binding component, which is also called LanT. The other protein consists of 250 - 300 aa and is a the membrane-spanning protein, which is termed as LanH. It is assumed, that the heterodimeric transporter functions as an exporter and immunity protein (Goto, Li et al. 2010, Iftime, Jasyk et al. 2015).

1.2.5 Lanthipeptide immunity and other accessory proteins

After the final steps of lanthipeptide secretion and maturation, the lanthipeptide is released without the LP to the extracellular space. In this active, mature form the lanthipeptide would immediately targeting the cell membrane or specific receptors of the producer strain. This especially hold true for antimicrobial active lanthipeptides (lantibiotics). Therefore, the expression of specific immunity proteins, which confer immunity to its own produced lantibiotic is indispensable.

In the most BGC of class I and II lanthipeptides at least one immunity system represented by LanI and LanFEG proteins is found. The LanI proteins are extracellular located lipoproteins, which are anchored in the membrane by an N-terminal Cys modification with a fatty acid chain (Takala, Koponen et al. 2004). The deletion of *lanI* reduces the immunity in the producer strain as shown for the nisin immunity protein NisI (Koponen, Takala et al. 2004). Other LanI proteins are for example found in the BGC of Pep5 (Reis, Eschbach-Bludau et al. 1994), epicidin 280 (Heidrich, Pag et al. 1998), subtilin (Stein, Heinzmann et al. 2005) and of the two-component lanthipeptides lacticin 3147 α and β (McAuliffe, Hill et al. 2000). For some LanI proteins the specific interaction with the lanthipeptide was shown (e.g. NisI and SpaI) (Stein, Heinzmann et al. 2003, Takala, Koponen et al. 2004, Stein, Heinzmann et al. 2005, Hacker, Christ et al. 2015). Furthermore, additional structural information about LanI proteins increased the knowledge of the immunity mechanisms. The NMR structure of SpaI and of NisI (additional an crystal structure is available (Jeong and Ha 2018)) shows a similar six/seven-stranded β-sheet fold with a hydrophobic patch in the C-terminal region (Christ, Bochmann et al. 2012, Hacker, Christ et al. 2015). There, NisI comprises of a N-terminal and a C-terminal domain, whereas SpaI has one domain. Both domains of NisI can be aligned to the SpaI structure, although the sequence identity is low. In the case of NisI a titration experiment with nisin was performed (Hacker, Christ et al. 2015). The comparison of the NMR structure with and without nisin showed chemical shifts of NisI in the C-terminal

domain. This indicates the importance of the C-terminus, which supports former mutational studies, where the deletion of the C-terminus of NisI decreased the immunity of *L. lactis* against nisin (Takala and Saris 2006, AlKhatib, Lagedroste et al. 2014). Additionally, other LanI protein like PepI also showed the importance of the C-terminal part to confer immunity (Hoffmann, Schneider et al. 2004). The possible interactions of LanI with its lanthipeptides are to inhibit membrane insertion and oligomerisation, which further hampers the pore formation (AlKhatib, Lagedroste et al. 2014). Thus, LanI proteins might interact with lanthipeptide receptors (e.g. membrane/lipid II for nisin) to circumvent receptor/LanA complex formation (Hacker, Christ et al. 2015, Khosa, Lagedroste et al. 2016, Jeong and Ha 2018).

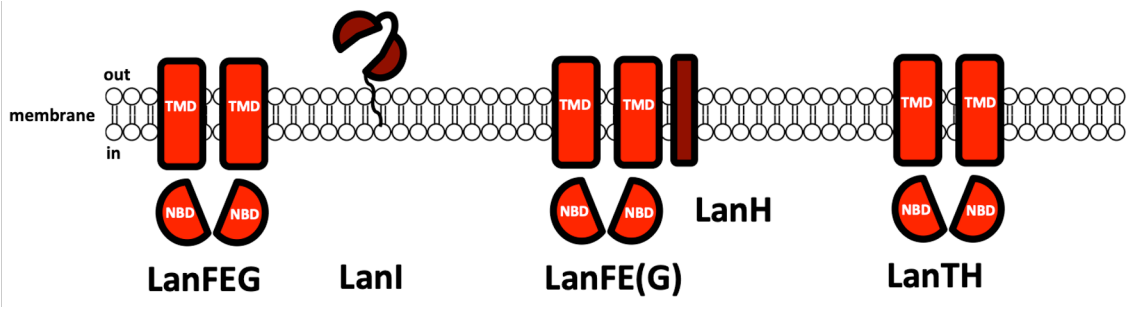


Figure 9: Scheme of lanthipeptide immunity systems.
Colour code: Transmembrane domain (TMD) and ATPase domain (NBD) of ABC transporter in light red. Lipoprotein LanI and accessory protein LanH are indicated in dark red.

The second immunity system are LanFEG proteins. They belong to the class of LanFEG-type ABC transporter, which confer immunity against their specific lanthipeptide by extrusion from the cytoplasmic membrane (Peschel and Gotz 1996, Otto, Peschel et al. 1998, Stein, Heinzmann et al. 2003). These ABC transporters consist of the membrane-spanning proteins LanE and G, whereas LanF is the cytoplasmic ATP-binding protein LanF. A fully functional LanFEG-type ABC transporter comprises of heterodimeric LanEG and a homodimeric LanF proteins. Interestingly, immunity systems are described, where either LanI or LanFEG are expressed and this seems to correlate with the mode of actions of the lanthipeptide.

In the cases, where the lantibiotic has a dual antimicrobial activity (e.g. pore formation and inhibition of peptidoglycan biosynthesis), LanI and LanFEG work in a cooperative manner to confer full immunity against the dedicated lantibiotic (Stein, Heinzmann et al. 2003, Stein, Heinzmann et al. 2005). Thus, LanFEG and LanI proteins are for example present in the BGC of nisin (Ra, Qiao et al. 1996), epidermin (Peschel and Gotz

1996), subtilin (Klein and Entian 1994) and streptin (Wescombe and Tagg 2003). Moreover, if the lantibiotic only forms pores, LanI but not LanFEG is found in the operon (e.g. Pep5 (Reis, Eschbach-Bludau et al. 1994, Pag, Heidrich et al. 1999) epicidin 280 (Heidrich, Pag et al. 1998) and lactocin S (Skaugen, Abildgaard et al. 1997). The LanFEG-type ABC transporter is especially found in operons, where the lantibiotic binds to specific receptor (epidermin) (Peschel and Gotz 1996), interferes with peptidoglycan biosynthesis (mersacindin) (Altena, Guder et al. 2000) or the lipid-composition of membranes (cinnamycin) (Marki, Hanni et al. 1991).

In the BGC of gallidermin and epidermin no lipoprotein is encoded but an accessory protein termed LanH. This accessory protein contributes to a higher immunity towards the lanthipeptide and also has an influence on the production level (Hille, Kies et al. 2001). Similarly, in *Staphylococcus warneri* ISK-1 the membrane protein NukH acts in cooperative manner with the LanFEG-type ABC transporter NukFEG to confer 100% immunity against nukacin ISK-1 (Aso, Okuda et al. 2005, Okuda, Aso et al. 2008). Another example of an operon encoding a lipoprotein, an ABC transporter and an accessory protein is found in the NAI-107 producer *Microbispora* ATCC PTA-5042. There, MlbQ is the lipoprotein, that confers immunity to NAI-107-like lanthipeptides analogously to LanI proteins (Pozzi, Coles et al. 2016). Additionally, an ABC transporter MlbYZ and the accessory protein MlbJ (function as LanH protein) located in the same operon seem to contribute to immunity in a cooperatively manner. In the class II lanthipeptides are two-component lanthipeptides (α- and β-mLanA) found, where one variant has a pore-forming mode of action and the other inhibits for example the peptidoglycan biosynthesis (McClerren, Cooper et al. 2006, Wiedemann, Bottiger et al. 2006). In their operons a LanI protein, a LanFEG-type ABC transporter and an accessory protein LanH are expressed and to confer immunity against both lanthipeptide variants (e.g. lichenicidin BGC (Dischinger, Josten et al. 2009, Caetano, Krawczyk et al. 2011)). Some operons like in the BGC of lacticin 3147 lack the LanH protein and only ltnI and LtnEF are found (McAuliffe, Hill et al. 2000). In many cases the components of the immunity system seem to be coevolved to the biological activity of the lanthipeptide and thereby different strategy for immunity systems are found.

In the BGC of class III and IV lanthipeptides the immunity proteins LanI and LanFEG are absent. But in class IV lanthipeptides the proteins LanTH seem to be important for substrate translocation and also for immunity (Goto, Li et al. 2010, Iftime, Jasyk et al. 2015).

However, detailed information's about the lanthipeptide activity are missing and thereby nothing is known about these ABC transporters.

1.2.6 Activity and applications of lanthipeptides

The antimicrobial activity is until now mostly dedicated to class I and II lanthipeptides, which are also know as lantibiotics. Many of these are highly active against Gram-positive bacteria from the genera of *Bacillus, Clostridium, Lactobacillus, Micrococcus, Streptococcus and Staphylococcus* (Crowther, Baines et al. 2013, Wang, Zhang et al. 2014, Maffioli, Monciardini et al. 2015, van Heel, Kloosterman et al. 2016). However, most lanthipeptides show no effect on Gram-negative bacteria or yeast under normal conditions (Kordel, Benz et al. 1988, Stevens, Sheldon et al. 1991). The reason is the additional outer membrane of Gram-negative bacteria or the cell wall of yeasts, which act as a barrier and are generally not permeable for these peptides.

Lantibiotics are antimicrobial active through many mechanisms, which are ubiquitous found in these AMPs. There, the pore-formation, inhibition of peptidoglycan synthesis or rather specific mode of actions (e.g. targeting certain receptor in the cell wall) can be identified. Nisin, subtilin and mersacidin are common members, which are inhibiting the peptidoglycan biosynthesis (Reisinger, Seidel et al. 1980, Brötz, Bierbaum et al. 1995, Parisot, Carey et al. 2008). The mechanism of inhibition was shown for nisin and was investigated on a molecular level. Nisin is interacting with the pyrophosphate of lipid II via ring A and B to from a membrane located complex with a ratio of 1:1 (Hsu, Breukink et al. 2004). Following, as a second mechanism (sequestering effect), the complex dissociates from the septum (cell division site) and the cell wall precursor lipid II is no loner available (Hasper, Kramer et al. 2006). Besides nisin also mersacidin (Brötz, Bierbaum et al. 1998), nukacin ISK-1 (Fujinami, Mahin et al. 2018), plantaricin C (Wiedemann, Bottiger et al. 2006), lacticin 3147 (Wiedemann, Bottiger et al. 2006), lichenicidin (Shenkarev, Finkina et al. 2010) are binding lipid II or other peptidoglycan precursors. Additionally, nisin has a pore-forming mode of action, where the membrane complex of nisin and lipid II subsequently assemble to a pore complex of four nisin and eight lipid II molecules with a diameter of 2-2.5 nm (Hasper, de Kruijff et al. 2004, Wiedemann, Benz et al. 2004, Medeiros-Silva, Jekhmane et al. 2018). A partly complex result in membrane perturbation, then alternation of the membrane potential and the final fully assembled complex lysis the cell (Ruhr and Sahl 1985, Gao, Abee

et al. 1991, Hasper, de Kruijff et al. 2004, Tol, Morales Angeles et al. 2015, Mulholland, Turpin et al. 2016, t Hart, Oppedijk et al. 2016). Further members with pore-forming or membrane depolarization mode of action are subtilin, Pep5 and epidermin (Kordel, Schuller et al. 1989, Schuller, Benz et al. 1989, Brötz, Josten et al. 1998, Parisot, Carey et al. 2008). For other lantibiotics the interaction with or the inhibition of specific receptors was observed. The lantibiotic cinnamycin or cinnamycin-like peptides (e.g. duramycin B/C) inhibit the phospholipase A2 by targeting phosphatidyl ethanolamine and thereby alternate the phospholipid composition of membranes (Fredenhagen, Fendrich et al. 1990, Marki, Hanni et al. 1991). By this mode of action the membrane permeability of bacterial cells can be increased (Sokolove, Westphal et al. 1989). Another mode of action of lantibiotics (e.g. nisin) is the inhibition of sporulation of *Bacillus cereus* strain T or other spore forming species (Morris, Walsh et al. 1984, Gut, Blanke et al. 2011).

Although, the class III and IV lanthipeptides are not associated with antimicrobial activity, there are some other bioactivities describe for some class III lanthipeptides. For the lanthipeptide NAI-112 has a low antimicrobial activity against *Staphylococci* and *Streptococci* but more interestingly a high antinociceptive effect was determined (Iorio, Sasso et al. 2014). The antiviral activity against the HIV and HSV is described for labyrinthopeptin A1 (Ferir, Petrova et al. 2013), whereas the lanthipeptides SapT and SapB have a morphogenic activity on the hyphae of Streptomycetes (Kodani, Hudson et al. 2004, Kodani, Lodato et al. 2005).

Most applications of lanthipeptides are derived form their antimicrobial activity, therefrom they are used in the fields of veterinary medicine, food and pharmaceutical industry (Delves-Broughton, Blackburn et al. 1996, Cotter, Hill et al. 2005, Dischinger, Basi Chipalu et al. 2014). Especially, in the food industry some lanthipeptides are used, because of their insensitive to proteolysis and their heat stability. There, they are used in dairy industry to prevent food spoilage, due to the high activity towards other Gram-positive bacteria (Denny, Sharpe et al. 1961). One example is nisin, which is used as a food preservative since over 50 years (Rayman, Aris et al. 1981, Delves-Broughton 1990). Its is commercial produced (Nisaplin , company DuPont) and used as a food additive (E234) for canned foods, cheese, milk, or other dairy products. In addition, lanthipeptide-producing strains can be used as a starter or adjunct strain in dairy industry or for fermented food (O' Shea, Cotter et al. 2013). In the veterinary medicine the two-component lanthipeptide

lacticin 3147 and nisin are used to prevent and tread mastitis in cattle (Ryan, Meaney et al. 1998, Twomey, Wheelock et al. 2000, Cao, Wu et al. 2007). Besides the use in the veterinary medicine some lantibiotics are investigated for the treatment of pathogenic bacteria. There, some are in the preclinical and clinical phases of the development as pharmaceutical. Examples are a derivative of the class II lanthipeptide deoxy-actagardine B (NVB302, company Novacta Biosystems Limited) (Li, Li et al. 2012), the class I lanthipeptide NAI-107 (company NIACON) (Castiglione, Lazzarini et al. 2008) or the synthetic class II lanthipeptide mutacin 1140-S (company Organics) (Ghobrial, Derendorf et al. 2009) to treat *Clostridiodes difficile*, methicillin-resistant *Staphylococcus aureus* (MRSA) or vancomycin resistant enterococci (VRE) infections (Sandiford 2019). Other lanthipeptides like mersacidin, the two-component lanthipeptides lichenicidin (Bliα and Bliβ) or lacticin 3147 (LctA/A2) and epilancin 15X are active against MRSA and VRE with partly higher potency than the antibiotic vancomycin (Brötz, Bierbaum et al. 1995, Ekkelenkamp, Hanssen et al. 2005, Lawton, Ross et al. 2007, Begley, Cotter et al. 2009). Interestingly, the cinnamycin-like lanthipeptide duramycin display another bioactivity and can be used in the therapeutic treatment of cystic fibrosis (Grasemann, Stehling et al. 2007, Grasemann 2012).

The lanthipeptide NAI-107 and its derivatives (e.g. brominated variant) produced by *Microbispora sp*. 107891 are hitherto the most active lanthipeptides and show the highest potential against clinical relevant pathogenic bacteria (Jabes, Brunati et al. 2011, Cruz, Iorio et al. 2015, Maffioli, Monciardini et al. 2015, Maffioli, Cruz et al. 2016, Brunati, Thomsen et al. 2018). All these examples of lanthipeptide usage as pharmaceutical applications show the high potency of lanthipeptides especially against pathogenic Gram-positive bacteria. Moreover, the engineering and rational design of lanthipeptides will lead to new properties and alternate target specificity, which will further extend the bioactivity spectrum against pathogenic bacteria (Zhou, van Heel et al. 2015, Zhou, van Heel et al. 2016, Baumann, Nickling et al. 2017, Burkhart, Kakkar et al. 2017, Montalban-Lopez, van Heel et al. 2017, Bartholomae, Baumann et al. 2018, Li, Montalban-Lopez et al. 2018). The high throughput screening of novel or engineered lanthipeptides can be achieved by a colony-based assay in *E. coli* (Si, Tian et al. 2018), a nano-scale Fleming platform (Schmitt, Montalban-Lopez et al. 2019) or by yeast- and phage-display libraries following an activity screen (Urban, Moosmeier et al. 2017, Hetrick, Walker et al. 2018)

Additional applications for lanthipeptides or parts of the biosynthetic gene cluster are biotechnology applications to overexpress recombinant peptides or proteins. Two example are the nisin-controlled expression (NICE) (Kuipers, de Ruyter et al. 1998) and subtilin-regulated expression (SURE) (Kleerebezem, Bongers et al. 2004) of proteins (homologous or heterologous) in Gram-positive bacteria hosts. Both systems work similar and it will be briefly described for the NICE system. This application is based on the TCS proteins NisK and NisR. They are encoded on the chromosomes of the *L. lactis* MG1363 derivative MG1614 (called NZ9000), where the genes for both proteins are included into the *pepN* locus (Kuipers, de Ruyter et al. 1997, Kuipers, de Ruyter et al. 1998). The expression of recombinant proteins is possible with plasmids, where the target gene is under the control of the *nisA* promotor. This promotor is tightly regulated by its TCS and the addition of nisin (inducer concentration range: 0.01-25 ng/ml) leads to protein overexpression (Kuipers, de Ruyter et al. 1997, Eichenbaum, Federle et al. 1998). Especially, the NICE system is widely used and it is commercially available to employ homologous and heterologous overexpression in lactic acid bacteria.

1.3 Lanthipeptide ABC Transporters

1.3.1 General aspects of ABC transporters

ATP-binding cassette (ABC) transporters are ubiquitous found in all kingdoms of life and represent a large superfamily of membrane proteins (MP) (Davidson, Dassa et al. 2008, Holland 2011). In these MPs the hydrolysis of a γ-phosphate from ATP molecule generates the energy for translocation of various substrates from the *cis*-side to the *trans*-side of a membrane (Anderson, Berger et al. 1991, Higgins 1992). The substrates are for example ions, polysaccharides, amino acids, siderophores, drugs and polypeptides from dipeptides to large proteins, where they differ in size, charge, hydrophobicity and display variable inherent properties. Therefore, ABC transporters are involved in many processes ranging from drug extrusion, nutrient uptake, substrate secretion and lipid homeostasis (Martinoia, Klein et al. 2002, Raetz, Reynolds et al. 2007, Rea 2007, Ruiz, Gronenberg et al. 2008, Seeger and van Veen 2009, Cuthbertson, Kos et al. 2010, Leprohon, Legare et al. 2011, Wong, Ma et al. 2014). As some processes are critical for any organism, an ABC transporter dysfunction especially in humans is related to known diseases and thereby they are of special interest in current research. Examples are the defect of ABCC7 (cystic fibrosis transmembrane

conductance regulator: CFTR), which causes cystic fibrosis (Riordan, Rommens et al. 1989) or the overexpression of ABCB1 (P-glycoprotein: PgP) in tumour cells (Germann, Pastan et al. 1993).

In general, ABC transporters have a two-domain organization consisting of a transmembrane domain (TMD) and a nucleotide-binding domain (NBD). There, the aa sequence of NBDs is more conserved than of the TMDs and are the hallmark of the ABC transporter family. The NBD has an ATPase function similar to P-loop NTPases, which binds and hydrolyses ATP (Vetter and Wittinghofer 1999). The TMD comprises of α-helical transmembrane (TM) segments, where two TMDs form a pathway for substrate translocation. In ABC transporters the number of TM helices can vary between 5 and 12 and TMDs share a low sequence identity (Biemans-Oldehinkel, Doeven et al. 2006, Rees, Johnson et al. 2009). The architecture of ABC transporters and its subdomains is mainly depended on the origin (prokaryotic or eukaryotic) and their function. Here, the superfamily of ABC transporters can be classified as exporters or importers and function as homo- or heterodimeric proteins (Higgins 1992, Davidson, Dassa et al. 2008).

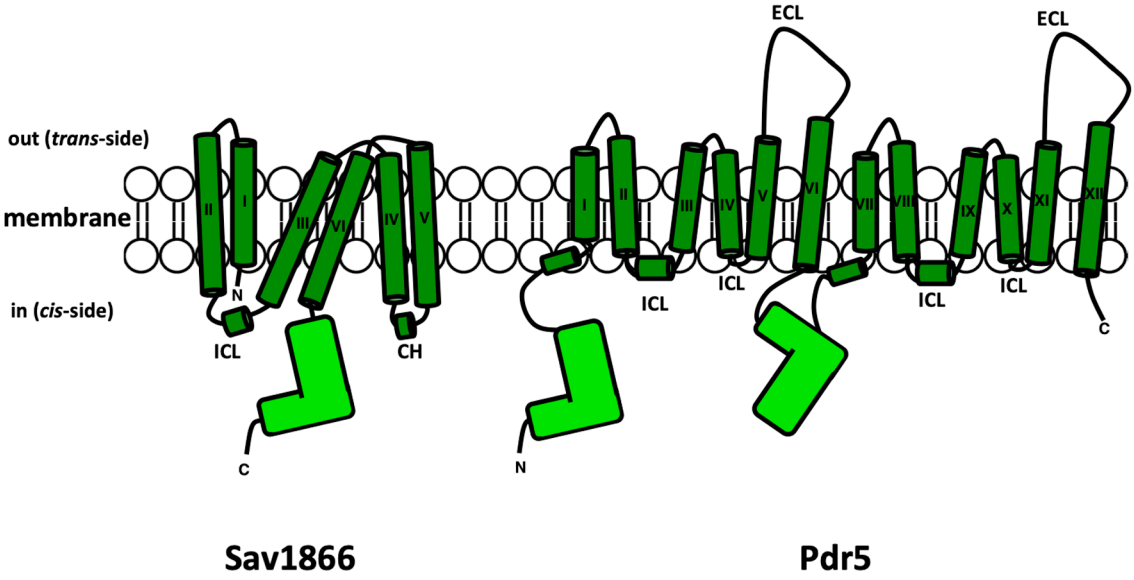


Figure 10: Topology of ABC transporters.
A) Multidrug ABC transporter Sav1866 is a half-size transporter with a pseudo-twofold symmetry between TM helix 1-3 and 4-6. The coupling helix (CH) is located between TM helix 4 and 5 (Dawson and Locher 2006). **B)** A revers topology is represented by the multidrug ABC transporter Pdr5, which is a full-size transporter. Additionally, Pdr5 has large extracellular loops (ECL) and intracellular loops (ICL), which function as connecting and coupling helices (Golin and Ambudkar 2015, Lee, Kinch et al. 2016). Colour code: TMDs are displayed in dark green and NBDs in light green.

Export systems can be found from bacteria to mammalians, whereas import systems are dedicated to prokaryote membranes. ABC transporter exporters are located in the cytoplasmic membrane (prokaryotes) and in the membrane compartments of organelles

(eukaryotes). The domain organization of exporter follows an N- to C-terminal orientation of TMD and NBD, which are often located on one polypeptide chain. This feature is a common organization in prokaryotic exporters (except MacB NBD-TMD (Okada, Yamashita et al. 2017)) and they are termed half-size transporter. If two polypeptide chains are fused to a domain organization of two TMDs plus two NBD, they are called full-size transporter and mainly found in eukaryotes (e.g. ABCB1). In the case of full-size transporter a domain organization of $(TMD\text{-}NBD)_2$ or *vice versa* is found. Nevertheless, there are also domain organizations possible, where all subdomains are synthesized as separate proteins. Additional some NBDs, which are not associated with the TMD, display divers, independent functions (Boel, Smith et al. 2014). Thus, the overall term of ABC proteins might be used to include these proteins.

The available crystal structures of the ABC transporter superfamily allows the identification of seven types, where three types are allocated to the group of importer and four to the group of exporter (Figure 11) (ter Beek, Guskov et al. 2014, Locher 2016, Rempel, Stanek et al. 2018).

The importers are found in the cytoplasmic membrane of prokaryotes and facilitate the uptake of nutrients from the *trans*-side to the *cis*-side of the membrane (e.g. ions, amino acids and sugars). The importers are in contrast to exporters associated with additional substrate-binding domains (SBDs) or substrate-binding proteins (SBPs). There, the SBD or SBP are located at the trans-side of the membrane and are necessary for the uptake of the substrates via delivery to the TMDs (Ames 1986, Higgins 1992). All prokaryotic importers can be distinguished into three types based on their structural arraignments. The type I importer transports substrates like sugars or amino acids, which are needed in high amounts for the growth of prokaryotes. Examples of this type are the ABC transporter $MalFGK_2$ (importer for maltose) (Mannering, Sharma et al. 2001) and HisPQM (importer for histidine) (Ames, Nikaido et al. 2001). The other importers from the type II and type III (energy-coupling factor; ECF) transport specific nutrients like vitamins (vitamin B12), metal chelates (e.g. heme) or metal ions (Co^{2+}) (Rempel, Stanek et al. 2018). It is assumed, that these importers transport their substrates in small quantifies with high specificity, whereas the type I importers transport in high quantities without being too specific for the substrate (ter Beek, Guskov et al. 2014). The vitamin B12 transporter BtuCDF represents the type II importer and consists of the TMD BtuC, the NBD BtuD and the SBP BtuF.

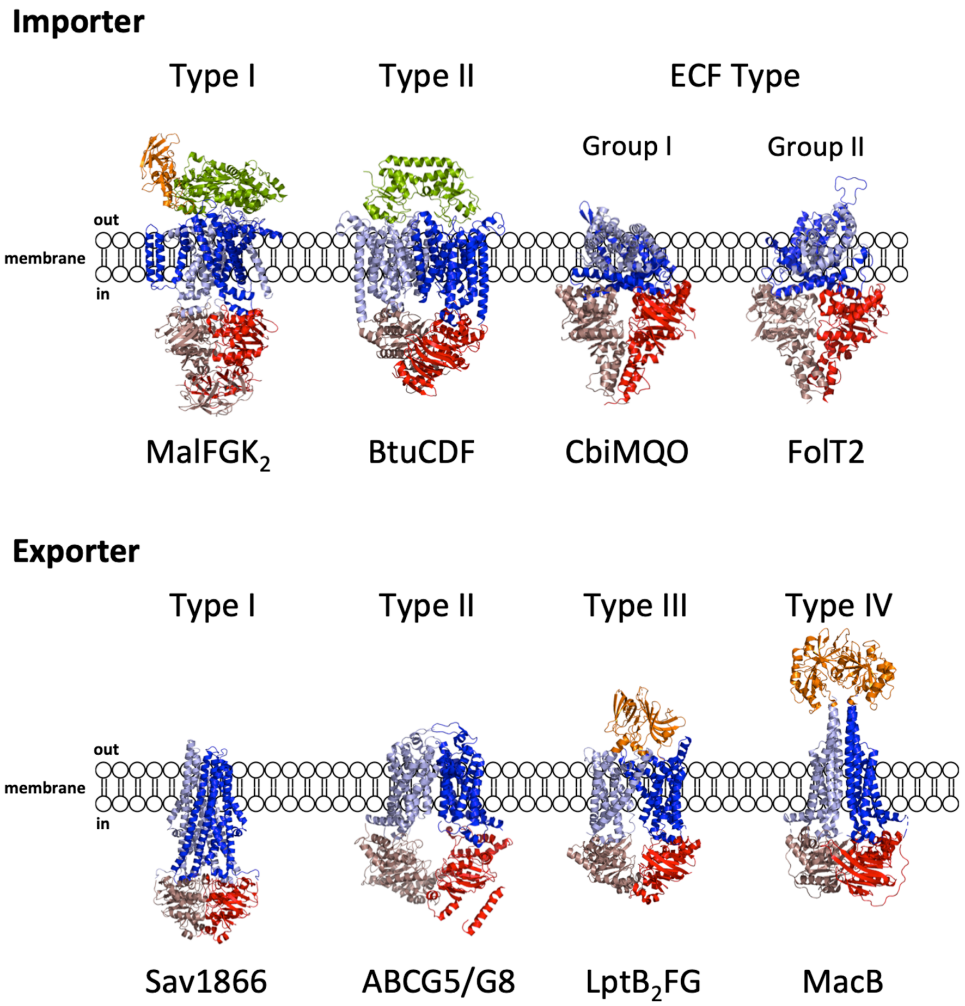


Figure 11: Structural representation of importer and exporter types of ABC transporters.
Seven groups represent the different types of ABC transporter based on their structure and their function. The class of importers (**A**) are constituted by three types: BtuCDF (PDB ID: 2QI9), CbiMNQO (PDB ID: 5X3X) and ECF-FolT2 (PDB ID: 5JSZ). The class of exporters (**B**) are constituted by four types: Sav1866 (PDB ID: 2HYD), ABCG5/G8 (PDB ID: 5DO7), $LptB_2FG$ (PDB ID: 5X5Y) and MacB (PDB ID: 5LIL). Colour code: TMDs in (light and dark blue), NBDs in (light red and red), SBP/S-components (green) and other domains (orange). The figure is adapted with modifications from Rempel *et al.* (Rempel, Stanek et al. 2018). The program PyMOL 2.1.1 was used to display the crystal structures in a cartoon representation.

Noteworthy, the ECF-type transporters lack the extracellular SBPs, which are found in type I and II importers and use a membrane-bound binding protein (S-components). Sometimes, this protein functions solitary and transport their substrate without the dedicated components of the group 1 and group 2 of ECF-type transporter. There, the fully assemble transport complex comprises of the NBD (also termed ECF-A component), TMD (also termed ECF-T component) and the S-component (Rempel, Stanek et al. 2018). One example for group 1 is the cobalt transporter CbiMNQO and for group 2 is the folate transporter ECF-FolT2, whereas ButM represents a S-component, which transports cobalamin (vitamine B12) without the ECF-A and ECF-T components.

In the class of exporter four types of ABC transporter are know, which differ in their overall fold, especially of the TMDs. The type I exporters were formerly termed B-family ABC exporters, where the structure of Sav1866 represented the general fold of the ABC transporter exporter family (Dawson and Locher 2006, Locher 2016). This exporter type is either a heterodimeric transporter like Tm287/288 (Hohl, Briand et al. 2012) or a homodimeric transporter like Sav1866 (Dawson and Locher 2006). Interestingly, most bacterial ABC transporters are homodimers (except Tm287/288 from *Thermotoga maritima*), whereas most eukaryotic ABC transporters are heterodimers. In some of these exporters one NBD has the normal consensus sequence and the other NBD comprises of a degenerated site, where conserved amino acids are mutated. In 2016, a novel fold of ABC exporter was discovered as the crystal structure of the heterodimeric ABC transporter ABCG5/G8 was solved (Lee, Kinch et al. 2016). This exporter represents a new type II exporter and other ABC transporters of the A- and G-family comprise of the same fold (e.g. ABCA1 (Qian, Zhao et al. 2017) and ABCG2 (Taylor, Manolaridis et al. 2017)). The ABC exporter ABCA1 has additional features to the typical type II exporter fold, which includes a larger extracellular domain (ECD) and a C-terminal extension (CTE). In 2017. another type of exporter (type III) was identified. The lipopolysaccharide (LPS) transporter $LptB_2FG$ consists of two NBDs LptB and the TMDs LptF /G. A striking feature of this transporter is the periplasmic β-jellyroll domain of LptF/G, which is requisite for transporting LPS (Dong, Zhang et al. 2017). A complete other fold reassembles the ABC transporter MacB from *Acinetobacter baumannii*, where the TMDs are not intertwining and a large periplasmic domain (PLD) is located between the TM helix 1 and 2 (Okada, Yamashita et al. 2017). Thus, the structure of MacB is the type IV exporter of ABC transporter. The exporter is part of the tripartite multidrug efflux transporter comprising of the periplasmic adaptor protein MacA, the transporter MacB and outer membrane porin (OMP) TolC and extrudes for example antibiotics or enterotoxins from the cell. A recent comparison of the bacterial MacB /MacB-like ABC proteins to the eukaryotic A-/G-family of ABC transporters showed their possible origin and how these exporters evolved (Ford and Beis 2019).

Although, the structural classification of ABC transporters proves to be a good reference to group the importers and exporters in distinct group, the common feature of all are the NBDs. This especially holds true as the TMDs share less sequence similarity than the NBDs with their conserved motifs, which are the hallmark of ABC proteins.

1.3.2 The hallmark of ABC proteins

The NBDs are the catalytic domain, which provides the translocation process with energy for conformational changes in the TMDs. Within the NBDs structural features and conserved motifs can be determined, which makes them a subgroup of P-loop NTPases (Vetter and Wittinghofer 1999). The overall structure of NBDs, notably HisP as their hallmark structure, is L-shape and consists of two arms (Hung, Wang et al. 1998). The first arm comprises of β-sheets as well as α-helices and harbours the nucleotide-binding pocket (RecA-like domain) (Story and Steitz 1992). The second arm, also called α-helical domain has many α-helices as secondary structure elements and is unique to ABC proteins (Ames and Lecar 1992, Schmitt, Benabdelhak et al. 2003).

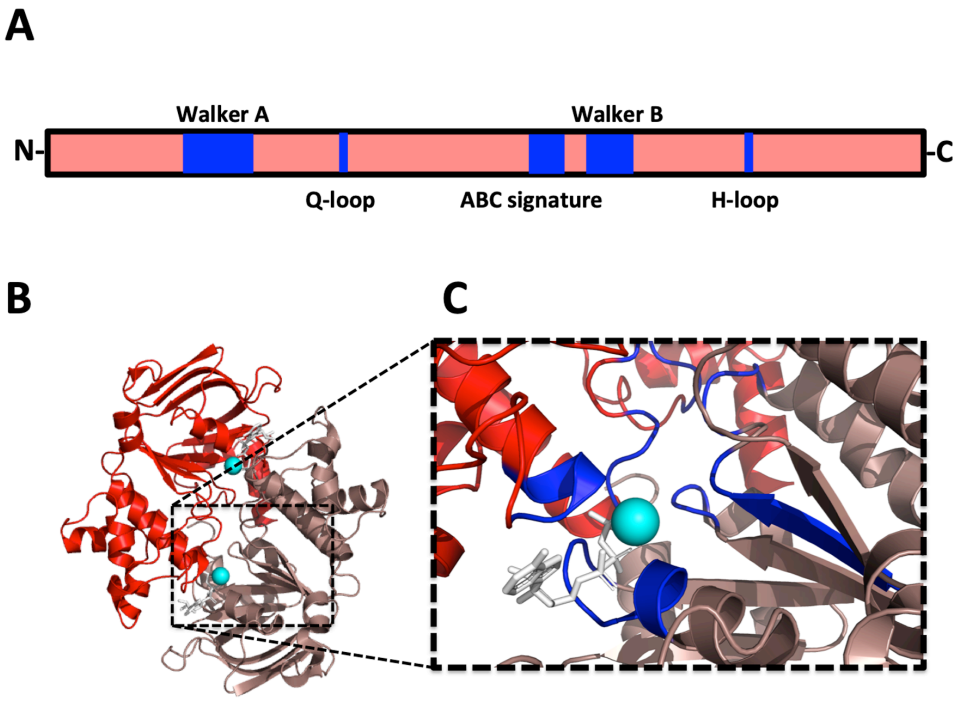


Figure 12: The ATP-binding domain of ABC transporters.
A) Scheme of a NBD with conserved motifs highlighted in blue. **B**) Crystal structure of HlyB NBD H662A mutant bound with two ATP molecules and magnesium ions (PDB ID: 1XEF). Colour code: NBD monomers (red and dark red) ATP molecule (white) and magnesium ion (cyan). Conserved NBD motifs are highlighted in blue. The program PyMOL 2.1.1 was used to display the crystal structure in a cartoon and the ATP molecule in stick representation.

The nucleotide-binding site of one NBD monomer comprises of the Walker A and the Walker B motif. The Walker A motif or P-loop harbours the conserved sequence GXXGXGKS/T (Walker, Saraste et al. 1982). There, a highly conserved lysine residue interacts with the β- and γ-phosphate of a ATP molecule and is important for the correct coordination. On the other hand is the Walker B motif (φφφφDE) important for the

coordination of the magnesium ion (Vetter and Wittinghofer 1999). The conserved aspartate residue is likely involved in coordinating the metal ion, while the glutamate residue is proposed to be the general base catalyst for polarisation of an attacking water molecule (Oldham and Chen 2011). C-terminal of this Walker B motif is a four aa sequence (SALD) termed D-loop. Upon ATP binding this motif is completed by the ABC signature motif (C-motif; LSGGQ) of the second NBD to form the nucleotide-binding site (Schmitt and Tampe 2002). The two NBDs are oriented head-to-tail and assemble a sandwich dimer, which provides the molecular basis for the cooperativity of the NBDs (Senior and Bhagat 1998, Oswald, Holland et al. 2006). The A-loop with its conserved aromatic residue (e.g. Tyr) also contributes to the nucleotide-binding site by π-π stacking with the adenine ring of the ATP molecule. During ATP hydrolysis another motif called the H-loop is necessary to assist in the positioning of the attacking water molecule, the Glu residue (Walker B) and the magnesium ion (Zaitseva, Jenewein et al. 2005). A further conserved motif in the NBD is the Q-loop, which completes the nucleotide-binding site via γ-phosphate interaction and is the switch between the NBDs and the TMDs (Gaudet and Wiley 2001, Dawson and Locher 2006). Additional conserved residues are the Pro-loop, which links the helical-domain with the RecA-like domain and the X-loop, which interacts with the coupling helix of the TMDs (Dawson and Locher 2006).

Generally, the NBDs of ABC transporters follow a similar mechanism of ATP binding and hydrolysis, but certain models has be proposed to explain the mechanism of ATP hydrolysis. First, two models are formerly suggested to explain the catalytic cycle without considering the translocation process. These suggested models are based on trapping experiments with beryllium-, aluminium-fluoride (BeF_x / AlF_x) or vanadate (VO_4^{3-}) and on observations of crystal structures from apo- and holo-proteins (Smith and Rayment 1996, Smith, Karpowich et al. 2002, Karpowich, Huang et al. 2003, van der Does and Tampe 2004). In the switch model (formerly: processive clamp mechanism) the two binding sites of the NBDs are loaded with ATP, which is sequential hydrolysed and followed by dimer dissociation as well as ADP/ATP exchange (Janas, Hofacker et al. 2003, Higgins and Linton 2004). The other is the constant contact model (formerly: alternating catalytic site mechanism), where only one ATP is hydrolysed and then ADP is exchanged to a new ATP, while ATP in the second binding site is hydrolysed consecutively (Senior, al-Shawi et al. 1995, Jones and George 2007, Jones and George 2009). Here, the NBDs do not dissociate and only

the open/closed conformation of the monomer alternates after ATP hydrolysis. In sum, these two models of the ATP cycle are supported by many biochemical data and structural observations without proving the other model to be wrong. Therefore, either the switch model or constant contact model might be correct, but the whole transport cycle of each ABC transporter has to be consider in the end, which might support one model.

Similarly, two models can explain the specific mechanism of ATP hydrolysis. There, either by the general base catalysis (GBC) (Moody, Millen et al. 2002) or by the substrate assisted catalysis (SAC) (Dall'Acqua and Carter 2000). In the GBC the conserved Glu residue of the Walker B motif is the general base, which attacks a water molecule generating a nucleophile to attack the γ-phosphate of ATP. This hypothesis was supported by mutational studies, where the Glu residue was exchanged to a Gln residue (E/Q mutant) in BmrA and other ABC transporters, which abolished ATPase activity (Orelle, Dalmas et al. 2003). In this model the rate limiting step is the generation of the nucleophile via the proton abstraction of the general base. Other findings are contrary and support the SAC as the mechanism for ATP hydrolysis. Some studies observed, that the mutation of the H-loop (conserved His residue) results in loss of ATP hydrolysis or even transport (Shyamala, Baichwal et al. 1991, Davidson and Sharma 1997). Additional, ATPase activity measurements in D_2O provided evidences for SAC, as the velocity of the reaction was not changed with increasing % of D_2O indicating that the nucleophilic attack is not the rate-limiting step (Zaitseva, Jenewein et al. 2005). Thus, binding and hydrolysis of ATP is stabilized by the H-loop and additional supported by the interaction of the H-loop with the D-loop. In the end, some biochemical data favour the GBC and other the SAC. But more importantly all motifs found in the NBDs either contribute to nucleotide binding, hydrolysis or are important for the communication with the TMDs to transfer conformation changes depended upon nucleotide binding/hydrolysis. These features make the NBDs indispensable for the transport mechanism.

1.3.3 Transport mechanism of ABC transporters

All ABC transporters are primary active transporter and thereby they translocate a substrate against a chemical gradient under the usage of ATP (Ames 1986, Hyde, Emsley et al. 1990). Furthermore, these transporters pump their substrate across the membrane unidirectional, at which the ATP hydrolysis in the NBDs provides the energy for conformational changes in

the TMDs in a tightly coupled process. How the coupling of ATP hydrolysis in the NBDs, the conformation changes in the TMD and subsequently translocation of the substrate is achieved, is still unknown for many ABC transporters. Hence, only biochemical and structural data would provide enough information to conclude about their mechanisms and additionally not all ABC transporters would have the same mechanism due to their different physiological functions. However, there are some accepted models, which might explain the transport cycle of ABC transporter. To simplify the possible mechanism of all ABC transporters (importers/exporters), the focus will be on ABC exporters.

The hallmark of substrate translocation is the alternating access model (Senior, al-Shawi et al. 1995), in which the transporter adopts a conformational change in the TMDs of inwards-facing (IF) to outward-facing (OF) conformation. Theses conformation changes start at a ground state, where the transporter adopts an IF conformation with a closed extracellular gate (Dawson and Locher 2006, Pinkett, Lee et al. 2007). The ATP-dependent dimerization of the NBDs leads an initiate conformation change, which is connected to the catalytic cycle of the NDBs. There, the catalytic cycle of the NBDs can either follow the switch or the constant contact model (see section above) (Higgins and Linton 2004, Sauna, Kim et al. 2007, Siarheyeva, Liu et al. 2010). The stoichiometry of ATP to substrate per transport cycle might support the switch model or the constant contact model, but this matter is still under debate. For an ABC importer (OpuA, the glycine/betaine transporter) a stoichiometry of two ATP per substrate was determined (Patzlaff, van der Heide et al. 2003). This however must not be true for all ABC transporters and beside the 2:1 also a 1:1 stoichiometry is possible (Shapiro and Ling 1997, Sauna and Ambudkar 2000, Zelcer, Huisman et al. 2003). The alternating access model requires a coupled communication between TMDs and NBDs during the catalytic cycle of ATP binding and hydrolysis (Dawson, Hollenstein et al. 2007). This coupling is achieved by the coupling helices (CH), which are located in the intracellular loops (ICL) of the TMDs, but the topology of the CH within the TMDs differs. The main reason is the different number of TM helices (TMH), which can account five, six or even ten helices. Thus, the location of the CH can be between TMH 4 and TMH 5 (e.g. Pgp) (Kim and Chen 2018) or between TMH 6 and TMH 7 (e.g. BtuCD) (Locher, Lee et al. 2002). Although, their sequence is not conserved the CH share a similar short α-helical structure to fulfil the communication between the TMDs and NBDs via the interface of the RecA-like domain/α-helical domain. With the movement of the CH after ATP

binding/hydrolysis the ABC transporter can undergo different conformational changes during one transport cycle. The binding of ATP converts the IF conformation of the exporter to the OF conformation. This allows the substrate to swap the substrate binding site at the TMDs from a high affinity (*cis*-side of the membrane) and a low affinity binding site (*trans*-side of the membrane) (van Veen, Margolles et al. 2000). The low affinity substrate binding site is exposed to the exterior and the release to *trans*-site of the membrane completes the translocation (Linton and Higgins 2007). The ATP hydrolysis and release of ADP and P_i leads to recovery of the ground state and reset of the transporter by the movement of the CH and TMDs, respectively (Lu, Westbrooks et al. 2005, Oldham, Khare et al. 2007). Afterwards, the binding of new ATP molecules allows a new transport cycle.

For ABC exporters like Sav1866 the transport cycle is mainly a simple alternation of IF to OF and OF to IF conformation (Dawson and Locher 2006, Seeger and van Veen 2009, Xu, Seelig et al. 2017). Although, variations of the OF and IF conformations as closed and open variants or even occluded conformations are described for ABC transporters (Oldham and Chen 2011, Choudhury, Tong et al. 2014, Srinivasan, Pierik et al. 2014, Lin, Huang et al. 2015). These conformations represent transition states of the transport cycle and can be observed under certain conditions such as a pre-hydrolysis state when ATP-γ-S/ AMP-PNP (Dawson and Locher 2007, Sauna, Kim et al. 2007) or post-hydrolysis state when Mg•ADP•V_i is bound (Reyes and Chang 2005).

Some novel findings even allude to other possible mechanism beside the alternating access model. For example for the bacterial peptide exporter McjD from *E. coli*, which transports a lasso peptide, an occluded-mechanism with transient opening was observed without adopting an OF conformation (Bountra, Hagelueken et al. 2017). In the case of the ABC transporter PglK an OF-only conformation was described during the translocation of lipid-linked polysaccharides at the lipid bilayer (Perez, Gerber et al. 2015). Another example is the ABC transporter MacB, which is part of a tripartite pump comprising of MacA, MacB and TolC. There, the substrate enters the translocation channel via the large periplasmic domain of MacB. Thus, the substrate is transferred from the periplasm to the fully assembled complex of MacAB-TolC without following the alternating access mechanism (Okada, Yamashita et al. 2017). Additionally, other transport mechanisms must be obviously present, as some ABC transporters from the B- or G-family have only one canonical ATPase site. There, the second ATPase site is degenerated and lacks for example the Walker B motif

or the ABC signature motif (Lubelski, van Merkerk et al. 2006, Procko, Ferrin-O'Connell et al. 2006, Gupta, Kueppers et al. 2014). This disruption leads to a certain asymmetry of ATP hydrolysis in some ABC transporter. Examples are the heterodimers Tm287/288 and ABCG5/G8 or in the homodimers CFTR (Hohl, Briand et al. 2012, Lee, Kinch et al. 2016, Zhang and Chen 2016).

Moreover, is the mechanism of transport depended on the nature of the substrate, which can have totally different properties (e.g. hydrophobic or hydrophilic) and may differ in size. Thus, ABC transporters might have a common motion and transmission strategy but they are specialized towards their substrate translocation mechanism.

1.3.4 Bacterial ABC transporters: peptide exporters

One group of specialized ABC transporter are bacterial ABC exporters, which translocate peptides (e.g. lanthipeptides, thiopeptides or unmodified bacteriocins) or proteinaceous substrates (e.g. glycopeptides) across a lipid bilayer independent of the Sec translocon pathway (Fath and Kolter 1993, Nes, Diep et al. 1996, Michiels, Dirix et al. 2001, Gebhard 2012). Generally, the substrates are often of ribosomal origin and are synthesized as precursor peptides with an N-terminal signal sequence (also termed leader peptide; LP), which is proteolytical removed upon the translocation process (Gilson, Mahanty et al. 1990, Havarstein, Diep et al. 1995). Their dedicated export systems can be found in Gram-negative as well as in Gram-positive bacteria, where the substrates exhibit various functions such as antimicrobial peptides or quorum-sensitive peptide pheromones.

The export systems in Gram-negative bacteria need additional accessory proteins (AP) and inevitable outer membrane factors (OMF) for the release of the substrate to the extracellular space (Gilson, Mahanty et al. 1990, Paulsen, Park et al. 1997, Michiels, Dirix et al. 2001). In Gram-positive bacteria is no need for OMF, as the substrates are secreted across one membrane. Hence, these export systems lack OMFs, but some ABC transporters need APs to secrete their substrates (Jack, Tagg et al. 1995, Franke, Tiemersma et al. 1999, Biemans-Oldehinkel, Doeven et al. 2006).

Many of these export systems comprise of ABC transporter, which have an additional N-terminal located domain (SunT-type). This domain is a C39 peptidase (C39P) belonging to the cysteine protease superfamily (Rawlings 2016). Thus, these exporters are termed AMS transporter, which secrete and maturate their substrates. The substrates of these ABC

transporters are either post-translational modified or unmodified peptides, which are under 12 kDa in size and are processed by the C39P domain by LP cleavage after the double Gly-motif (Havarstein, Diep et al. 1995). Examples of theses ABC transporters are SunT (sublancin 168), NukT (nukacin- ISK-1), CvaB (colicin V), ComAB (ComC) and PCAT1 (Cthe_0535) (Hwang, Zhong et al. 1997, Paik, Chakicherla et al. 1998, Aso, Sashihara et al. 2004, Lin, Huang et al. 2015, Wang, Patel et al. 2018).

Other export systems comprise of ABC transporter, which lack the peptidase domain and are classified as NisT-type exporter (Gebhard 2012). These exporters are mainly found in Gram-positive bacteria but some examples are found in Gram-negative bacteria, which not necessary belong to the NisT-type exporter (e.g. McjD from *E. coli*) (Choudhury, Tong et al. 2014). Similar to the other exporter group, they transport post-translational modified or unmodified peptides, whereas the LP is processed independently from the ABC transporter by specific leader peptidases. Noteworthy, some transporters like the lantibiotic gallidermin exporter GdmT need membrane associated APs (GdmH) to secrete the substrate (Hille, Kies et al. 2001). Moreover, a combined activity of export and immunity is also described or proposed for some peptide exporter like for the lasso peptide exporter McjD (Choudhury, Tong et al. 2014), a putative bottromycin transporter (Huo, Rachid et al. 2012) and a class IV lanthipeptide transporter VenTH (Goto, Li et al. 2010).

1.3.5 Lanthipeptide exporters

In all lanthipeptide BGC at least one ABC transporter is found, which is involved in the export of the lanthipeptide. The exporters secrete their LanA precursor peptides in a LP-dependent manner, where the N-terminal located LP is the signal sequence, which is indispensable for the secretion process (Kuipers, de Boef et al. 2004). In most cases the substrate size seem to be limited to 10 kDa, in which the peptides are only partial folded (secondary structure) and have post-translational modifications like lanthionine/labionin-rings or dehydrated aa. Nevertheless, there is an example described, where the size of the substrate exceeds 10 kDa and translocation was still observed. Here, the $SpaS_{LP}$ was attached to the alkaline phosphatase (PhoA) and the exporter SpaT secreted the LP-fusion protein to the extracellular space (Izaguirre and Hansen 1997). In general, the exporter systems of lanthipeptides can be classified into three types of ABC transporter, but in every class of lanthipeptides exceptions of this rule can be found (Figure 13).

The first type is the LanT-type ABC transporter, which is also referred as NisT-type ABC transporter (Gebhard 2012). They comprise of 540-630 aa and differ in size between 61-71 kDa. These exporters have a two-domain organisation consisting of a TMD and NBD and are functional homodimers. The TMDs consists of five to six membrane-spanning helices with the NBD at the C-terminus. In the 90s it was shown by gene disruption experiments, that these LanT-type transporters are important for substrate secretion. The disruption of a *spaT* gene lead to decreased cell growth of the subtilin producer and the subtilin precursor was accumulated inside the cell (Klein, Kaletta et al. 1992). Other *lanT* disruptions or deletions showed similar results (Quiao and Saris 1996). But in the case of PepT (Meyer, Bierbaum et al. 1995), EpiT (Schnell, Engelke et al. 1992) and MutT (mutacin 1140 transporter) (Escano, Stauffer et al. 2015) other transporters can compensate the function of the LanT exporter or they are dispensable. The interaction of the exporter and the lanthipeptide via the LP is mainly unknown, but an importance of the FN/DLD-box during substrate recognition has been proposed (Plat, Kluskens et al. 2011). Additional chimeric LPs of nisin and subtilin were secreted by the LanT-type transporter SpaT and NisT (Kuipers, Rollema et al. 1993, Chakicherla and Hansen 1995). Especially, the LP of class I lanthipepitdes shared some sequence similarity, from which a related recognition motif for the exporter can be postulated.

Besides the standard LanT-type transporter, other export systems can be found in the BGC of class I lanthipeptides, which will be mentioned in the following. For example In the gallidermin producer *Staphylococcus gallinar*um Tü3928 the exporter GdmT need the AP GdmH, which is similar to the epidermin producer (EpiT/EpiH) (Peschel, Schnell et al. 1997). There, only higher production of the lanthipeptide is achieved, when the AP is co-expressed with the LanT-type transporter.

A second type of lanthipeptide exporter, which is termed LanTU-type transporter, can be found in the BGC of Microbiospora sp. and Planomonospora sp., which consists of separate ATPase (LanU) and permease (LanT) proteins. The LanT proteins have a size of ~34 kDa and LanU of 28-30 kDa, which would be in sum the size of a LanT-type transporter (61-71 kDa). Thus, the class I lanthipeptides NAI-107 produced by *Microbiospora corallina* is secreted by a presumably heterodimeric exporter MibT/U (Castiglione, Lazzarini et al. 2008). Similarly, planosporicin is secreted by the heterodimer PspT/U (Sherwood, Hesketh et al. 2013). There, each exporter comprises of a two LanT and two LanU proteins to assemble a

functional transporter for substrate secretion. To date no other exporter for class I lanthipeptides are described to be LanTU-type exporter.

Interestingly, the ABC transporter of the antifungal, class I lanthipeptides pinensin A/B is an exporter with peptidase function and is thereby not a LanT-type transporter (Mohr, Volz et al. 2015). This exporter belongs to the AMS transporter and has a size of 83 kDa. Furthermore, the secreted lanthipeptide is produced by the Gram-negative bacterium *Chitinophaga pinensis* and the substrate needs to be translocated across two membranes. Thus, the transport through PinT would result in a periplasmic intermediate, which needs to be further translocated by an export system (e.g. TolC-dependent efflux pumps).

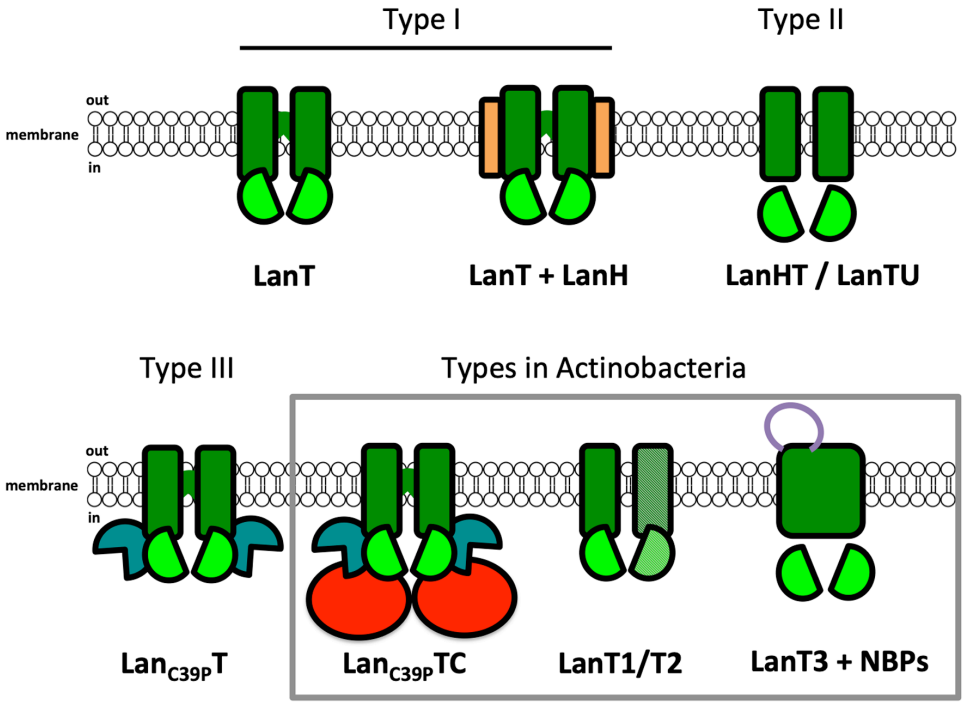


Figure 13: Types of lanthipeptide exporters.
In the BGC of lanthipeptides different types of ABC transporters can be found, which are involved in precursor peptide secretion. There are three main types (I-III) and additional variants originated in the phylum of Actinobatceria. Colour code: TMDs are displayed in dark green, NBD/NBPs in light green, peptidases in grey, accessory proteins in orange, LanC-like domain in red and FtsX-domain in violet.

In the BGC of class II lanthipeptides is a third type of exporter located, which have an additional N-terminal domain comprising of 100-200 aa. These exporters were termed LanTP or LanT$_P$ and belong to the group of AMS transporter (Sahl and Bierbaum 1998, Lin, Huang et al. 2015, Repka, Chekan et al. 2017). In order to refer to their N-terminal C39 peptidase (C39P) domain another nomenclature for these ABC transporters is be proposed and will be hereupon used and termed Lan$_{C39P}$T. The Lan$_{C39P}$T exporters are bifunctional ABC transporter, where LP proteolysis is tightly coupled to the ATPase activity in a cooperative

manner (Nishie, Shioya et al. 2009). The lanthipeptide carry a double-glycine motif (GG/GA/GS) at the C-terminus of the LP, which is the cleavage site for the C39P domain. The LP is cleaved prior to the translocation process as shown for NukT (Nishie, Shioya et al. 2009, Zheng, Nagao et al. 2017). Noteworthy, the LP of class II lanthipeptides exhibits a secondary, α-helical structure, which is in contrast to class I lanthipeptides. This α-helical structure is an important recognition motif for the C39P domain of the $Lan_{C39P}T$ (Furgerson Ihnken, Chatterjee et al. 2008, Nagao, Morinaga et al. 2009). Moreover, the exporter seems to be stimulated by mLanA peptide and recognizes the unmodified LanA less efficient (Zheng, Nagao et al. 2017). The deletion of the $Lan_{C39P}T$-type transporter (similar to LanT-type) led to lanthipeptide accumulation or no lanthipeptide production and provided evidence for their importance during lanthipeptide secretion (Aso, Nagao et al. 2004, Uguen, Hindre et al. 2005). The *in vitro* activity of isolated N-terminal C39P domain from LctT, NukT, and BovT was described (Furgerson Ihnken, Chatterjee et al. 2008, Lin, Teng et al. 2011, Nishie, Sasaki et al. 2011). Additional, the *in vitro* activity of a full-length $Lan_{C39P}T$-type transporter was described for NukT, which is further the only *in vitro* characterized lanthipeptide exporter until now (Zheng, Nagao et al. 2017).

Although, $Lan_{C39P}T$-type transporter exhibit a peptidase function, some BGC of class II lanthipeptides harbour a LanP enzyme for further LP trimming. One example is found in the operon of the two-component peptides α-lichenicidin and β-lichenicidin produced by *Bacillus licheniformis*. In this BGC are LicT and also LicP located, which process a hexapeptide from β-lichenicidin (Caetano, Krawczyk et al. 2011). Further examples of a $Lan_{C39P}T$ exporter and a LanP peptidase are found in the BGC of haloduracin and cytolysin (Booth, Bogie et al. 1996, McClerren, Cooper et al. 2006).

In contrast to the $Lan_{C39P}T$-type transporter, which are normally found in the BGC of class II lanthipeptides, a few exceptions are found in some producer strains of class II lanthipeptides. The exporter system of cinnamycin (and presumably of duramycin) comprises of a LanT and LanH protein (Kaletta, Entian et al. 1991). CinT is the NBP and CinH the TMP, which is similar to the exporter organisation of the class I lanthipeptide NAI-107 and planosporicin (LanTU-type transporter). Additional in the BGC of the lanthipeptide mathermycin produced from *Marinactinospora thermotolerans* also a LanT/H (MaT/H) export system is found (Chen, Chen et al. 2017). Another exception is the exporter (LasT) of

lactocin S, which is a LanT-type transporter and has no C39P domain (Mortvedt, Nissen-Meyer et al. 1991).

Since the 90s, it is postulated, that the secretion of class I lanthipeptides is coupled with the modification enzymes LanB and LanC in a multimeric modification/secretion complex (suggested for SpaTBC and NisBTC) (Siegers, Heinzmann et al. 1996, Kiesau, Eikmanns et al. 1997). This hypothesis was later supported by the observation, that mNisA was only efficiently secreted in presents of NisBC and thereby a channelling mechanism for substrate translocation was proposed (van den Berg van Saparoea, Bakkes et al. 2008). A similar multimeric enzyme complex consisting of NukM and NukT is also postulated for the class II lanthipeptide nukacin ISK-1 (Nagao, Aso et al. 2005). Interestingly, in the Gram-positive bacteria of the genus Streptomyces a special BGC can be found, where a Lan_{C39P}T-type ABC transporter is fused to a LanC-like protein (Lan_{C39P}TC-type). In *Streptomyces bingchenggensis* BCW-1 the lanthionine-ring formation, the proteolytic activity and the export seems to be performed by one ~182 kDa protein (Wang, Yan et al. 2010, Singh and Sareen 2014). Presumably, this tri-functional protein is a homodimer, which assemble a large multimeric enzyme complex with the putative LanM-like enzyme. Similarly, in the strain *Streptomyces sparsogenes* DSM 40356 a tri-functional protein with the same domain organisation is located in the BGC. Thus, this fusion protein of a LanC-like protein and exporter could be a hind for the general strategy of a membrane-associated multimeric enzyme complex in lanthipeptide producer strains.

A few studies dealt with the exporters of class III and IV lanthipeptides so far and none of these exporters are functionally characterized. Nevertheless, by investigating BGC databases from genome mining tools like BAGEL4 or anti-SMASH in combination with BLAST search (www.blast.ncbi.nlm.nih.gov/Blast.cgi) or the SMART database (www.smart.embl-heidelberg.de) additional information's about these exporters can be gathered (Blin, Medema et al. 2017, van Heel, de Jong et al. 2018).

In the BGC of class III lanthipeptides generally two copies of the LanT-type ABC transporter are found, which are termed LanT1 and LanT2 (sometimes also termed LabT1 and LabT2) (Meindl, Schmiederer et al. 2010, Krawczyk, Voller et al. 2012, Voller, Krawczyk et al. 2012). These ABC transporters are either heterodimeric (T1/2) or homodimeric (2xT1 and 2xT2) proteins with a length of 560-640 aa and a size of 58-65 kDa. Presumably, they are only involved in the export of the lanthipeptide. The BGC of these lanthipeptides harbours

normally no ABC transporter with a putative immunity function as the lanthipeptides exhibit no antimicrobial activity. Additionally, no peptidase domain is associated with these exporters like in the class I lanthipeptides exporter. Examples are the exporters RamA/B of SapB and LabT1/2 of labyrinthopeptins (Kodani, Hudson et al. 2004, Meindl, Schmiederer et al. 2010).

Interestingly, in the BGC of the low antimicrobial active lanthipeptide NAI-112 produced by *Actinoplanes sp.* another transporter organisation can be found. There, a NBP (AplT1) a TMP (AplT2) and an FtsX-like permease (AplT3) are located on the BGC, which are involved in export and immunity (Chen, Xu et al. 2019). The NBP shows similarity to FtsE ATPases and the TMP to daunorubicin resistance ABC transporter membrane proteins. The FtsX-like permease lacking an ATPase domain has ten TM-helices, with an FtsX domain located at aa position 287-383 and shows a similar domain organisation to a putative macrolide exporter from *Corynebacterium glyciniphilum*. Unfortunately, the dedicated function of export and immunity of the transporter system can only be assumed. Presumably, two NBP and two TMP function as heterodimeric transporter involved in immunity, whereas the permease and two NBPs assemble a functional heterodimeric transporter, which exports the lanthipeptide to the exterior.

In class IV lanthipeptides little is known about the transporter, but in the BGC two proteins involved in export and immunity can be found (Goto, Li et al. 2010, Zhang, Doroghazi et al. 2015, Hegemann and van der Donk 2018). One protein, termed LanT is the ATPase and consists of 330-380 aa with an average size of 35-40 kDa. The other protein, termed LanH is the TMP and consists of 250-300 aa with an average size of 27-32 kDa. There, the LanT protein shows similarity to the daunorubicin resistance ATPase subunit and the LanH protein with the daunorubicin resistance ABC transporter membrane protein. Probably, the ABC transporter is a functional heterodimeric transporter involved in exporter and immunity of the lanthipeptide (Goto, Li et al. 2010, Iftime, Jasyk et al. 2015). Examples are the streptocollin exporter StcT/H from *Streptomyces collinus* Tü365 and the venezuelin exporter VenT/H from *Streptomyces venezuelae* ATCC 10712 (Goto, Li et al. 2010, Iftime, Jasyk et al. 2015).

1.3.6 Lantibiotic exporters of immunity and resistance

In the BGC of lanthipeptide producer a specialized immunity system is located, which mediates protection against the secreted lantibiotics. There, the immunity system consists of a membrane-associated lipoproteins (LanI) and/or of the ABC transporter LanFEG (Alkhatib, Abts et al. 2012). In many BGC of class I and II lanthipeptides these LanFEG-type transporters can be found as a second export system besides the LanT/Lan$_{C39P}$T-type transporter (Klein and Entian 1994, Siegers and Entian 1995, Peschel and Gotz 1996, Rince, Dufour et al. 1997, Hille, Kies et al. 2001, Guder, Schmitter et al. 2002, Aso, Nagao et al. 2004). Throughout the lanthipeptide BGC the LanFEG-type transporters are homologous to each other and share the same domain organization (Figure 14).

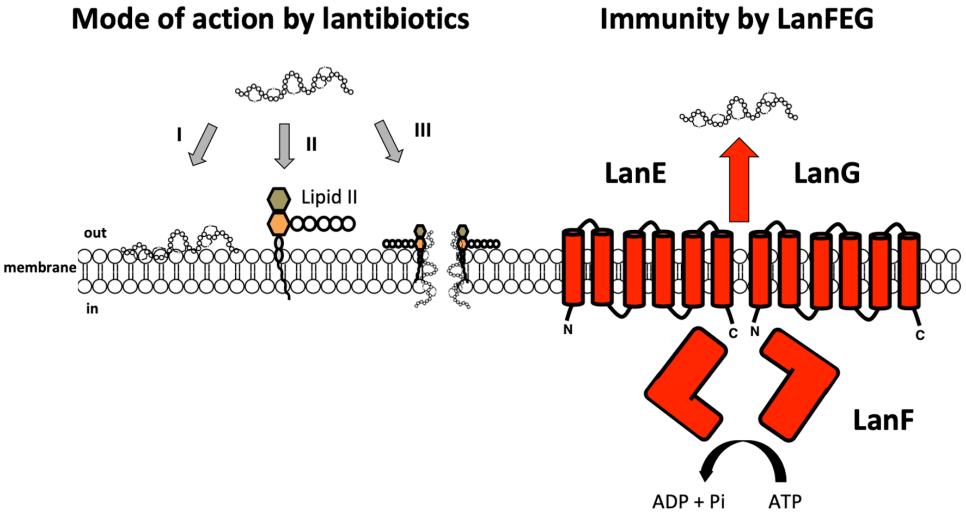


Figure 14: Lantibiotic immunity by LanFEG.
The ABC transporter LanFEG (red) is conferring immunity against lantibiotics by extruding the peptides from the membrane. Thus, the antimicrobial activities of lantibiotics such as membrane perturbation (I), lipid II binding (II) or pore formation (III) are hampered.

In contrast to the LanT-type ABC transporters, the domains of the LanFEG-type ABC transporters are synthesized on separate polypeptide chains with a fully assembled transporter in a proposed stoichiometry of 2:1:1 (LanF$_2$EG) (Siegers and Entian 1995). There, the LanE and LanG are the integral membrane proteins. They have a similar size of 25-30 kDa, a high hydrophobicity and they all have six putative TM helices (Stein, Heinzmann et al. 2005). The TMPs are functional heterodimers as shown for NisEG and SpaEG and are important for substrate binding and translocation (Stein, Heinzmann et al. 2003, Stein, Heinzmann et al. 2005, AlKhatib, Lagedroste et al. 2014). The LanF proteins are cytoplasmic, dimeric proteins with a size of 25-28 kDa. They are the NBP and share the common conserved ATP-binding cassette motifs of the ABC protein superfamily. The NBPs are the

energy-coupling proteins and provide the energy for substrate translocation by hydrolysing ATP. Within the conserved ATPase motifs an E-loop instead of the Q-loop can be found, that is highly conserved in LanFEG-type ABC transporters. It is involved in communication of the NBPs with the TMPs and therefore important for lantibiotic translocation (Okuda, Yanagihara et al. 2010).

The LanFEG-type ABC transporters belong to the ABC-2 subfamily of multidrug resistance (MDR) proteins, which are involved in the efflux of macrolides, antibiotics, toxins or other hydrophilic and hydrophobic compounds (Saier, Paulsen et al. 1998, Lubelski, Konings et al. 2007). The LanFEG-type transporters are specific for their dedicated lantibiotic and confer immunity against them without cross-immunity (Otto, Peschel et al. 1998). Although, the exact mechanism is unknown LanFEG-type transporters might function like other MDR proteins as a hydrophobic vacuum cleaner (Bolhuis, van Veen et al. 1997). Generally, the exporter extrudes the hydrophobic substrate from the inner/outer leaflet of the membrane to the *trans*-side of the membrane and extracellular space, respectively. This efflux mechanism was proposed and shown for the lantibiotics nisin, subtilin epidermin and nukacin ISK-1 (Otto, Peschel et al. 1998, Stein, Heinzmann et al. 2003, Stein, Heinzmann et al. 2005, Okuda, Aso et al. 2008). The fast extrusion from the membrane seems to hinder the pore formation of the lantibiotics as shown for nisin (AlKhatib, Lagedroste et al. 2014). Important to note is the suggested cooperative function of the LanFEG-type transporter with associate membrane-bond immunity proteins (LanI or LanH) to confer full immunity against the lantibiotic (Ra, Beerthuyzen et al. 1999, Takala, Koponen et al. 2004, Takala and Saris 2006, Okuda, Aso et al. 2008, Draper, Grainger et al. 2009, Geiger, Korn et al. 2019). However, an independent action of the LanFEG-type transporters were also observed for NisFEG and SpaFEG (Stein, Heinzmann et al. 2003, Stein, Heinzmann et al. 2005) but the literature seems to be in favour for the cooperative function.

Although, resistance against lantibiotics is rare observed, still some resistance mechanism are described, which negate the antimicrobial activity of lantibiotics. This resistance arise for example from modifications in the cell wall or membrane and the formation of biofilms or endospores (Draper, Cotter et al. 2015). In all cases, the lantibiotic sensing is connected to a specific detection system (e.g. two-component systems; TCS), which alters the gene regulation and thereby the expression of enzymes involved in peptidoglycan modification, lipid biosynthesis or resistance proteins (e.g. efflux pumps).

Some resistance mechanisms comprise of a resistance system consisting of a TCS associated with an ABC transporter. These resistance systems can especially be found in Firmicutes and are involved in detoxification of the bacteria. There, the ABC transporter either belongs to the group of CprABC-type transporter or to BceAB-type transporter (Figure 15) (Gebhard 2012, Revilla-Guarinos, Gebhard et al. 2014, Clemens, Zaschke-Kriesche et al. 2017).

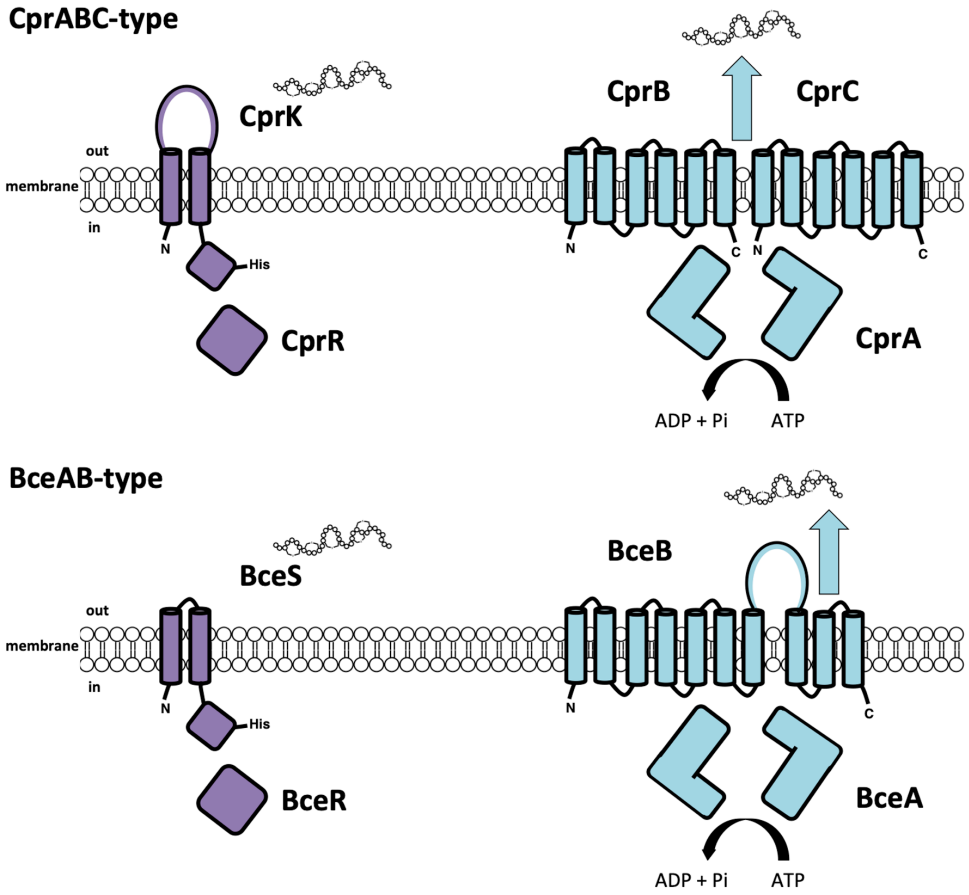


Figure 15: Lantibiotic resistance transporter in bacteria.
In general, there are two types of lantibiotic resistance transporter termed CprABC-type and BceAB-type transporter. They function with the corresponding TCS (CprK/R and BceS/R) to confer resistance against lantibiotics or other AMPs. Colour code: The TCS is displayed in violet and the ABC transporter in light blue. Figure is adapted and modified from (Dintner, Heermann et al. 2014, Clemens, Zaschke-Kriesche et al. 2017).

The first group are CprABC-type transporters (Cpr: cationic AMP resistance), which are similar to LanFEG-type transporters (McBride and Sonenshein 2011, Suarez, Edwards et al. 2013). All proteins are located on one operon and expressed separately under the control of the corresponding TCS, which is responsible for lantibiotic sensing. The functional transporter consists of two NBPs and of the heterodimeric TMPs to exhibit resistance against lantibiotics. For example the ABC transporter $CprA_2BC$ of the Gram-positive bacterium *Clostridioides difficile* confers resistance against nisin and gallidermin (McBride and Sonenshein 2011). In contrast to LanFEG-type transporter of lantibiotic producer, these ABC

transporters have a border substrate specificity and are not specific for one lantibiotic. Another CprABC-type transporter is LctFEG from *Streptococcus mutans*, which confers resistance against nukacin ISK-1 and lacticin 481 under control of the TCS LcrRS (Kawada-Matsuo, Oogai et al. 2013). Interestingly, on a different gene locus another TCS NsrRS regulates the expression of an ABC transporter $NsrFE_1E_2G$, which confers resistance against nisin but not against other lantibiotics like nukacin ISK-1. There, NsrF is the ATPase, whereas NsrE1/E2 (split TMP: E1 has two and E2 four TM helices) and NsrG are the permease. In the Gram-positive bacterium *Streptococcus pyogenes* SF370 a TCS SrtRK and an ABC transporter SrtFEG confers also only resistance against nisin (Kawada-Matsuo, Tatsuno et al. 2016). Whether, this observation is a specific resistance to one lantibiotic or a broader substrate specificity to more lantibiotics needs to be further investigated.

The second group are BceAB-type transporters, which are part of a broad stress response system (Gebhard and Mascher 2011). These exporters have a less strict substrates specificity and they response to lantibioics, bacteriocins or other AMPs, glycopeptides, and even antibiotics (e.g. bacitracin or β-lactam antibiotics) (Gebhard 2012). The domain organization of these ABC transporters is completely different in comparison to the LanT-type or LanFEG-type transporter and they are homologous to a MacB protein, which is part of a tripartite efflux pump (TEP) (Greene, Kaplan et al. 2018). The BceA protein is the NBP and provides the energy for substrate translocation. The BceB protein (TMP) comprises of ten TMHs and has an additional large extracellular subdomain (ECD) between TMH 7 and TMH 8. Interestingly, the topology of the C-terminal four TMHs and the EDC is similar to MacB although BceAB-type transporters do not assemble to a TEP. The EDC domain is important for sensing the putative substrate together with the dedicated histidine kinase (HK) of the TCS. It is proposed, that these transporters have coevolved with their TCS, as the HK lacks the sensor domain of other putative HKs (Rietkotter, Hoyer et al. 2008, Dintner, Staron et al. 2011). The presumed functions of BceAB-type transporters are either the translocation of the substrate (bacitracin or AMP) and/or the flipping of a receptor (e.g. undecaprenyl pyrophosphate or lipid II in complex with the substrate), which is sensed by the associated TSC (Bernard, Guiseppi et al. 2007, Dintner, Heermann et al. 2014, Kingston, Zhao et al. 2014). Subsequently, the TSC is triggering the expression of the ABC transporter to higher levels, which leads to increased resistance against the substrate. The suggested stoichiometry of a BceAB-type transporter is 2: 1, as shown for the ABC transporter BceAB

from *Bacillus subtilis*, where the HK BceS interacts with BceB (Dintner, Heermann et al. 2014). This transporter is mainly important for bacitracin export and functions together with the bacitracin sensing TCS BceRS *(Ohki, Giyanto et al. 2003, Rietkotter, Hoyer et al. 2008).* Additional, this transporter confers resistance against plectasin, actagardine and mersacidin (Staron, Finkeisen et al. 2011). Similarly, the BceAB paralog PsdAB from *B. subtilis* confers resistance against the lantibiotics mersacidin, actagardine, gallidermin, nisin and against the peptide antibiotic bacitracin *(Staron, Finkeisen et al. 2011).* Further exemplary BceAB-type transporter are MbrAB from *Streptococcus mutants (Tsuda, Yamashita et al. 2002)*, VraDE in *Staphylococcus aureus (Hiron, Falord et al. 2011)*, YsaBC from *L. lactis* IL1403Nis^{r} *(Kramer, van Hijum et al. 2006)* and AnrAB from *Listeria monocytogenes*, which confers resistance against nisin, bactitracin and some β-lactam antibiotics (e.g. oxacillin and penicillin G) (Collins, Curtis et al. 2010).

A special variation of the standard operon of BceAB-type transporter can be mainly found in pathogenic strains such as *S. aureus*, *Streptococcus agalactiae*, *Enterococcus faecium* and *Streptococcus sanguinis* (Khosa, AlKhatib et al. 2013). These operons consist a TCS, a BceAB-type transporter and additional of a lipoprotein. Interestingly, strains containing such an operon do not produce AMPs like lantibiotics, but they are resistant against them. For example in the human pathogen *S. agalactiae* COH1 an operon for nisin resistance proteins can be found. It comprises of the lipoprotein *Sa*NSR, which is a serine protease belonging to the S41 protease family, a BceAB-type transporter *Sa*NsrFP and the TCS *Sa*NsrRK (Khosa, AlKhatib et al. 2013, Khosa, Frieg et al. 2016, Khosa, Hoeppner et al. 2016, Khosa, Lagedroste et al. 2016).

1.4 Lanthipeptide peptidases

1.4.1 General aspects of peptidases

Peptidases are enzymes that catalyse the hydrolysis of the peptide bond between two amino acid residues in proteinaceous substrates. Endopeptidases cleave their substrate at specific cleavage sites within a polypeptide sequence that mostly results in the processing of the precursor. Other peptidases, which are termed exopeptidase, process their substrates by cleaving single aa residues from the N- or C-termini of a polypeptide chain (trimming activity). In general, peptidases are enzymes with a high diversity in function, structure and mechanism, which are classified in 273 different families and 64 clans (June 2019; MEROPS

database). In the MEROPS database, peptidases are dissect into seven major groups based on their catalytic type of their proteolytic activity. There, the groups with the most members are the serine peptidases (1835), the metallopeptidases (1154), the cysteine peptidases (1004) and carboxyl (aspartic/glutamic) peptidases (331) (Rawlings 2016, Rawlings, Barrett et al. 2018).

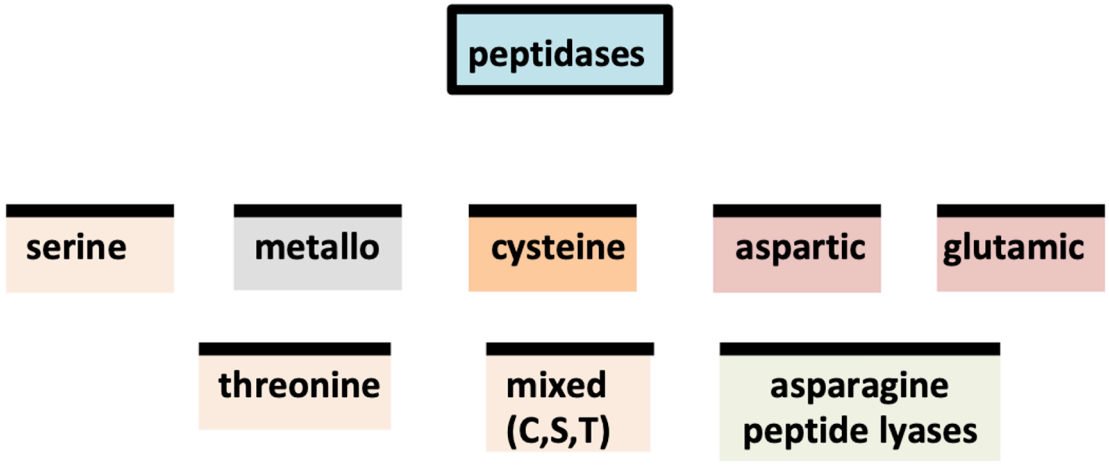


Figure 16: Classification of peptidases.

A special nomenclature was defined to name the residues of the peptidase and the substrate, which are interacting with each other. There, the substrate residues facing the N-terminus are the non-prime site and termed Pn…P4, P3, P2, P1. The prime site residues are termed P1', P2'...Pn'. On the other hand the residues of the substrate follow the same nomenclature but the P is replaced by S (Schechter and Berger 1967). Each peptidase is more or less specific for a certain cleavage site within the substrate, where peptidases with one binding pocket (mostly S1 pocket) show an apparent higher specificity to one cleavage site (Rawlings 2016). But there is no clear rule, if all peptidases of all groups are taken into account. A known unspecific peptidase with endo- and exopeptidase activity is proteinase K from the Ascomycota *Engyodontium album* and it is used for degrading proteins. Examples of less specific and promiscuous peptidases are trypsin or chymotrypsin, which cleave after Arg/Lys and hydrophobic aa at P1, respectively. Other peptidase like aminopeptidases do not have a binding pocket for the P4-P2 residues, because the cleavage site is directly at position P1 and they trim the polypeptide chain (Rawlings 2016). Other peptidases are highly specific for a recognition/cleavage site, like for example the enterokinase ($(Asp)_4$-Lys|X; cleavage is indicated by |) or the TEV protease (Glu-Asn-Leu-Tyr-Phe-Gln|Ser).

Many peptidases are produced as pro-enzymes (zymogen) and they became active after auto-cleavage activity to process their own sequence (Neurath and Walsh 1976). The auto-cleavage (limited proteolysis) of the pro-sequence induces the final correct folding of the enzyme after translocation. There are two general mechanisms for their proteolytic activity, which is mainly depended on the catalytic type. The carboxyl (aspartic/glutamic) peptidases and metallopeptidases generate a nucleophile by activating a water molecule, which performs a nucleophilic attack on the peptide bond at the cleavage site. The other peptidases (e.g. serine, threonine and cysteine) generate a nucleophile by their catalytic centre, which performs the attack on peptide bond. In the case of serine peptidase a catalytic triad of a serine, a histidine and an aspartate residue (Ser-His-Asp) is the active centre (Kraut 1977, Polgar 2005). There, the Ser residue is the nucleophile and attacks the substrate to form an acyl-enzyme intermediate with tetrahedral coordination. Then, a nucleophilic attack of a water molecule leads to deacylation of the enzyme and release of the cleaved substrate and the serine peptidase. Here, either the acylation or the deacylation reaction can be the rate-limiting step of this reaction and therefore influence the k_{cat} or K_m values of the peptidase towards the substrate (Perona and Craik 1995). For serine peptidases even other catalytic triads consisting of Ser-His-His or dyads of Ser-Lys are possible to obtain proteolytic active peptidases (Ekici, Paetzel et al. 2008).

1.4.2 Lanthipeptide processing peptidases

The removal of the LP is important for lanthipeptides to yield the mature, active form of the peptide. This task is performed by dedicated leader peptidases, which are located within the BGC of most lanthipeptides (class I, II and III). Here, three types of peptidases can be found, which are either classified as serine protease, as metalloprotease or as cysteine protease (Figure 17). The latter are the bifunctional AMS transporter ($Lan_{C39P}T$), which secrete and process the lanthipeptides with the double Gly-motif in the LP (see section above 3.5). The other lanthipeptide peptidases LanP belong either to the clan of subtilisin-like serine proteases (clade S8) or to the clan MA metalloproteases (clade M1). The serine proteases have an endopeptidase activity and cleave the LP at specific cleavage sites (van der Meer, Polman et al. 1993, Siezen and Leunissen 1997), whereas metalloproteases are bifunctional peptidases with endo- and aminopeptidase activity (Chen, Xu et al. 2019).

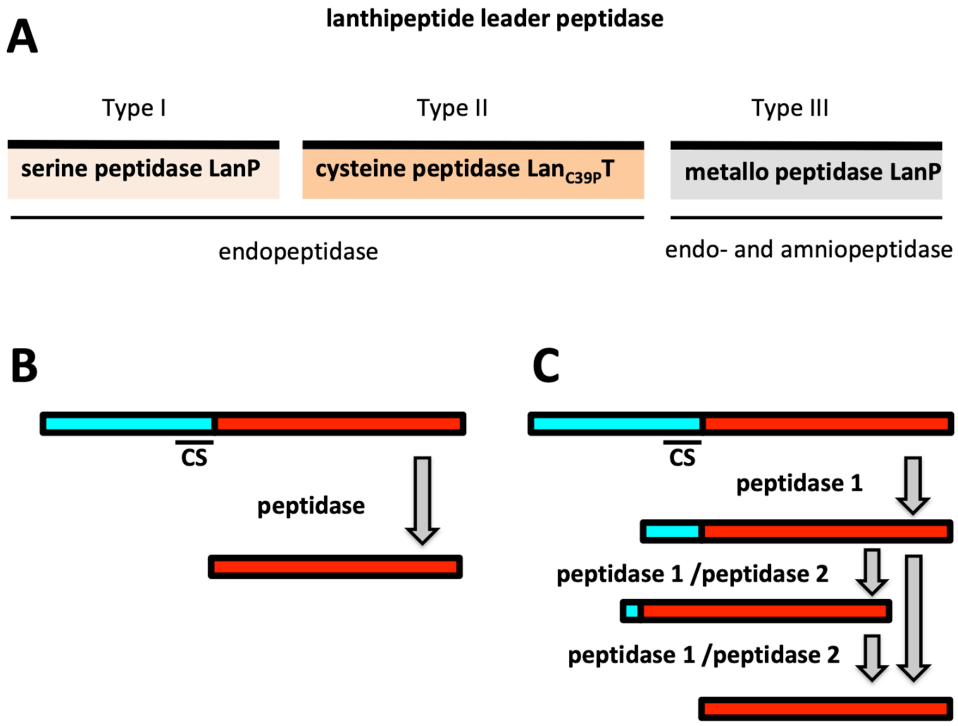


Figure 17: Different types of lanthipeptide leader peptidases.
A) Presentation of three types of lanthipeptide leader peptidase based on their catalytic type. The serine and cysteine peptidases have only endopeptidase activity, whereas the metallopeptidases display endo- and aminopeptidase activity. **B**) The leader peptide is cleaved by one step or **C**) at least two steps. In the latter case the leader peptide is often trimmed by one peptidase and finial matured by another peptidase. Colour code: The core peptide is highlighted in red and the leader peptide in cyan.

In many BGC of class I and in some of class II lanthipeptides, the LanP serine proteases are the dedicated leader peptidase. The LanP enzymes are either located in the cytoplasm like ElxP (Ortega, Velasquez et al. 2014), PepP (Meyer, Bierbaum et al. 1995) and LasP (Skaugen, Abildgaard et al. 1997) or extracellularly like EpiP (Geissler, Götz et al. 1996), NisP (van der Meer, Polman et al. 1993) and LicP (Caetano, Krawczyk et al. 2011). The extracellular located LanP enzymes have an N-terminal *sec*-dependent signal peptide (SP) and undergo an autocatalytic processing after translocation via the SEC translocon. There, the SP is removed by the signal peptidase I and the autocatalytic cleavage of the pre-sequence (prodomain) lead to protease folding. This autocatalytic cleavage activity of LanP enzymes was first shown for CylA, but it was observed before for other extracellular located LanP proteins, which also lack the N-terminal prodomain (Tang, Bobeica et al. 2018). Like many extracellular located proteins of Gram-positive bacteria, LanP proteins have a C-terminal located LPXTGX-motif for peptidoglycan (PG) anchoring of the enzyme (Schneewind and Missiakas 2014). This anchoring is mediated by a sortase and prohibits the release of the protease. Interestingly, in the case of NisP another autocatalytic cleavage site in front of the

LPXTGX-motif was found and a NisP variant, which escapes the anchoring was observed (Xu, Li et al. 2014). Additional, a C-terminal extension like in NisP and the LPXTGX-motif are absent in EpiP, thus also a anchor-free peptidase is possible (Geissler, Götz et al. 1996). Hence, a detailed investigation about LanP anchoring to PG and their exact extracellular localisation are necessary.

The overall structure of LanP is similar to other subtilisin-like serine proteases (e.g. thermitase or subtilisin BPN'), where LanP crystal structures are available of EpiP (PDB ID: 3QFH), NisP (PDB ID: 4MZD) and LicP (PDB ID: 4ZOQ) (Kuhn, Prachi et al. 2014, Xu, Li et al. 2014, Tang, Dong et al. 2015). The LanP structure is heart-like shape with a cleft near the catalytic centre, where aa are located that are supposed to be important for substrate recognition and binding (Xu, Li et al. 2014). In the case of EpiP and LicP the N-terminal non-covalently bound prodomain was also crystalized, while the prodomain of NisP is absent in the finial crystal structure (Kuhn, Prachi et al. 2014, Xu, Li et al. 2014, Tang, Dong et al. 2015). The catalytic important residues of LanP enzymes are located in the C-terminal catalytic domain. There, the canonical catalytic triade of serine proteases consisting of Ser-His-Asp and additional an oxyanion-hole represented by an Asn residue are found (Siezen, de Vos et al. 1991, Siezen, Rollema et al. 1995). Interestingly, the calcium binding sites, which are found in subtilisin-like serine proteases are missing in LanP proteins but a calcium ion dependency on activity, stability or folding was not shown (Xu, Li et al. 2014, Tang, Dong et al. 2015).

In general, LanP enzymes have a high substrate specific, where their activity is firstly dependent on the cleavage site and secondly on the modification state of the CP. Here, only mLanA was shown to be the optimal substrate for LanPs and exhibited efficient LP cleavage, although even processing of unmodified precursor peptides is described (Plat, Kluskens et al. 2011, Ortega, Velasquez et al. 2014, Lagedroste, Smits et al. 2017, Montalban-Lopez, Deng et al. 2018). As the primary recognition site for LanPs is the cleavage site, there are some variations depending on the LP sequence. In the class I lanthipeptides at least two different groups of C-terminal recognition site can be identified. The first group are nisin-like LPs (without subtilin and subtilin-like LPs), where the cleavage site comprising of $GA(X)_2R|I$ (cleavage is indicated by |). There, especially the LP residue pairs of Arg-1/ Ala-4 (P1 and P4) and Arg-1/Glu-3 (P1 and P3) are important for peptidase activity as shown for NisP and EpiP, respectively (van der Meer, Rollema et al. 1994, Geissler, Götz et al. 1996). Other examples

of this group are the LPs of gallidermin, epidermin and salivaricin D. In the LanP enzymes the S1 and S4 binding pocket defines, which substrate sequence at position P1 and P4 are still allowed and where efficient cleavage is still observed. The positively charge aa Arg-1 is requisite for the electrostatic interaction with S1 in LanP (Siezen, Rollema et al. 1995). The second group are Pep5 (or ElxA)-like LPs, which have an other cleavage site consisting of E/DL/V$(X)_2$Q|S/T (Ortega, Velasquez et al. 2014).There, an alanine mutations of Asp, Leu and Gln identified these residues to be important for efficient LP cleavage. Noteworthy, the LanPs corresponding to these lanthipeptides (e.g. Pep5, ElxA and EciA) are located in the cytoplasm and not extracellularly.

The LPs of some class II lanthipeptides (mostly two-component lanthipeptides) are processed by the bifunctional $Lan_{C39P}T$ and an extracellular located peptidase LanP (Booth, Bogie et al. 1996, Caetano, Krawczyk et al. 2011, Wang, Zhang et al. 2014). For example the precursor peptide of $LicA_{\beta}$ is first processed by the AMS transporter $Lic_{C39P}T$. Here, the cleavage after the double Gly-motif results in a peptide with six aa overhang at the N-terminus. These are further trimmed by the extracellular located serine protease LicP to yield the mature lanthipeptide (Caetano, Krawczyk et al. 2011, Tang, Dong et al. 2015). Similarly, the cytolysin precursor peptides $CylL_S$ and $CylL_L$ are finally processed by CylA to the mature cytolysin (Tang, Bobeica et al. 2018).

Most BGCs of class III and IV lanthipeptides lack a peptidase, which is normally found on the BGCs of class I and II lanthipeptides. Moreover, it was observed, that the length of the remaining LP was variable and had different quantity of aa residues at the N-termini (Meindl, Schmiederer et al. 2010, Krawczyk, Voller et al. 2012, Voller, Krawczyk et al. 2012, Iftime, Jasyk et al. 2015, Zhang, Doroghazi et al. 2015, Jungmann, van Herwerden et al. 2016). Therefore, an N-terminal trimming activity of putative aminopeptidases was suggested, although first no peptidases were identified. In 2013, Völler *et al.* described a propyl oligopeptidase FlaP with endopeptidase activity, that specifically cleaves FlaA at position Pro-12 (Voller, Krawczyk et al. 2013). This resulted in a precursor peptide ($FlaA_{-11-22}$) that need further trimming by an aminopeptidase. Nevertheless, it showed a possible strategy in class III lanthipeptides maturation. Recently, a Zn-dependent metalloprotease (M1-class) AplP with endo- and aminopeptidase activity was described (Chen, Xu et al. 2019). This peptidase is located in the BGC of the class III lanthipeptide NAI-112, but other AplP-like peptidases are normally found outside of the BGC (e.g. SapP from the lanthipeptide SapB,

EryP from erythreapeptin and AciP from catenulipeptin). All peptidases harbour the conserved HEXXH(X)$_{18}$E- and the AMEN-motif, which are the putative Zn-binding residues and the catalytic motif. First, the peptidases cleaves at the E(I/L)(L/Q) motif within the LP, before the substrate tolerant aminopeptidase activity trims the LP to yield the mature lanthipeptide. There, both functions are utilized by one active site as shown by mutations of the Zn-binding pocket (Chen, Xu et al. 2019). In the BGC of class IV lanthipeptides a dedicated peptidase is still unknown, but similarly to the BGC of class III lanthipeptides a peptidase with endo-/aminopeptidase activity is found outside of the BGC.

Additionally, in some BGCs of class I lanthipeptide no LanP or Lan$_{C39}$T proteins are found and the processing activity is carried out by other unspecific proteases. Examples are the lanthipeptides subtilin and the subtilin-like peptide ericin A/S, entianin and geobacillin I. For example in the subtilin producer *B. subtilis* ATCC 6633 the precursor peptide is cleaved by extracellular located serine proteases. These proteases are AprE, WprA and Vpr, which cleave off the LP of mSpaS to yield mature subtilin (Corvey, Stein et al. 2003).

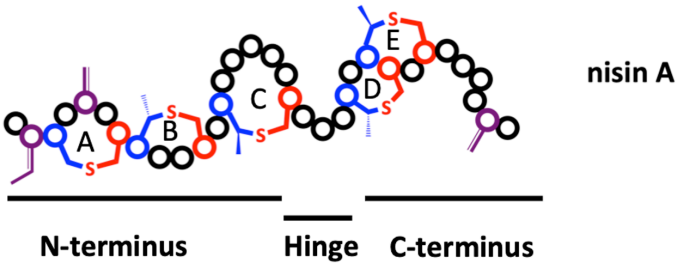
1.5 The model lanthipeptide nisin

1.5.1 The expression, biosynthesis and modification of nisin

Since the discovery in 1928, nisin is the best-studied lanthipeptide and therefore it is used as a model system to study the biosynthesis, modification and general properties of lanthipeptides. Nisin is classified as a lanthipeptide based on special post-translational modifications within the core peptide. Most prominent PTMs are the Lan and MeLan rings, which are the origin of their class name (Newton, Abraham et al. 1953, Ingram 1969, Gross and Morell 1971, Kellner, Jung et al. 1988). In total the CP harbours the dehydrated amino acids (Dha and Dhb), one (2S, 6R)-lanthionine ring (ring A) and four (2S, 3S, 6R)-3-methyllanthionine rings (rings B-E) (Figure 18A). The NMR structure of nisin shows, that it consists of two domains connected by a flexible region (Van de Ven, Van den Hooven et al. 1991). The N-terminal domain is from aa residues 3-19 including rings A-C, which has a hydrophobic (opposite to thioether cross links) and a hydrophilic side (Lubelski, Rink et al. 2008). Residues from 23-34 with the intertwined rings D and E are the C-terminal domain. The flexible region between ring C and rings D/E is called hinge region. The C-terminal domain is important for membrane perturbation and pore formation (Hooven, Doeland et al. 1996, Breukink, van Kraaij et al. 1997, Kraaij, Breukink et al. 1997, Wiedemann, Breukink

et al. 2001), whereas the rings A/B from the N-terminal domain are interacting with the peptidoglycan precursor lipid II (Hsu, Breukink et al. 2004). Nisin is active in a nM range, due to the high-affinity binding to lipid II and shows a high antimicrobial activity mostly against other Gram-positive bacteria (e.g. *Enterococci, Staphylococci and Streptococci*) (Kuipers, Rollema et al. 1995, Sahl, Jack et al. 1995, Breukink 1999). Furthermore, nisin or engineered nisin-variants are heat-stable and acid tolerance with a highly solubility at acidic pH (Lui and Hansen 1990, Rollema, Kuipers et al. 1995, Lu, Jiang et al. 2010). Additional nisin is less accessible to proteolytic cleavage (e.g. trypsin) due to the Lan and MeLan rings (Rink, Arkema-Meter et al. 2010). But it is sensitive against the activity of α-chymotrypsin (Jarvis and Mahoney 1969). Nevertheless, these properties make nisin interesting to use it in the food industry, for veterinary medicine or for pharmaceutical purposes (Delves-Broughton, Blackburn et al. 1996, van Kraaij, de Vos et al. 1999, Cao, Wu et al. 2007, Piper, Cotter et al. 2009, Shin, Gwak et al. 2016).

A



B

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NisinO4          ITSQHSFCTPNCLTGFLCP-PKTQLTCTCKLKGQ-
NisinH           -FTSISMCTPGCKTGALMTCNYKTATCHCSIKVSK
NisinQ           -ITSISLCTPGCKTGVLMGCNLKTATCNCSVHVSK
NisinF           -ITSISLCTPGCKTGALMGCNMKTATCNCSVHVSK
NisinA           -ITSISLCTPGCKTGALMGCNMKTATCHCSIHVSK
NisinZ           -ITSISLCTPGCKTGALMGCNMKTATCNCSIHVSK
NisinO1          -YKSKSACTPGCPTGILMTCPLKTATCGHITGK--
NisinO2          -YKSKSACTPGCPTGILMTCPLKTATCGHITGK--
NisinO3          -YKSKSACTPGCPTGILMTCPLKTATCGHITGK--
NisinU2          -VTSKSLCTPGCKTGILTGCPLKTATCGCHFG---
NisinP           -VTSKSLCTPGCKTGILMTCAIKTATCGCHFG---
NisinU           -ITSKSLCTPGCKTGILMTCPLKTATCGCHFG---
                   . .  *  ***.*  **  *      .   **
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Figure 18: The lanthipeptide nisin.
A) Scheme of the lanthipeptide nisin. It comprises of three major region and has five (methyl-)lanthionine rings (rings A-E). **B**) Multiple sequence alignment with Clustal Omega of natural nisin variants. The red box marks the conserved Cys residues for thioether formation with dehydrated aa (Dha or Dhb). Colour code of scheme: Dehydrated aa are highlighted with violet, former dehydrated aa with blue and former Cys residues with red.
Similar amino acids (.); conserved amino acids (:); highly conserved amino acids (*)

Nisin belongs to the class I lanthipeptides and to date nine natural nisin variants are known. There, nisin A, F, Q, and Z are produced by *L. lactis* strains, nisin O1-4 by *Blautia obeum* strain A2-162, nisin P by *Streptococcus gallolyticus* subsp. *pasteurianus*, nisin U by *Streptococcus uberis*, nisin U2 by *Streptococcus agalactiae* and nisin H by *Staphylococcus hypointestinalis (Gross and Morell 1971, Mulders, Boerrigter et al. 1991, Zendo, Fukao et al. 2003, Wirawan, Klesse et al. 2006, de Kwaadsteniet, Ten Doeschate et al. 2008, Zhang, Yu et al. 2012, O'Connor, O'Shea et al. 2015, Hatziioanou, Gherghisan-Filip et al. 2017)*. These variants share common features like an FN/DLD-box in the LP or the number of Lan/MeLan rings, except nisin O4 that has four Lan/MeLan rings. Most intrudingly is the aa sequence of the CP, which differs at certain positions (Figure 18B). For example the variant nisin A differs from nisin Z at pos. 27, where a His is exchange by an Asn (Mulders, Boerrigter et al. 1991). The nisin F variant differs at two positions (pos. 27 and 30), whereas nisin Q differs at four (pos. 15, 21, 27 and 30) and nisin H at five positions (pos. 1, 6, 18, 21 and 31). The nisin variants from Streptococci (U, U2 and P) as well as nisin O from *Blautia obeum* show a higher diversity in the sequence of the LP and CP. There, the Streptococci are more similar especially in the CP sequence. The nisin O variants 1-4 are more similar to the subtilin precursor (SpaS) and to each other, where the nisin O4 variant is the peptide with the highest diversity. The origin of the different nisin BGC in different microorganisms is not clear, but for example transposase-like sequences downstream of the *nsoA* gene would suggest a horizontal gene transfer (Hatziioanou, Gherghisan-Filip et al. 2017).

In Gram-positive bacteria the gene transfer is conducted through conjugative elements like conjugative plasmids or transposons (Johnson and Grossman 2015). In *L. lactis* nisin producer strains NIZO R5 and FI5876 the conjugative transposons Tn5276 (Rauch and de Vos 1992) and Tn5301 (Dodd, Horn et al. 1990, Horn, Swindell et al. 1991) are described. This 70 kbp large mobile element harbours the BGC of nisin and the ability to ferment sucrose. At the beginning of the 90s it was inserted into the chromosome of a plasmid-free, nisin non-producer strain (MG1614) resulting in the strain NZ9700 (Rauch and de Vos 1992, Kuipers, Beerthuyzen et al. 1993). In total the BGC comprises of 11 genes, which encode for the NisA precursor peptide, the proteins that are relevant for nisin modification, secretion, regulation and immunity. The proteins for modification are the dehydratase NisB and the cyclase NisC. The peptide is secreted by the ABC transporter NisT and then finally processed by the LP peptidase NisP. The mature nisin activates the TCS of NisR / NisK and induces its

own transcription. The lipoprotein NisI and a second ABC transporter NisFEG are important for the self-protection of the producer.

The ribosomal synthesized precursor peptide of nisin (NisA) consists of 57 residues (Figure 19) (Kaletta and Entian 1989). There, the N-terminal LP has 23 aa residues and includes the FNLD-box for recruiting NisB/NisC and the cleavage site (GASPR) for NisP. The CP comprises of 34 aa residues and it is the PTM site for the modification enzymes NisB and NisC (van der Meer, Polman et al. 1993). The dehydratase NisB is presumably associated with the membrane and catalyses the dehydration of Ser and Thr residues in the CP (Engelke, Gutowski-Eckel et al. 1992, Karakas Sen, Narbad et al. 1999). In the dehydration reaction NisB utilizes glutamyl-tRNA^{Glu} to transfer the glutamyl-group onto the hydroxyl-group of the Ser and Thr side chain (Ortega, Hao et al. 2015). The activation of serine or threonine residues is performed by the glutamylation domain of NisB. The place of the β-elimination of the glutamylated intermediate is the N-terminal elimination domain and results in the dehydrated aa Dha (originate from Ser) and Dhb (originate from Thr). During this process the LP has one binding site, where especially the FNLD-box of NisA$_{LP}$ is the main recognition site for NisB (Mavaro, Abts et al. 2011, Repka, Hetrick et al. 2018). Any alternation of the FNLD-box has an influence on the dehydration activity and thereby on the dehydration pattern of nisin (Plat, Kluskens et al. 2011, Khusainov, Moll et al. 2013). Furthermore, flaking amino acids at the PTM sites influence the dehydration activity of NisB, where the aa should be rather hydrophobic and nonaromatic (e.g. alanine or valine) instead of hydrophilic and charged (e.g. glutamate or aspartate) (Rink, Kuipers et al. 2005). Mutagenesis analysis of residues in the catalytic domains of NisB identified Tyr80, R83, R87, T98, D121, D299 and R464 to be important for glutamylation, where Tyr80 is postulated to shuttle the glutamyl-group from glutamyl-tRNA^{Glu} (Khusainov, van Heel et al. 2015, Repka, Chekan et al. 2017). Other important residues (R786, R826 and H961) are found in the elimination domain. A single point mutation of H961A lead to unmodified NisA, which cannot be released from the NisB/NisC modification complex (Khusainov, van Heel et al. 2015).

The Zn-dependent cyclase NisC catalyses the formation of Lan/MeLan rings in an alternated fashion with the dehydration activity of NisB (Koponen, Tolonen et al. 2002, Lubelski, Khusainov et al. 2009). The zinc ion in the active centre activates the thiol-group of a cysteine residue in the CP by lowering the pKa value of the sulphur atom (Okeley, Paul et

al. 2003). The thiolate attacks neighbouring Dha or Dhb residues in an intramolecular Michael-like addition. The resulting bond is a thioether bond and the Lan/MeLan rings are formed. Here, the cyclisation reaction catalysed by NisC is highly stereo- and regiospecific, where in the end only one isoform is produced. The crystal structure of monomeric NisC displays of two domains. A bowl formed α-toroid domain of seven α-helices and an SH2-like domain of three β-sheets and two α-helices. In the middle of the formed shallow bowl is the catalytic centre with the coordinated zinc ion. The zinc ion is bond by two Cys residues (pos. 284 and 330), one His residue (pos. 331), one water molecule in a tetrahedral coordination (Li, Yu et al. 2006). Further conserved residues (also among other LanC) are the residues His212 and Asp141 that are important for the correct cyclisation. The residues Arg280 and Tyr285 are not involved in the correct cyclisation but in the general acid-base reaction (Li and van der Donk 2007). For the SH2-like extension a substrate binding function is discussed as it is near the active centre and could support the cyclisation process (Bradshaw and Waksman 2002, Li, Yu et al. 2006). But until now there is no evidence to prove such function of the SH2-like domain in NisC.

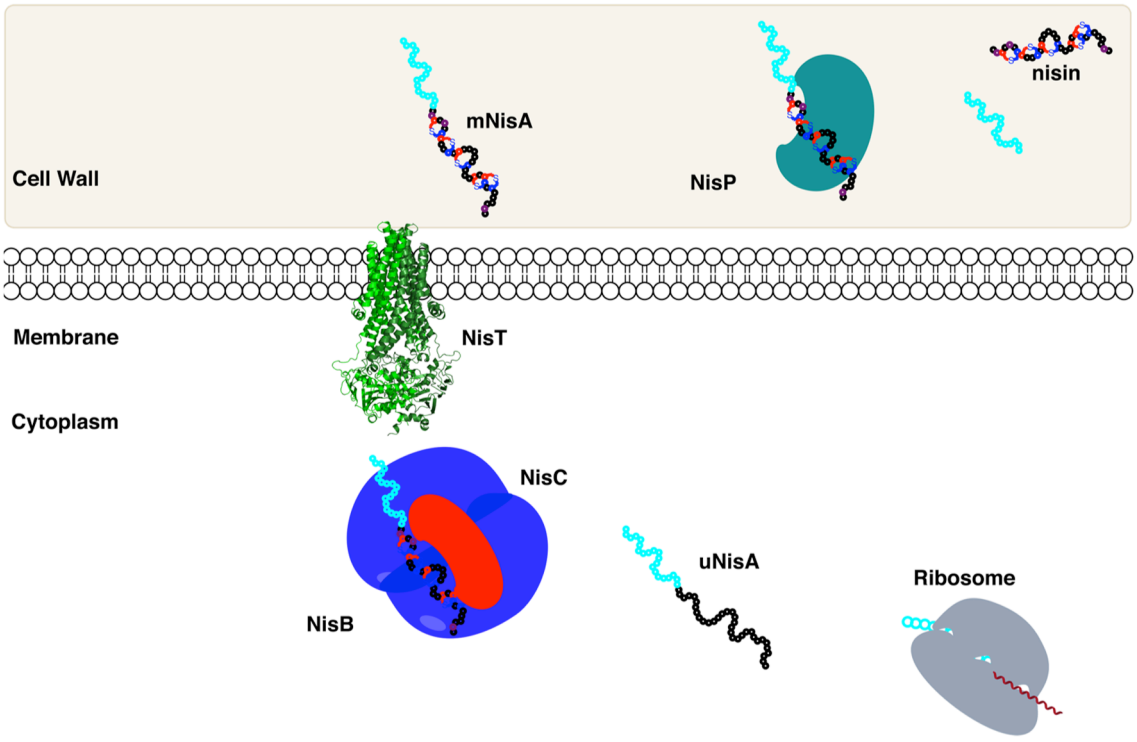

Figure 19: Scheme of the nisin biosynthesis, secretion and maturation.
Colour code: Dehydratase NisB (blue), cyclase NisC (red), exporter NisT (green) and peptidase NisP (light blue). Homology model of NisT (green) based on PCAT1 modelled with Phyre2 (Kelley, Mezulis et al. 2015). The program PyMOL 2.1.1 was used to display the homology models in a carton representation.

The modification of the NisA precursor peptide is based on the interaction of NisB with NisC, which form a modification complex. The first evidence for NisB and NisC interaction was obtained in the 1990s, where the interaction was shown by yeast two-hybrid and co-immunoprecipitation experiments (Siegers, Heinzmann et al. 1996). The proposed multimeric lanthionine synthetase complex was later supported by pull-down experiments of NisB and NisC from the cytoplasm of *L. lactis* with hexa-histidine tagged NisA (Khusainov, Heils et al. 2011). Eventually, the modification complex consisting of NisB/NisC/NisA was observed in solution by MALS-SEC and SAXS experiments. There, a stoichiometry of 2:1:1 (NisB, NisC and NisA) with a molecular weight of 291 kDa was determined (Reiners, Abts et al. 2017). Interestingly, the multimeric enzyme complex of $NisB_2C$ and the substrate mNisA was shown to dissociate and the complex formation was abolished. Therefore, the ring E seem to be a release signal, which is induced due to conformational changes through the intertwined rings D and E (Van de Ven, Van den Hooven et al. 1991, Reiners, Abts et al. 2017). The process of dehydration and cyclisation are coupled events with N- to C-termius directionality, which are probably performed in alternate pattern of dehydration and cyclisation (Kuipers, Meijer-Wierenga et al. 2008, van den Berg van Saparoea, Bakkes et al. 2008, Lubelski, Khusainov et al. 2009, Reiners, Abts et al. 2017).

After complete modification mNisA is translocated by the ABC transporter NisT (1.5.2) across the membrane, where the LP is cleaved off by the leader peptidase NisP (1.5.3) (van der Meer, Polman et al. 1993, Kuipers, de Boef et al. 2004). The enzymes for transport and proteolysis function independently from each other, thus the translocation of the precursor is not directly coupled to the processing like in $Lan_{C39P}T$-type exporter (Kuipers, de Boef et al. 2004). The procession by NisP yields active, mature nisin, which would directly interact with lipid II in the membrane of the producer strain. To circumvent this mode of action, the producer strain expresses an immunity system to overcome the antimicrobial activity of nisin. This immunity system comprises of the lipoprotein NisI and the ABC transporter NisFEG (1.5.4).

The release of nisin triggers the transcription of the nisin BGC as nisin acts as an autoinducer. Here, the TCS, consisting of the histidine kinase NisK and the response regulator NisR, controls the expression via the *nisA* promotor and *nisF* promotor in a quorum-sending manner (Kuipers, Beerthuyzen et al. 1995, de Ruyter, Kuipers et al. 1996). In contrast to the transcripts of the *nisR* promotor (*nisRK*) and *nisI* promotor (*nisIP*), where

the transcription is constitutive and independent from the nisin signal (de Ruyter, Kuipers et al. 1996) (Li and O'Sullivan 2006). NisK is the histidine kinase and the sensor protein, which is essential for the signal transduction pathway (Engelke, Gutowski-Eckel et al. 1994). It was shown, that the rings A/B of nisin are necessary for the interaction with NisK and the hydrophobic interaction activates NisK (Kuipers, Beerthuyzen et al. 1995, Dodd, Horn et al. 1996, Spiess, Korn et al. 2015). The HK is activated after nisin binding via an ATP-dependent autophosphorylation of a conserved histidine residue (Mascher, Helmann et al. 2006). Consequently, the phosphoryl-group is transferred to a conserved aspartate residue in the response regulator NisR, which binds the promotor region of the BGC to regulate the transcription (van der Meer, Polman et al. 1993, Eichenbaum, Federle et al. 1998, Kuipers, de Ruyter et al. 1998). Even sub-inhibitory amounts of mature nisin are enough to activate the TCS and thereby the transcription of the operon (Kuipers, Beerthuyzen et al. 1995). There, a linear dose-response between nisin amount and protein expression can be observed, where the promotors are tightly coupled to inducer addition (Eichenbaum, Federle et al. 1998, Kuipers, de Ruyter et al. 1998).

1.5.2 Nisin secretion – The ABC transporter NisT

The ABC transporter NisT is the exporter to secrete the lanthipeptide NisA to the exterior. Like other prokaryotic half-size ABC transporter, NisT is expressed as one polypeptide chain that consists of two domains (Figure 20A). The N-terminal located domain is the six-helix spanning TMD and important for substrate transport by forming the translocation pathway. The other domain is the C-terminal NBD, which energies the export by ATP hydrolysis. NisT is a functional homodimer, has a length of 600 aa and a molecular weight of 69 kDa.

NisT shows a low sequence similarity to other lanthipeptide exporters such as ElxT (21.3%) but higher similarity to EntT (44.0%) or to the NisT homolog NshT (75.3%), where the substrate is classified to the nisin-group (Table 1). The main sequence similarities originate from the NBDs, where the highly conserved residues motifs (e.g. Walker A/B and ABC signature motif) of ABC proteins are found. However, the TMDs display generally a low sequence similarity, although the membrane topology are six TM helices (I-VI) with the N- and C-termini located in the cytoplasm. Presumably, NisT, like other ABC transporters, will adopt different conformations during a transport cycle such as inward-facing, occluded and outward-facing conformation (Figure 20B).

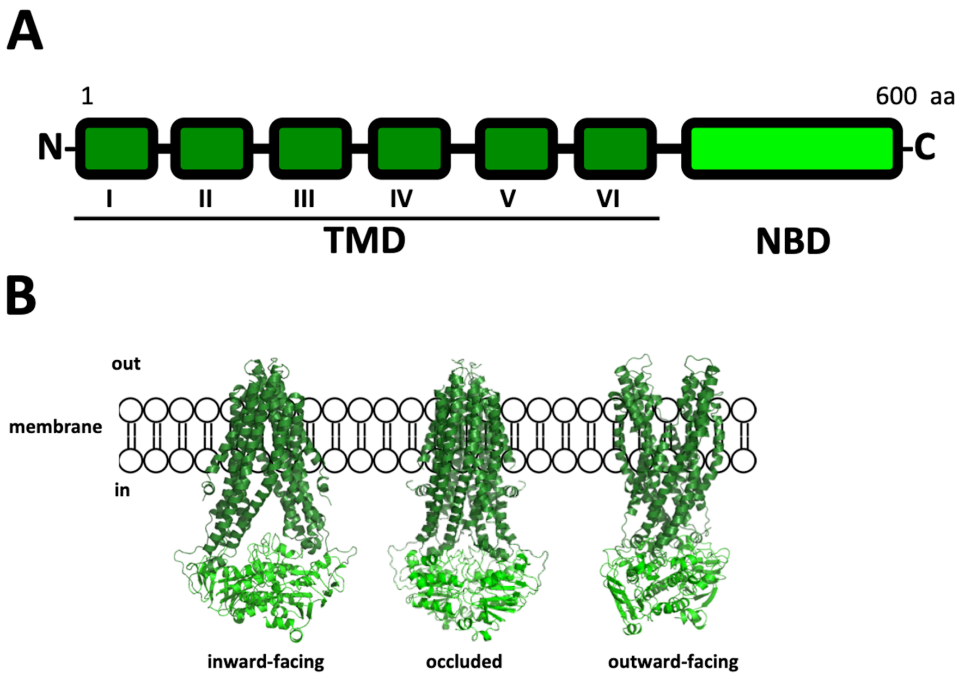

Figure 20: The ABC transporter NisT.
A) Domain organisation of the ABC transporter NisT. **B**) Homology model of NisT in different conformations from Phyre2 (Kelley, Mezulis et al. 2015) based on Tm287 (PDB ID: 3QF4), McjD (PDB ID: 5EG1) and Sav1866 (PDB ID: 2ONJ). Colour code: TMDs are highlighted in dark green and NBDs in light green. The program PyMOL 2.1.1 was used to display the homology models in a carton representation.

In 1996, it was shown, that NisT is the exporter of NisA precursor peptide. The disruption of the *nisT* gene lead to accumulation of mNisA inside the cell and no mNisA was observed in the supernatant (Quiao and Saris 1996). Later, it was shown, that NisT can export NisA in any modification state and secretion of substrate can function independently from the modification complex (Kuipers, de Boef et al. 2004). Furthermore, Kuipers *et al.* showed that NisT is able to export non-lanthipeptide peptides, by creating LP fusions with enkephalin, vasopressin, angiotensin and various hexa-peptides (Kuipers, de Boef et al. 2004, Rink, Wierenga et al. 2007). This showed the possible application of NisT as a secretion system for therapeutic peptides with or without Lan/MeLan rings and the rather broad than narrow substrate spectrum (Rink, Kuipers et al. 2005, Kluskens, Nelemans et al. 2009). The secretion of peptides with a length between 24-203 aa was observed, where the long polypeptides are stabilized by a C-terminal transmembrane segment and a positively charged sequence (KRKQREE) (Bosma, Kuipers et al. 2011).

In general, the secretion of NisA as the natural substrate is depended on the modification enzymes NisC and especially NisB. The production of NisA is enhanced by NisB and NisC, thus a channelling mechanism of substrate translocation was postulated (van den Berg van Saparoea, Bakkes et al. 2008, Lubelski, Khusainov et al. 2009).

Table 1: Sequence similarity of NisT to other LanT-type transporters.
The sequence similarity of NisT to LanT-type transporters was determined with the SIM alignment tool (Huang, Hardison et al. 1990). EpiT and ElkT are only available as partial sequence and were not aligned (n.a.).

name	similarity to NisT (%)	accession number	reference
EtnT	44.0	G0Z2H2	(Fuchs, Jaskolla et al. 2011)
EpiT	n.a.	O07860	(Allgaier, Jung et al. 1986)
ElxT	21.3	I6ZHK9	(Ekkelenkamp, Hanssen et al. 2005)
ElkT	n.a.	Q57072	(Kamp, Hooven et al. 1995)
EriT	44.5	Q93GH7	(Stein, Borchert et al. 2002)
GdmT	27.8	O07475	(Kellner, Jung et al. 1988)
GeoTI	36.7	WP_008881438	(Garg, Tang et al. 2012)
LasT	39.2	Q48851	(Skaugen and Nes 1994)
MutTI	25.3	AAF99583	(Qi, Chen et al. 2000)
MutIII	25.3	AAD56148	(Qi, Chen et al. 1999)
NshT	75.3	AKB95121	(O'Connor, O'Shea et al. 2015)
NsuT	58.3	ABA00880	(Wirawan, Klesse et al. 2006)
PaeT	33.8	K7U6S3	(Lohans, Huang et al. 2012)
PaenT	31.6	M1FLR0	(Huang and Yousef 2015)
PenT	34.9	ALR96371	(Baindara, Chaudhry et al. 2016)
PepT	20.7	Q54121	(Kaletta, Entian et al. 1989)
SlvT	61.2	H2D758	(Birri, Brede et al. 2012)
SrtT	37.9	BAB08163	(Karaya, Shimizu et al. 2001)
SpaT	43.6	P33116	(Klein, Kaletta et al. 1992)
SubT	23.7	AGL93176	(Phelan, Barret et al. 2013)

In the absence of NisB and NisC or only NisB the production level is decreased to 2%. The coexpression of NisB and NisT leads to a production level of 30% and only the coexpression of NisB and NisC with NisT increases the production level back to 100%. Therefore, a multimeric lanthionine synthetase complex consisting of NisBTC, similar to SpaTBC located at the cytoplasmic membrane, was suggested (Siegers, Heinzmann et al. 1996, Kiesau, Eikmanns et al. 1997). Although, the interaction of NisB with NisC and NisC with the NBD of NisT was observed by a yeast two-hybrid assay and co-immunoprecipitation experiments,

the isolation of a full assembly complex was unsuccessful (Siegers, Heinzmann et al. 1996). Only the isolation and the *in vitro* assembly of the modification complex comprising of NisB, NisC and NisA is described so far (Khusainov, Heils et al. 2011, Reiners, Abts et al. 2017). Additionally, no crystal structure of NisT and no *in vitro* characterization of NisT regarding the ATPase or the secretion activity are available. Moreover, the lack of detailed information about the modification/secretion complex makes it difficult to elucidate the exact molecular mechanism of NisA translocation.

1.5.3 NisA maturation – The serine protease NisP

The nisin leader peptidase NisP belongs to the family of subtilisin-like serine protease (Pfam entry: Peptidase S8). Like the majority of these serine proteases, NisP has an endopeptidase function and cleavages the LP of NisA at a specific cleavage site (GASPR|I) (van der Meer, Polman et al. 1993). The peptidase is ribosomally synthesized as a pro-protein with an N-terminal *sec* signal peptide. NisP is translocated via the SEC translocon to the extracellular space and the SP is cleaved by a signal peptidase I (Schneewind and Missiakas 2014). Furthermore, after translocation the pro-sequences (N- and C-terminal) are cleaved by an autocatalytic reaction and the folding is induced (Figure 21A). The mature form of the extracellular located NisP is a 54 kDa protein and due to a LPXTG-motif at the C-terminus anchoring to the peptidoglycan by sortase A is possible (van der Meer, Polman et al. 1993, Dieye, Oxaran et al. 2010, Schneewind and Missiakas 2014). Although, Xu *et al.* described an additional auto-cleavage site in the C-terminal region and self-cleavage would prohibit an anchoring (Xu, Li et al. 2014).

The extracellular located peptidase cleaves off the LP of mNisA as a final maturation step of nisin biosynthesis (van der Meer, Polman et al. 1993). First, *in vivo* and *in vitro* assays indicated a narrow substrate specificity to precursor peptides with at least one (methyl-)lanthionine ring (van der Meer, Polman et al. 1993, Kuipers, de Boef et al. 2004, Plat, Kluskens et al. 2011). Further investigations revealed that uNisA could be cleaved, if the cleavage site of the LP is changed to the auto-cleavage site of NisP (Plat, Kluskens et al. 2011). Recently, it was shown that the substrate specificity of NisP is independent from the modification state but the catalytic efficiency of the reaction is influenced. NisP is highly active against its natural substrate mNisA, but depending on the modification state the k_{cat} values and thereby the catalytic efficiency are strongly reduced (Lagedroste, Smits et al.

2017). There, the catalytic parameter for the substrates uNisA, dNisA, a Cys-less variant $dNisA_{AAAAA}$ and mNisA for a secreted variant of NisP were determined. This *in vitro* characterisation showed, that NisP is primary a sequence based peptidases but the catalytic efficiency of the reaction is depended on the Lan/MeLan rings for proper substrate recognition.

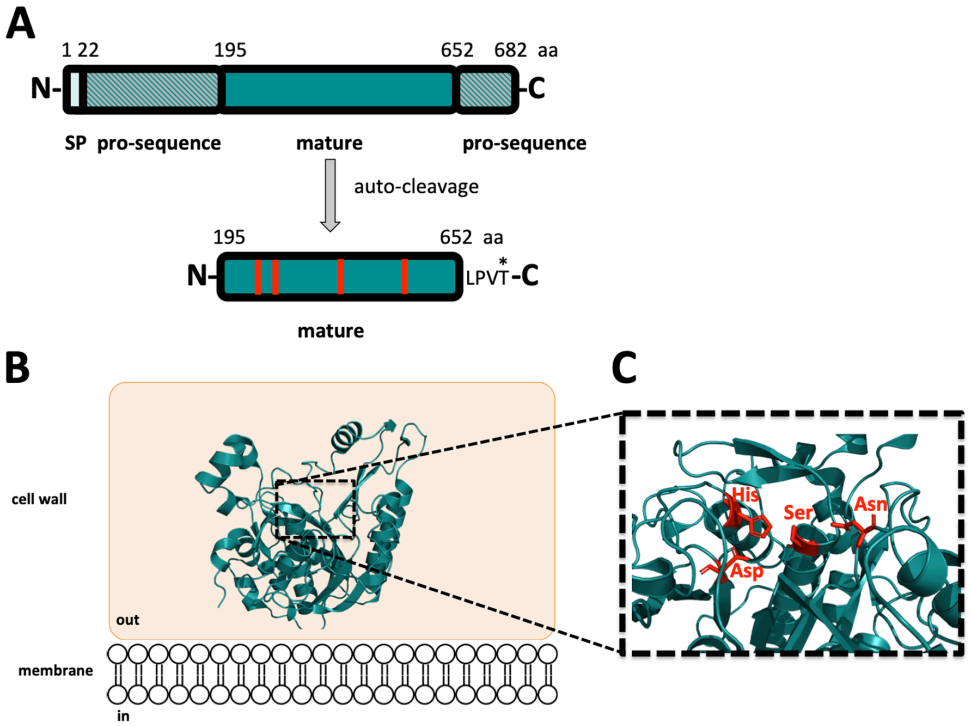

Figure 21: The leader peptidase NisP.
A) Domain organization and maturation of NisP. After cleavage of the signal peptide (SP) and auto-cleavage of the pro-sequences, the mature protein consists of aa 195 to 652. At the C-terminus is sequence (LPVTGD) located, which allow the peptidoglycan anchoring via the Thr (*) residue by a sortase. **B**) The crystal structure of NisP (PDB ID: 4MZD) display the fold of a subtilisin-like serine protease. **C**) Within the active centre is the catalytically tirade of Ser512, His306 and Asp259 located (highlighted in red). The oxyanion hole (Asn407) lies in close proximity to the Ser512. The program PyMOL 2.1.1 was used to display the crystal structure in a cartoon representation.

In 2014, the first crystal structures of lanthipeptide leader peptidase were solved, which were EpiP from *S. aureus* and NisP from *L. lactis* (Kuhn, Prachi et al. 2014, Xu, Li et al. 2014). The structure of NisP was determined at a resolution of 1.1 Å and contained the aa residues 224-566, where aa residues 196-223 and 567-655 are missing in the crystal structure (Figure 21B). Only aa residues 635-647 are visible at the active centre and were termed C-peptide. The peptidase shows the regular fold of a subtilisin-like serine protease with a heart-like shape comprising of 16 β-sheets and 11 α-helices (Xu, Li et al. 2014). There, the helices 6-8 as well as the β-sheets 6,8,12 and 13 might be involved in substrate binding, because of their location at the open cleft at the surface of NisP. The catalytic triade consisting of the residues Asp259, His306 and Ser512 is located on the β-strand 1 and at the

N-termini of α-helix 3 and 9, respectively (Figure 21C). The corresponding oxyanion hole is the Asn407 residue and it is located in a random coiled loop after the β-strand 7. Interestingly, the C-peptide is interacting with important residues of the substrate binding pocket, which is located near the active site. In 1995, an early homology model of NisP based on the subtilin BPN' with its inhibitor R45-eglin showed the putative binding site of a peptide at the active centre of NisP (Siezen, Rollema et al. 1995). There, a sequence corresponding to residues P6-P2' of mNisA is interacting with the substrate binding pocket (S6-S2') of NisP. The predominate interaction is an electrostatic interaction of the P1 residue (Arg) and the S1 binding site (Asp408 and Asp436). Especially, the importance of residues P1 (Arg-1) and P4 (Ala-4) was shown by a mutagenesis study of NisA and the cleavage activity of NisP (van der Meer, Rollema et al. 1994). Now, the interaction of the C-peptide with the S1 and S4 substrate binding pocket is confirmed in the crystal structure. In S1 the side chain of Asp436 interacts with an Arg residue via electrostatic interaction, which mimics the actual interaction with Arg-1 of mNisA. A mainly hydrophobic interaction is observed at the S4 binding site, where Phe 335, Leu 339 and Val 345 are interacting with Val and Ala residues of the C-peptide. Further interactions with the binding site are stabilized via hydrogen bonds between Ser340, Asp376 and Thr378 residues to the C-peptide (Xu, Li et al. 2014). Unfortunately, a co-crystal structure of NisP with its substrate is missing, but it would elucidate the interaction of NisP with the modified NisA peptide and how the modification state is influencing the catalytic efficiency.

1.5.4 Nisin immunity proteins – NisI and NisFEG

In the nisin producer strain the antimicrobial action of nisin is antagonized by the immunity system consisting of the lipoprotein NisI and the ABC transporter NisFEG (Kuipers, Beerthuyzen et al. 1993, Engelke, Gutowski-Eckel et al. 1994, Siegers and Entian 1995). This self-protection mechanism is supposed to be cooperatively as full immunity is only conferred by both proteins (Ra, Beerthuyzen et al. 1999, Takala, Koponen et al. 2004, Takala and Saris 2006).

The lipoprotein NisI is the first line of defence against nisin and confers immunity specifically to nisin (Stein, Heinzmann et al. 2003, Takala, Koponen et al. 2004). NisI has a molecular weight of 25.8 kDa and consists of two domains, which a structural similar to each other and to SpaI (Figure 22 A) (Hacker, Christ et al. 2015). One domain is the N-terminal

domain (ND), where at the N-terminus a signal peptide (residues 1-19) with lipobox consensus sequence (residues 16-21) is located. Within this sequence the Cys20 residue gets lipidated with diacylglycerol and NisI is anchored extracellularly to the membrane (Qiao, Immonen et al. 1995). The signal sequence is removed during this post-translational modification resulting in the mature protein of 226 aa. A membrane-binding experiment of NisI to unilamellar liposomes showed, that especially the ND binds to the membrane via its highly positive charged N-terminus (Hacker, Christ et al. 2015).

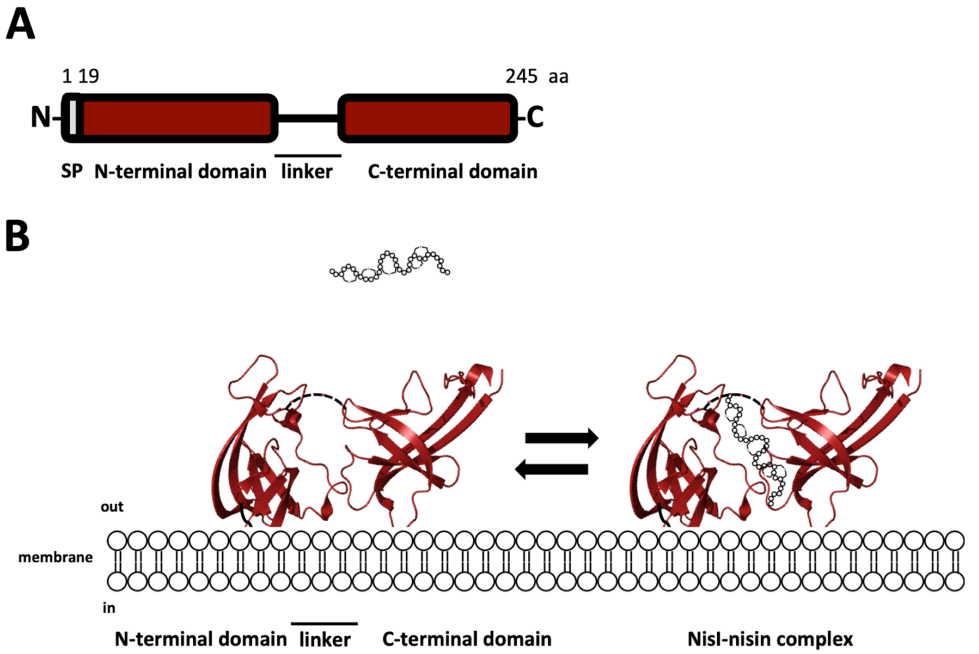

Figure 22: The nisin immunity protein NisI.
A) Domain organization of the lipoprotein NisI in a N-terminal and C-terminal domain (aa 20-245). The signal peptide (SP) is cleaved by a signal peptidase after translocation and the Cys20 undergoes a lipidation. The mature NisI is anchored to the membrane and extracellular located. **B**) The crystal structure of NisI (PDB ID: 5XHB) display the symmetric fold of the N- and C-terminal domain. The proposed binding site of the nisin molecule is between both domains. The program PyMOL 2.1.1 was used to display the crystal structure in a cartoon representation.

The other domain is the C-terminal domain (CD), which is important for nisin binding and displays a negatively charged surface with some hydrophobic patches. A deletion of 21 amino acids at the C-terminus of the CD decreased the level immunity to 14% of wild-type NisI (Takala and Saris 2006). Similarly, a truncation of the last 22 aa resulted in a decreased immunity to nisin, where the IC_{50} value was only 1/3 of the WT value (AlKhatib, Lagedroste et al. 2014). The explanation of the decreased immunity is a partially unfolded CD of the truncated $NisI_{2\text{-}204}$, where normally the C-terminal 22 aa form two β-strands (Hacker, Christ et al. 2015).

The overall crystal structure of full length $NisI_{22\text{-}245}$ consists mainly of β-strands, where the ND has a β-barrel structure of seven antiparallel β-strands and one β-sheet

(Figure 22 B) (Hacker, Christ et al. 2015, Jeong and Ha 2018). The CD has the same fold and shows also strong similarity to the SpaI structure. Between the two domains a flexible linker region connects the ND and the CD. There, SAXS data and additional biophysical experiments showed, that the interdomain interaction of the ND and CD is weak and dynamic (Hacker, Christ et al. 2015). In the crystal structure this linker region displays a putative binding site for nisin or even lipid II by creating a shallow groove between the two domains. In this region the aa residues Tyr172, Asp174, Glu232 and Asn234 located, which build a negatively charge patch and are involved in nisin binding (Hacker, Christ et al. 2015). A docking of a farnesyl diphosphate backbone containing three isoprene repeats was performed into the deep cleft, while on the opposite site the negatively charge patch was not occupied and free for a possible nisin interaction (Jeong and Ha 2018). This clearly shows the possible function of both domains in NisI. The ND binds to the membrane and enables an open conformation of NisI, which primes the protein for lantibiotic binding. There, nisin binds mainly to the CD in the groove between ND and CD. Interestingly, the K_D of nisin binding was determined to be in micromolar range, which is surprising as nisin acts at nanomolar concentration (Takala, Koponen et al. 2004, Hacker, Christ et al. 2015). Thus, an other mechanism, in which a nisin-lipid II complex is bound to NisI might be a possible explanation. This would also explain, why NisI is inhibiting the pore formation action of nisin and the change of the *L. lactis* phenotype, which might be a result of a decreased peptidoglycan biosynthesis after lipid II binding (AlKhatib, Lagedroste et al. 2014, Geiger, Korn et al. 2019).

The ABC transporter NisFEG is the second line of defence, which expels nisin from the membrane (Stein, Heinzmann et al. 2003). NisFEG belongs to the group of LanFEG-type transporters and consists of three proteins. The membrane-spanning proteins (TMPs) NisE and NisG form the translocation channel of the ABC transporter, where also the putative binding site for nisin might be located. The NBP is NisF and forms a homodimer to be a functional ATPase, which provides energy for conformational changes in the TMPs. Thus, the whole ABC transporter is a complex of $NisF_2EG$. Knockouts experiments showed, that NisFEG confers immunity against nisin and might cooperative interact with NisI to confer full immunity against nisin (Siegers and Entian 1995, Ra, Beerthuyzen et al. 1999). There, the exporter is depended on the ATPase activity of NisF and mainly recognizes the C-terminus (ring D/E) of nisin (AlKhatib, Lagedroste et al. 2014). A deletion of these rings or truncated variants of nisin ($nisin_{1-22}$ and $nisin_{1-28}$) led to a reduced fold of immunity in comparison to

wild-type nisin. Hence, these variants are less suitable substrate for the ABC transporter and are not recognized properly. Moreover, the action of the exporter has a certain concentration limit, which was determined by an *in vivo* study. There, a fluorescence-based assay was used to measure cell lysis of *L. lactis* after pore formation and a concentration limit of 60 nM nisin was observed (AlKhatib, Lagedroste et al. 2014). Above this limit inhibition of pore formation was not observed anymore.

Similar to the subtilin immunity system (Geiger, Korn et al. 2019), the lipoprotein NisI provides a fast response by inhibiting the pore forming action of nisin, whereas the ABC transporter NisFEG expels nisin from the membrane and contributes via a synergetic effect to full immunity.

1.5.5 Nisin resistance proteins – *Sa*NSR and *Sa*NsrFP

Lantibiotic resistance is rarely observed until now, but non-lantibiotic producing strains have obtained resistance systems against lantibiotics like nisin or gallidermin. These resistance systems are based on ABC transporter as detoxification system, on the alternating of the cytoplasmic membrane or peptidoglycan properties (Kramer, van Hijum et al. 2006, Gebhard and Mascher 2011, Draper, Cotter et al. 2015). Moreover, enzymes involved in nisin inactivation are for example described in *Bacillus* species and in *L. lactis* subsp. *diacetylactis* DRC3 (Jarvis 1967, Jarvis and Farr 1971, Froseth and McKay 1991). The first characterization of such a nisin resistance protein (NSR) was done by Sun *et al.* in 2009. They could show, that NSR, without the N-terminal signal peptide for membrane localization, processes an endopeptidase activity. It cleaves the last six aa of nisin at the C-terminus and resulting in inactivate nisin1-28 variant with a reduced antimicrobial activity (Sun, Zhong et al. 2009, Liang, Sun et al. 2010).

In the human pathogen strain *S. agalactiae* CHO-1 a homolog of NSR was reported and termed *Sa*NSR (Khosa, AlKhatib et al. 2013). There, *Sa*NSR is located on an operon comprising of five genes, which encode for *Sa*NSR, the TCS NsrRK and the BceAB-type transporter NsrFP (Figure 23). *Sa*NSR belongs to the S41 protease family, which are C-terminal processing endopeptidases. It confers resistance towards nisin by processing the C-terminus of nisin, where it cleaves the peptide bond between ring E and Ser29 leading to an antimicrobial less active nisin (AlKhatib, Lagedroste et al. 2014, Khosa, Frieg et al. 2016). It

was shown, that the heterologous expression of *Sa*NSR in *L. lactis* NZ9000 strain lead to a 20 fold higher resistance against nisin (Khosa, AlKhatib et al. 2013).

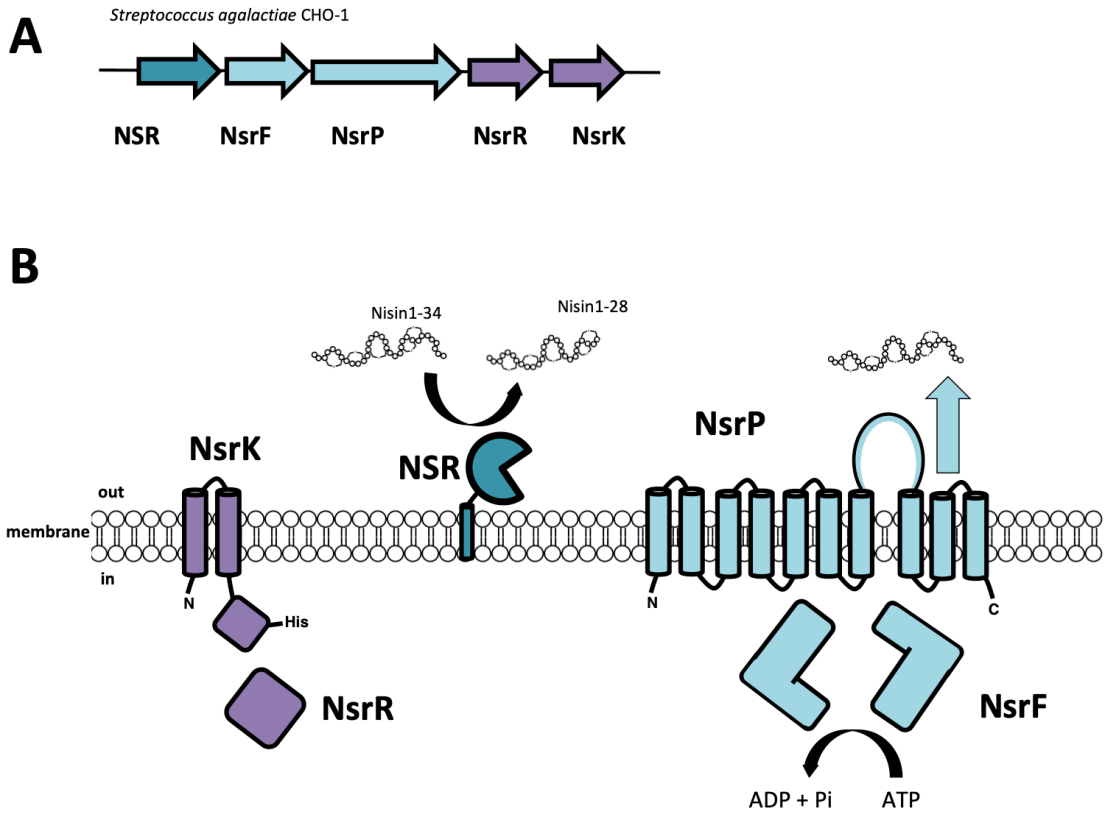

Figure 23: Nisin resistance proteins from *S. agalactiae*.
A) The operon for the nisin resistance proteins from *S. agalactiae* comprises of five genes and encodes fro the protease NSR (blue), the ABC transporter NsrFP (light blue) and the TCS NsrRK (violet). **B**) NsrK, NSR and NsrP are membrane located, whereas NsrR and NsrF are cytosolic proteins. The ABC transporter expels nisin from the membrane and NSR inactivates nisin (1-34) by cleaving the last six aa yielding in nisin1-28.

The crystal structure of *Sa*NSR was solved with a resolution of 2.2 Å and comprises of eleven β-strands and eleven α-helices (Figure 24)(Khosa, Hoeppner et al. 2015). The protein can be dissected into three domains, an N-terminal helical bundle, the catalytic domain and in a subdomain the protease cap domain. The latter mentioned domains form a tunnel, which is the binding site for the nisin molecule as shown by molecular docking simulations of nisin and *Sa*NSR (Khosa, Frieg et al. 2016). In the catalytic domain is a highly conserved TASSAEM sequence, which forms the active centre consisting of the dyad Ser236 and His98.

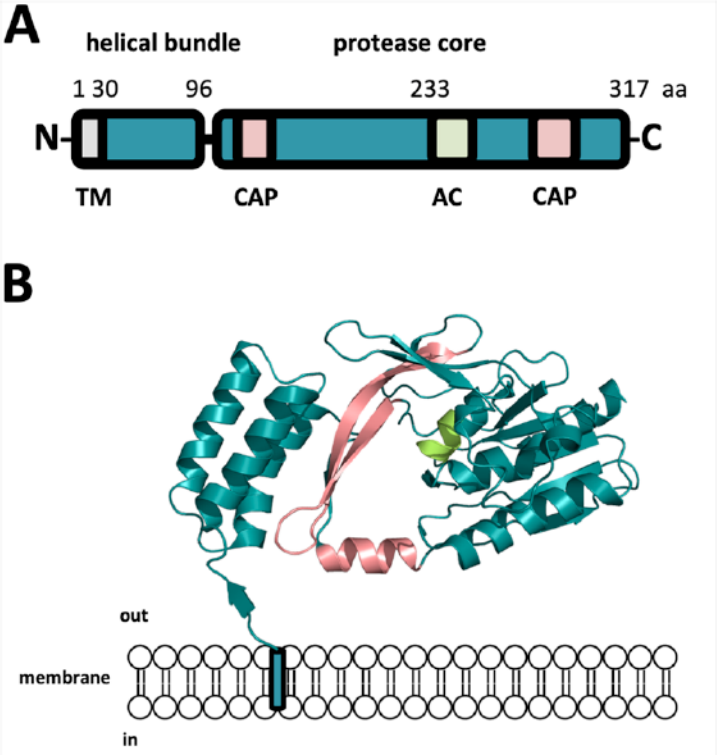

Figure 24: The nisin resistance protein *Sa*NSR.
A) Domain organization of the protease *Sa*NSR is dissect into three domains: an N-terminal helical bundle (aa 30-96) and a protease core domain with a subdomain (CAP) (aa 97-317). Additionally *Sa*NSR has a transmembrane helix (TM; aa 1-30) at the N-terminus and is located extracellular. **B**) The crystal structure of *Sa*NSR (PDB ID: 4Y68) display the three domain organization. The protease cap (CAP; red) and the active centre (AC; green) are highlighted. The program PyMOL 2.1.1 was used to display the crystal structure in a cartoon representation.

The nisin molecule is presumably primed via ring D and E into the tunnel, so that the active centre is at the cleavage site. The importance of ring D and especially rings E was shown by growth inhibition experiment in *L. lactis*. In this *in vivo* experiment nisin variants with ring D and/or E deletions were used to show the substrate specificity of *Sa*NSR. There, the deletion and truncation variants of nisin displayed a low fold of resistance and thereby were less recognized by *Sa*NSR as substrates (Khosa, Frieg et al. 2016). Thus, the peptidase mainly recognizes the C-terminus of nisin to prime the substrate into the binding site for cleavage.

The *Sa*NSR is highly specific for nisin, whereas the BceAB-type transporter *Sa*NsrFP has a broader substrate specificity. Recently, it was shown, that beside nisin A the transporter recognizes also nisin H and gallidermin as substrates and confers resistance in *L. lactis* NZ9000 (Reiners, Lagedroste et al. 2017). Like other BceAB-type transporters *Sa*NsrFP is conferring resistance to AMPs by expelling them from the membrane, which was shown in a peptide release assay for nisin A. Unlike the other BceAB-type transporters, *Sa*NsrFP confers resistance to nisin A without the associated TCS *Sa*NsrRK by expelling the lantibiotic

from the membrane and inhibiting pore formation (Reiners, Lagedroste et al. 2017). The importance of the N-terminus, especially of lanthionine-rings A/B, was shown for substrate recognition by *Sa*NsrFP and also observed for the transporter CprABC (Suarez, Edwards et al. 2013).

The ABC transporter *Sa*NsrFP has presumably a similar stoichiometry as other BceAB-type transporter of two NBPs (NsrF) and one TMP (NsrP). Within this complex, the ATPase NsrF hydrolyses ATP to provide the energy for confirmation change in the TMP, whereas NsrP forms the translocation pathway. Similarly, to BceB an EDC of 221 aa is present between TMH 7 and 8, that is assumed to be important for substrate sensing (Clemens, Zaschke-Kriesche et al. 2017). The overall nisin resistance mechanism conferred by *Sa*NsrFP and *Sa*NSR is suggested to be cooperative, where *Sa*NSR is the first line of defence. It lowers the antimicrobial activity of nisin by C-terminal processing, which results in $nisin_{1-28}$. This variant is still recognized by the transporter *Sa*NsrFP, which exports the lantibiotic from the membrane as a second line of defence (Clemens, Zaschke-Kriesche et al. 2017, Reiners, Lagedroste et al. 2017).

2 Aims of the thesis

Nisin is the best-studied lanthipeptide regarding its biosynthesis, modification, secretion and maturation. Starting in the 90s, many studies investigated the production of nisin originated from the biosynthetic gene cluster, that harbours the genetic determinants for the nisin system (Kaletta and Entian 1989, Dodd, Horn et al. 1990, Horn, Swindell et al. 1991, Kuipers, Beerthuyzen et al. 1993). These studies discovered, that the nisin system comprises of 11 genes, which encode for the nisin precursor peptide (NisA) and the proteins that are relevant for nisin modification, secretion, maturation, regulation as well as immunity.

Since then, much effort was made to investigate the nisin modification mechanism on a molecular level (Koponen, Tolonen et al. 2002, Okeley, Paul et al. 2003, Li, Yu et al. 2006, Lubelski, Overkamp et al. 2008, Lubelski, Khusainov et al. 2009, Khusainov, Heils et al. 2011, Garg, Salazar-Ocampo et al. 2013, Ortega, Hao et al. 2015, Repka, Hetrick et al. 2018). The identification of a conserved recognition motif within the leader peptide of NisA enabled us to study the interaction of modification enzymes with its dedicated substrate (Mavaro, Abts et al. 2011, Plat, Kluskens et al. 2011, Abts, Montalban-Lopez et al. 2013). In 2017, the *in vitro* assembly of the modification enzymes allowed a structural glimpse on the modification complex in solution and gave further evidence for the proposed multimeric, membrane-localized lanthionine synthetase complex (Siegers, Heinzmann et al. 1996, Reiners, Abts et al. 2017).

However, we lack of detailed information about the ABC transporter NisT and its role in the ensemble of NisB, NisC and NisA. *In vivo* studies of NisA secretion indicated, that NisT can function independently of the modification enzymes, but only in the presents of NisB and NisC an efficient secretion was observed (Kuipers, de Boef et al. 2004, van den Berg van Saparoea, Bakkes et al. 2008, Lubelski, Khusainov et al. 2009). The exact crosstalk between NisT, NisB and NisC in present of the substrate NisA remains elusive until now.

The overall aim of the thesis was to investigate the secretion and processing of NisA. Therefore, one aim was to focus on NisA secretion in presents of NisT and determine a putative recognition motif within the leader peptide of NisA. Next, the importance of NisB and NisC for the secretion process was investigated. Finally, the major aim of the thesis was the biochemical characterisation of the LanT-type transporter NisT to get insights into the molecular mechanism of lanthipeptide secretion.

Although, the maturation of NisA by the serine protease NisP is described in general, the substrate specificity and reaction kinetics remain ambiguous or unknown (van der Meer, Polman et al. 1993, Plat, Kluskens et al. 2011). Hence, one additional aim of the thesis was to investigate the maturation reaction and biochemical characterise the leader peptidase NisP. This would further enlighten the mechanism behind the maturation process of NisA in respect of future applications of the peptidase to activate lanthipeptides.

3 Publications

The chapter I, II and III are manuscripts to be submitted, whereas the chapter IV, V and VI of the present thesis were published in peer-review journals. In addition the following publications are emanated from the present thesis.

Khosa, S.; **Lagedroste, M.**; Smits, S. H. J. (2016). "Protein Defense Systems against the Lantibiotic Nisin: Function of the Immunity Protein NisI and the Resistance Protein NSR." Front Microbiol **7**: 504.

AlKhatib, Z.; **Lagedroste, M.**; Zaschke,J.; Wagner, M.; Abts, A.; Fey, I.; Kleinschrodt, D.; Smits, S. H. J. (2014). "The C-terminus of nisin is important for the ABC transporter NisFEG to confer immunity in Lactococcus lactis." Microbiologyopen **3**(5): 752-763.

AlKhatib, Z.; **Lagedroste, M.**; Fey, I.; Kleinschrodt, D.; Abts, A.; Smits, S. H. J. (2014). "Lantibiotic immunity: inhibition of nisin mediated pore formation by NisI." PLoS One **9**(7): e102246.

3.1 Chapter I – The maturation of lanthipeptides

Title A structural view on the maturation of lanthipeptides

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#Authors contributed equally

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A structural view on the maturation of lanthipeptides

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Summary

Lanthipeptides are ribosomally synthesized and post-translationally modified peptides, which display diverse bioactivities (e.g. antifungal, antimicrobial and antiviral). One characteristic of these lanthipeptides is the presence of thioether rings, which are termed (methyl-)lanthionine rings. The modifications are installed by corresponding modification enzymes in a two-step modality. First, serine and threonine residues are dehydrated followed by a subsequent catalyzed cyclization reaction, where the dehydrated serine and threonine residue are condensate with a cysteine residue. The dedicated enzymes are encoded by one or two genes and the classification of lanthipeptides is pending on them. The modification steps form the basis of distinguishing the different classes of lanthipeptides and further reflecting also important mechanistic differences. Here, we will summarize recent insights into the mechanisms and the structures of the participating enzymes focusing on these two core modification steps – dehydration and cyclization.

1. Introduction

Ribosomally synthesized and post-translationally modified peptides (RiPPs) are a large family of natural compounds with diverse biological functions [1]. Among the RiPPs, lanthipeptides form the largest sub-family [2], which is characterized by the presence of multiple lanthionine or (methyl-)lanthionine rings ((Me)Lan), that restrict the conformational flexibility of the peptides and give rise to their high biological stability [3, 4]. Common to lanthipeptides is the ribosomal biosynthesis of a precursor peptide that is composed of an N-terminal leader peptide (LP) and a C-terminal core peptide (CP), termed LanA [1, 5]. While all post-translational modifications (PTMs) are introduced only in CP, the LP targets the lanthipeptide to its PTM machinery and keeps the peptide in an inactive state prior to translocalization [6-8]. The fully modified lanthipeptide is termed mLanA [1]. After all PTMs have been introduced the LP is proteolytically removed either before or after secretion to the extracellular space and the active lanthipeptide is released [7, 9, 10].

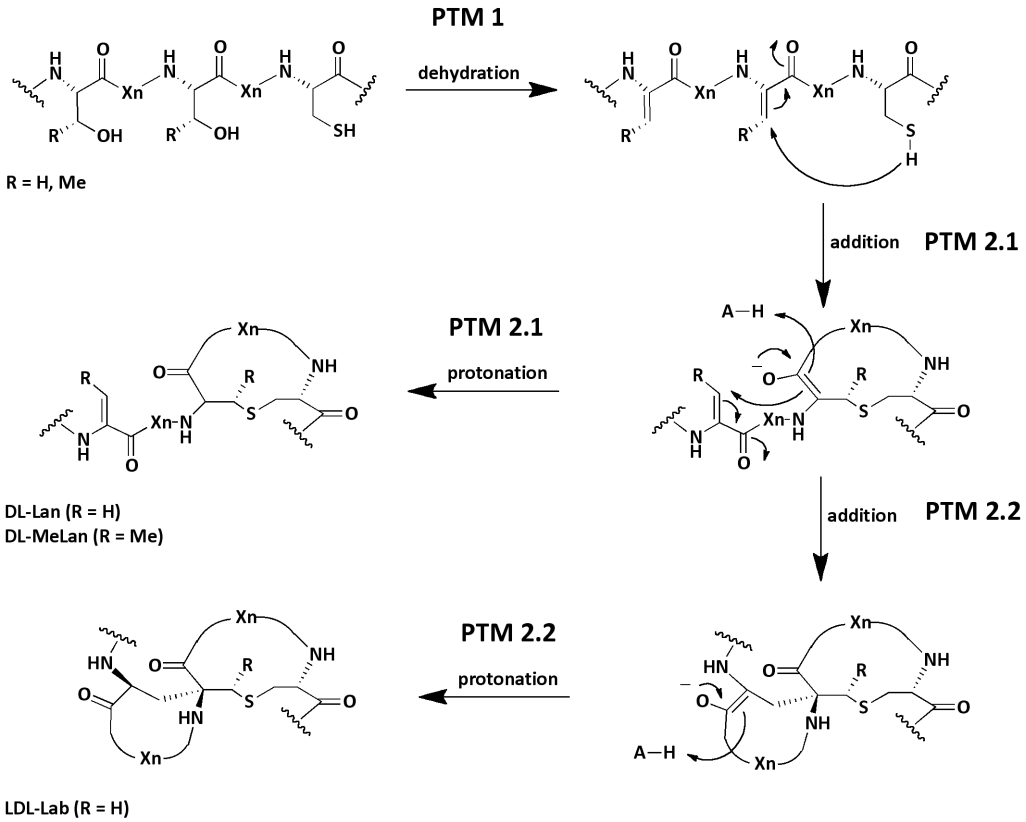

Figure 1: Reaction and structure of common PTMs in lanthipeptides.
During a first modification step (PTM 1) a dehydratase catalyze the dehydration of serine and threonine residues (Dha, Dhb). In a second step (PTM 2.1) a cyclase catalyzes the Michael-type addition of a cysteine residue to a dehydrated amino acid. Within the active center an acid (H-A, e.g. His) protonates the enolate. Finally, the Lan or MeLan ring are formed. In some lanthipeptides an additional condensation reaction (PTM 2.2) is catalyzed by the cyclase yielding a Lab amino acid. In the scheme the stereochemistry of the chiral center in the final products are exemplary (e.g. DL-Lan; but LL-Lan is also possible). The acronym X_n stands for n-quantity amino acids. The scheme is modified and based on [11]

In general, all lanthipeptides share at least two common PTMs. The first one is the dehydration of serine and threonine residues forming 2,3-didehydroalanine (Dha from Ser) and 2,3-didehydrobutyrine (Dhb from Thr) (Figure 1) [12-14]. This reaction is catalyzed by the dehydratase LanB or dehydratase domains depending on the classification of the lanthipeptide (see next section) [15-17 18, 19]. The second common PTM is the Michael-type condensation of a cysteine side chain and the dehydrated amino acids yielding *meso*-lanthionine (from Dha) or (3-methyl-) lanthionine (from Dhb) (Figure 1) [12-14, 20-22]. Additional to these two PTMs that are the foundation of lanthipeptides, many additional modifications like labionin (Lab) ring formation (Figure 1) [23, 24] or tailoring reactions such as halogenation of tryptophan residues, decarboxylation or acylation have been observed [25-34].

However, these reactions are not the topic of this review and will not be discussed any further and the reader is referred to excellent reviews covering these aspects [1, 23-38].
In 2013 a new nomenclature was suggested that subdivides lanthipeptides based on their modification machinery in four families, classes I to IV (Figure 2) [1].

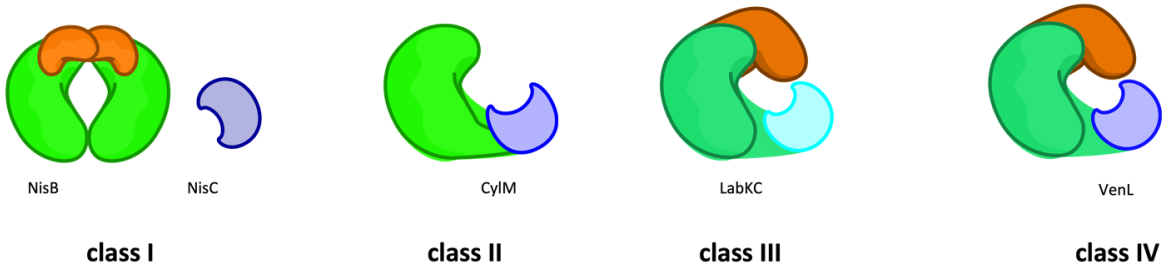

Figure 2:**Classes of lanthipeptides based on the modification machineries.**
The classification of lanthipeptides depends on their modification enzymes and their domain organization as well as those PTM mechanisms. In class I (e.g. NisB) the first PTM (dehydration) is catalyzed by a glutamylation/elimination-domain (green/orange). The dehydration in class II (e.g. CylM) take place in a kinase-domain (green). In class III (LabKC) and IV (VenL) this modification is catalyzed by a lyase/kinase-domain (dark green/dark orange). The second PTM (cyclization) are derived by the LanC enzyme (e.g. NisC; blue) or LanC-like domain (light blue), whereas in class III the LanC-like domain is independent from a zinc-ion (cyan). For further details see text.

In this review, we will follow this new nomenclature. Furthermore, we will restrict ourselves to the two common maturation steps that occur in the cytosol of lanthipeptide producing strains.

2. Lantibiotics – specialized lanthipeptides

The hallmark of lanthipeptides is the presence lanthionine or (methyl-)lanthionine rings. In cases that lanthipeptides possess antimicrobial activity they are called lantibiotics [1, 39]. However, other activities such as antifungal, antiviral, morphogenetic or antinociceptive have been described [24, 40-43]. Concerning the antimicrobial activity, which is mainly directed against Gram-positive bacteria, the target of most lantibiotics is the membrane and/or a specific receptor. For example is nisin, a lantibiotic produced by *Lactococcus lactis*, targeting the peptidoglycan precursor lipid II. Nisin contains five (Me)Lan rings, where the first two bind to the pyrophosphate moiety of lipid II and directly inhibit the cell wall synthesis. Additionally, nisin and lipid II form pores in the cell membrane of the target in a stoichiometry of four nisin and eight lipid II molecules [44-58]. Despite its usage in the food industry for almost 50 years [59], this dual mode of action explains why hardly any acquired resistances has been described in the literature.

3. The fist maturation step – the dehydration reaction

The major discriminators among the four classes of lanthipeptides are the lanthipeptide synthetases. Here, four different routes corresponding to the four subfamilies (LanB, LanM, LanKC and LanL) have evolved, which mainly differ in the mechanism of dehydrating Ser and Thr residues (Figure 2).

Class I family dehydratases (LanB) contain the well-studied NisB or SpaB enzymes that dehydrate nisin (NisA) or subtilin (SpaA) [12, 13]. NisB adopted a dimeric state in solution and interestingly interacted with the different maturation states of NisA and not only with its cognate substrate (unmodified NisA) in the low micromolar range [60]. Co-expression studies of NisB and the cyclase NisC without purification resulted in dehydration and cyclization of NisA indicating functional enzymes [15, 61-63]. In 2013, Garg *et al.* demonstrated *in vitro* activity of purified NisB by using extracts of *E. coli and* subsequently identified the cytosolic extract to be the key element for glutamylation of NisA [64]. Finally, the use of purified recombinant NisB and addition of glutamyl-tRNA^{Glu} derived from glutamyl-tRNA synthetase, tRNA^{Glu} and glutamate reconstituted *in vitro* activity and poly-glutamylated intermediates were identified by MS analysis [65]. This highlighted that the hydroxyl group of Ser and Thr in the CP of NisA became esterified with the side chain of glutamate, where a cognate tRNA is the glutamyl-donor. Then, the elimination of these activated residues result

in the dehydrated residues Dha and Dhb. Of course, this reconstitution allowed a detailed study of the catalytic activity and the identification of essential amino acids of this LanB dehydratase.

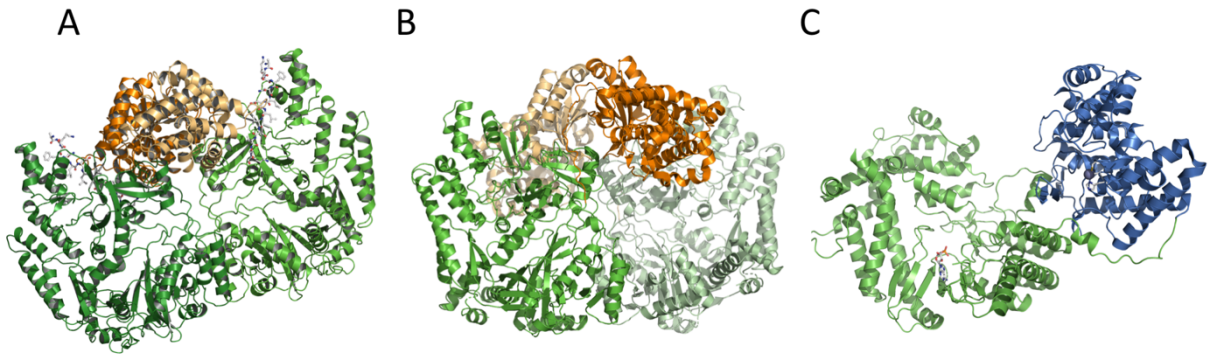

Figure 3: Comparison of LanB and LanM crystal structures.
(A) Crystal structure of dimeric NisB (PDB ID: 4WD9; [65]), the first example of a class I LanB dehydratase. The glutamylation domain is highlighted in green and light green, the elimination domain in orange and light orange. The part of the LP of NisA is shown in ball-and-stick representation. (B) Crystal structure of dimeric MibB (PDB ID: 5EHK; [66]). Color-coding is as in (A). (C) Crystal structure of CylM (PDB ID 5DZT; [67]), the first example of a LanM enzyme of class II lanthipeptides. The kinase-domain is shown in green and the cyclase-domain in blue. A bond AMP molecule within the kinase-domain is displayed as a ball-and-stick representation.

In 2015, the laboratory of Wilfred van der Donk also reported the crystal structure of NisB (Figure 3A). Similar to *in solution* observations, NisB crystallized as a dimer [65, 68]. Importantly, NisB was co-expressed with NisA and parts of the LP including the FNLD box, which is pivotal for the interaction with NisB, was visible in the final electron density (shown in ball-and-stick representation in Figure 3A). This resulted in a 2 : 2 stoichiometry of NisB : NisA, which is in contradiction to the *in vitro* data, which determined a 2 : 1 ratio of NisB : NisA using surface plasmon resonance [60]. In the crystal structure, the LP interacts with twisted ß-strand resulting in an antiparallel, four-stranded ß-sheet. Furthermore, NisB can be sub-divided into an N-terminal glutamylation domain (green and light green cartoons in Figure 3A of approximately 800 amino acid residues) and a C-terminal elimination domain (orange and light orange cartoons in Figure 3A of approximately 350 amino acid residues). This two-domain structural architecture was also found within MibB (Figure 3B), the class I LanB enzyme of NAI-107 [66]. Equally important, MibB like NisB required $tRNA^{Glu}$ to catalyze the dehydration reaction. Two further examples of related LanB enzymes, so-called split LanB (one protein is involved in aminoacylation and the other protein in the elimination of activated aa residues) [43, 69], also depend on the presence of $tRNA^{Glu}$ for dehydration, clearly

demonstrating that class I LanB enzyme use this rather unexpected mechanism to dehydrate Ser and Thr residues in the CP of lanthipeptides.

In contrast to NisB [65], MibB [66] was crystallized in the absence of a substrate and displayed the same overall dimeric architecture composed of an approximately 800 amino acids large N-terminal glutamylation domain (green and light green in Figure 3B) and an approximately 350 amino acid large C-terminal elimination domain (orange and light orange in Figure 3B). The absence of the natural substrate, allows a comparison with NisB to highlight structural changes that occur concomitant with substrate binding (Figure 4).

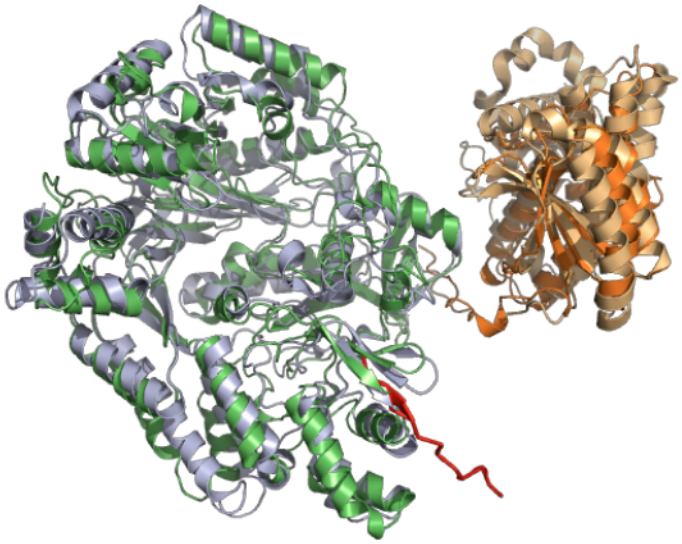

Figure 4: Superposition of NisB and MibB crystal structures.
The superposition is based on the elimination domain (residues 713- 961 of NisB and 800- 1048 for MibB with a RSMD of 3.6 Å over 618 Cα atoms). The glutamylation domain is shown in green (NisB) and gray (MibB), while the elimination domain is shown in light orange (NisB) and orange (MibB). For simplicity only monomers of NisB and MibB are shown. The bound LP of NisA in NisB is shown in red. NisB (PDB ID: 4WD9; [65]), MibB (PDB ID: 5EHK; [66]).

As evident from the structural superposition of both proteins using the C-terminal elimination domain as anchor point, the glutamylation domain undergoes a translational and rotational motion resulting in a more compact shape of the LanB enzyme (Figure 4). This transition might be reminiscent of the conformational selection proposed for class II LanM enzymes. Here, the LanM enzyme is in equilibrium between an inactive and an active conformation. In the absence of substrate, more precise the LP, the equilibrium is shifted towards the inactive state, while binding of the LP shifts it towards the catalysis-competent state. This model is supported by experiments, in which the LP was added *in trans* or fused

to the LanM enzyme [70-72]. In both cases, the isolated CP was modified although the fusion of the LP resulted in a more efficient system. Whether a similar scenario holds for class I LanB enzymes as suggested by the structural comparison of NisB and MibB has to be verified experimentally.

Structural information is also available for class II LanM enzymes [73, 74] that encode the dehydration, elimination and cyclization domains on a single gene (Figure 3C). In clear contrast to LanB enzymes, these dehydratases require ATP and Mg^{2+} as cofactors as shown experimentally for LctM in 2005 [75]. The crystal structure of CylM [67] revealed the expected two-domain organization, an N-terminal dehydration domain (green in Figure 3C) and a C-terminal cyclization domain (blue in Figure 3C), which resembled the structure of NisC [76], a class I LanC enzyme (see below). Not anticipated, the N-terminal domain displayed structural similarities to eukaryotic lipid kinase domains bearing a novel, secondary structure topology.

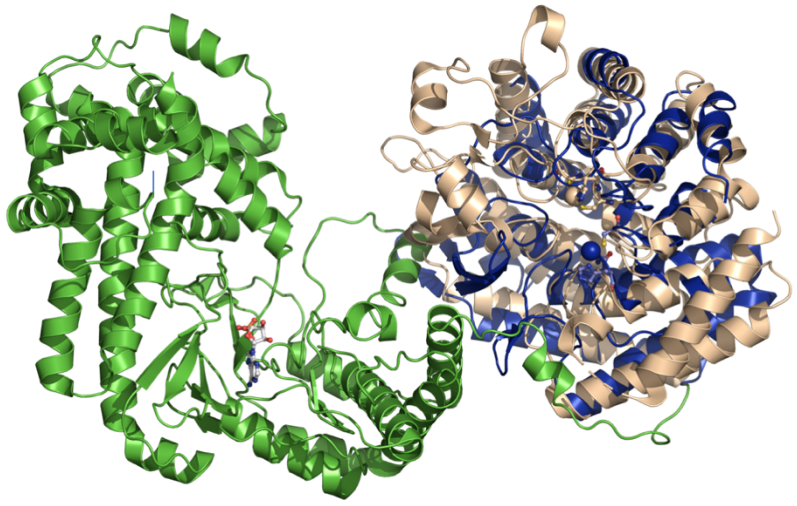

Figure 5: Superposition of NisC and CylM crystal structures.
The superposition is based on the cyclase domain of CylM (residues 641-992 of CylM). NisC is shown in blue and CylM in green (dehydratase domain) and light brown (cyclase domain). The bound AMP of CylM is shown in ball-and-stick representation. The two Zn^{2+} ions and the coordination amino acids are shown as blue sphere (NisC) and light brown sphere (CylM). The coordination amino acid residues are highlighted in ball-and-stick representation in blue (NisC) and light brown (CylM). CylM (PDB ID 5DZT; [67]); NisC (PDB ID: 2G02; [76]).

The activation loops of Ser / Thr kinases including the P-loop are present as well as characteristic helices. Nevertheless, also a novel kinase activation domain is present, which function was determined by mutational studies. This obviously explains the dependence of LanM enzymes on ATP and Mg^{2+} and in contrast to LanB enzyme [64], the dehydration relies on phosphorylation of serine and threonine residues of the substrate, the presence of phosphorylated instead of glutamylated intermediates and the elimination of inorganic

phosphate [75]. However, only AMP was observed in the structure and conclusions on the molecular mechanism of LanM function are not available at the moment.

In general, share the cyclization domain of CylM and NisC a high degree of structural identity (Figure 5). This strongly suggests that the mechanism of cyclization is shared between LanC and LanM enzymes. Noteworthy, a SH2-like domain[76, 77], which is found in NisC and might be involved in substrate binding, is not present in CylM. There, an additional subdomain is included in the cyclization domain, that seem to be important for substrate binding [67].

Class III and class IV lanthipeptide modification enzymes, which have been discovered only recently, display a three-domain organization composed of a lyase and kinase domain and a C-terminal cyclization domain, which differs among the two classes (Figure 2) [23, 78, 79]. While the cyclase domain of LanL is apparently similar to the C-terminal domain of LanM enzymes or LanC and its activity clearly relies on Zn^{2+}, the cyclase of LanKC is apparently not Zn^{2+}-dependent as it does not contain the highly conserved residues that are required for the coordination of this ion. Thus, two classes depending on the ability to coordinate Zn^{2+} or not were defined and the generic names LanKC (Class III) or LanL (class IV) were introduced [40, 78]. Structural information is so far not available and insights into these lanthipeptide modification enzymes depend solely on genetic and functional data. Sequence analysis revealed similarities to Ser / Thr kinases and effector proteins from Gram-negative pathogens that catalyze the elimination of phosphorylated serine and threonine residues (phosphor-lyases) in the N-terminal part of the protein [80-82]. In contrast to class II LanM enzymes that are strictly ATP-dependent, the kinase domains of LanKC and LanL have no real specificity for the phospho-donor. Depending on the enzyme under investigation, specificities for GTP/dGTP, ATP, ATP/GTP/CTP/TTP or any NTP/dNTP was discovered [83-87]. However, based on the sequence similarities of the lyase and kinase domains, one can assume that the mechanism of phosphorylation and elimination is shared between LanKC and LanL enzymes. Another important aspect within class III lanthipeptides was the identification of another type of cyclization, the labionin or methyllabionin (MeLab) [23, 24]. After the initial condensation of a cysteine residue and Dha, the system undergoes another condensation reaction with a second Dha residue that results in the methylene moiety based ring, a (Me)Lab ring. However, one has to wait for structural insights into the LanKC family before final mechanistic conclusions can be drawn.

We have now a fairly detailed understanding of how the dehydration reactions are catalyzed in the different classes of lanthipeptides synthetases. Nevertheless, class I enzymes represent a special case as the dehydratase LanB and the cyclase LanC are separate expressed enzymes that can act on their own [62-64, 76, 88]. However, *in vivo* both enzymes are present. An elegant set of experiments using plasmid-based expression of the possible combinations of maturation enzymes demonstrated an astonishing inter-dependence. Here, ring formation and dehydration acted in concert resulting in protection of certain positions of dehydration with respect to ring formation [5, 15, 62-65, 76, 88-95]. This resulted in the proposal that a strict N- to C-directionality is operational in NisA maturation, suggesting that dehydration and ring formation is an intertwined process [65]. Consequently, such a directionality would also suggest a sort of channeling of the substrate that is bound to a LanB / LanC complex forcing the PTM reactions to start at the N-terminus and proceed all the way to the C-terminus before finalizing the maturation reactions. In clear contrast are *in vitro* studies of NAI-107 (MibA, classI), which suggested the absence of a N- to C-directionality, rather a C- to N-directionality, after dehydration of the N-terminus was observed [66]. This obviously raises the question whether a unique mechanism is operational and what the molecular ruler underlying these mechanisms actually is.

4. The second maturation step – the cyclization reaction

LanC enzymes or the cyclization domain of classes II – IV enzymes catalyze the thiolate (from Cys) attack to dehydrated aa, where they facilitate the regio- and stereoselectivity to form thioether rings with the correct ring topology. Although, the lanthionine ring formation can occur spontaneously at basic pH values (pH >7.5) the stereochemistry of Lan/MeLan is erroneous [8, 96, 97]. The analysis of purified LanC enzymes (NisC and SpaC) revealed the presence of stoichiometry amounts of Zn^{2+} suggesting that the metal ion plays an essential role in deprotonating the thiol group of the cysteine residue by decreasing the pK_a value of the cysteine side chain [98]. Such a deprotonation or at least polarization accelerates the rate of Michael-type addition in the formation of the (Me)Lan rings. A detailed glimpse on the mechanism was possible, as the crystal structure of NisC was reported in 2006 [76]. It displays an α,α toroid consisting of six helices each, a SH2-like domain and one Zn^{2+} ion (Figure 6). The Zn^{2+} ion is coordinated by two highly conserved cysteine residue (Cys284 and Cys330) and one histidine residue (His331).

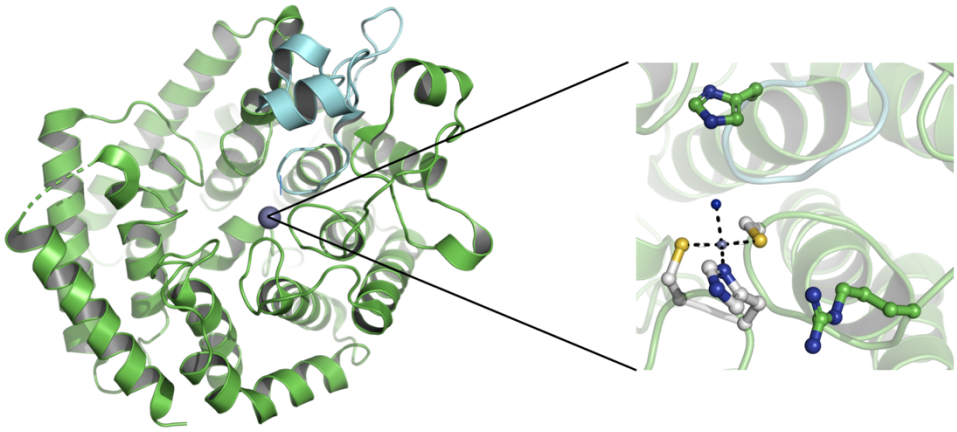

Figure 6: Crystal structure of NisC.
Left: The overall crystal structure (PDB ID: 2G02; [76]) comprises an α,α toroidal fold (green) and an domain extension. This extension a SH2-like domain (cyan) is located close to the catalytic center. Within the active site a Zn^{2+} ion, shown as a gray sphere, is bond. Right: Zoom-in into the active site of NisC. Residues important for the coordination of the Zn^{2+} ion (Cys284, His330 and Cys331) or function (His212 and Arg284) are shown in ball-and-stick representation.

Additional the tetragonal coordination sphere is complemented by a water molecule (inset of Figure 6). The presence of the SH2-like domain suggests that this domain interacts with dehydrated NisA, however there are no experimental evidence supporting this hypothesis and the functional role of this domain remains elusive. Based on the crystal structure of NisC, some residues conserved among LanC proteins were mutated [88]. There, the mutation of Cys284, Cys330 and His331 lead to inactive NIsA. Interestingly, the ability to bind Zn^{2+} was preserved by mutating other active site residues (e.g. mutation H212N, H212F and D141N) but no cyclization was detected. Thus, we slowly obtain a mechanistic picture of how NisC, but also LanC proteins in general, guide the formation of lanthionine rings. These insights will likely also hold for the cyclization domains of class II and class IV enzymes due to the structural conservation (Figure 5) or the conservation of residue identified to be essential for Zn^{2+} coordination or enzyme function. Nevertheless, we still do not have structural information of the LanA - LanC complex and only this information will result in a final and complete picture.

5. A concerted action during maturation

The individual domains of LanKC and LanL enzymes are capable of catalyzing their individual reactions also in the absence of the other domains [99]. This is especially holds true for class I enzymes. However, early on, functional studies based on co-immunoprecipitation, yeast two hybrid approaches or mutational studies demonstrated that at least LanB and LanC act synergistically [90, 100, 101]. Moreover, a maturation complex consisting of not only NisB and NisC but also the ABC transporter NisT apparently exists during the modification of NisA [100]. Further support of a concerted action came from studies of subtilin, which suggested also the presence of such a complex composed of the dehydratase SpaB, the cyclase SpaC and the ABC transporter SpaT [101].

First insights into the assembly and architecture of a full class I lanthipeptide maturation complex was obtained for the lanthipeptide nisin. Reiners *et al.* [68] used purified components to assemble the NisB / NisC / NisA maturation complex *in vitro*. Using size exclusion chromatography combined with multi-angle light scattering (SEC-MALS), they demonstrated that the complex was composed of a dimer of the dehydratase NisB, a monomer of the cyclase NisC and one molecule of the substrate, NisA resulting in a stoichiometry of 2 : 1 : 1 and a molecular weight of approximately 291 kDa. Importantly, the formation of the maturation complex was strictly dependent on the presence of the – FNLD – box within the LP as shown previously by *in vivo* and *in vitro* studies. Mutation of the four amino acids of the – FNLD – to – AAAA – completely prevented complex assembly [60, 91, 93, 94, 102, 103]. From a mechanistic point of view it was also important that a molecular signal was identified in this study that triggered disassembly of the maturation complex. Using a series of (Me)Lan ring mutants, e.g. Cys-Ala exchanges that prevented ring formation at the corresponding position, proved that the presence of the last, C-terminal MeLan ring represented the ‘disassembly signal’. This obviously goes nicely in hand with the *in vivo* situation as a maturation complex should continue the PTM reaction and only release the fully modified product. In other words, the maturation complex is capable of reading out the stage of modifications and only the terminal modification state, the fully modified product is released, ready to be secreted by the cognate ABC transporter. Of course the exact molecular role of the transporter within such a maturation complex is currently completely unknown and requires further experiments addressing its precise role.

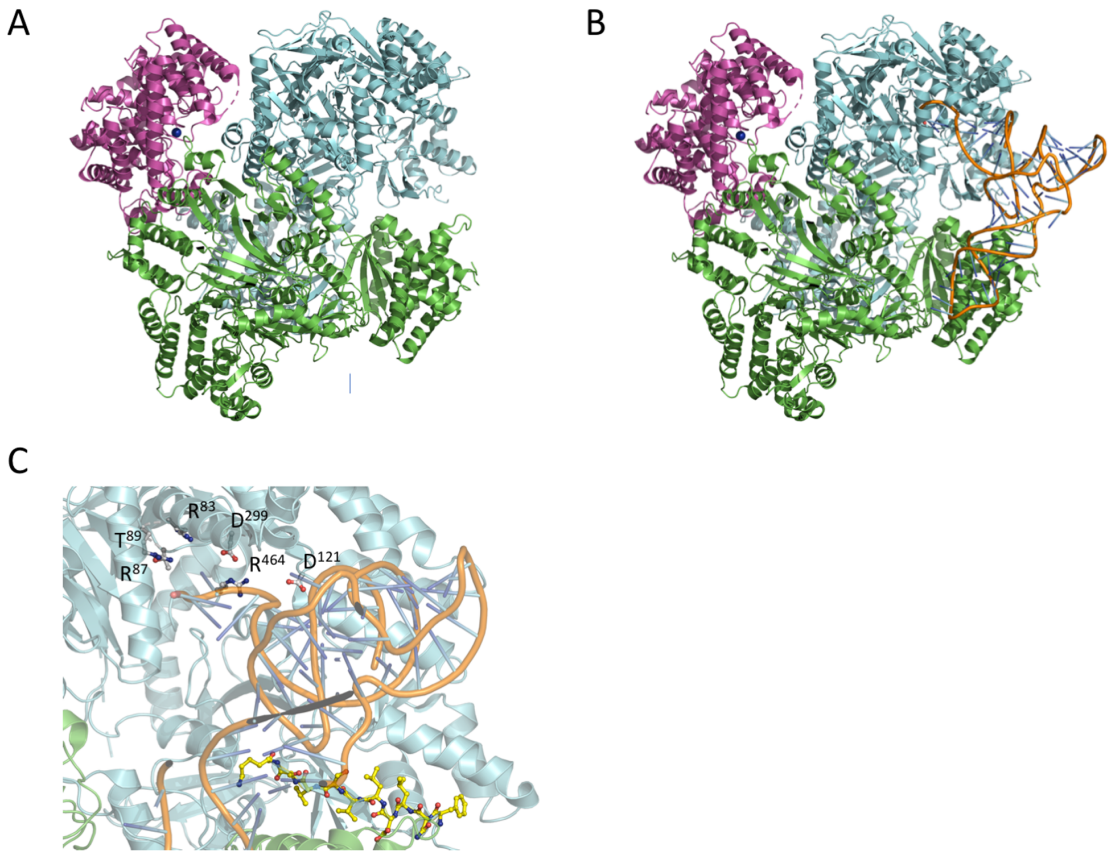

Figure 7: Composition of the NisA maturation complex NisB and NisC and tRNA docking.
(A) Crystal structures of NisC and NisB docked into the SAXS envelope [68]. (B) Docking of $tRNA^{Glu}$ into the NisA maturation complex composed of dimeric NisB (cartoon representation in green and cyan) and monomeric NisC (cartoon representation in magenta). The LP bound to NisB is shown in yellow ball-and-stick representation. (C) Zoom-in into the tip region of bound $tRNA^{Glu}$. Residues of NisB [64, 65, 93], which resulted in abolished dehydration upon mutation (Arg87, Thr89, Asp121, Asp299 and Arg464), are highlighted in ball-and-stick representation.

Moving one step further, small-angle X-ray scattering (SAXS) was used to produce a low resolution envelope that could be used to dock the individual high resolution crystal structures of NisB and NisC into the SAXS envelope (Figure 7A) [68]. A comparison of apo-NisB and NisA-saturated NisB suggested the presence of a tunnel and therefore provided an idea for the actual substrate-binding site within the complex. This allowed therefore a first molecular glimpse on the molecular architecture of a maturation complex of a class I lanthipeptide (Figure 7A).

Following the protocol of Ortega *et al.*,[65] we docked the crystal structure of $tRNA^{Glu}$ (extracted from PDB entry: 1N78) using the HDOCK server (http://hdock.phys.hust.edu.cn) employing standard settings [104]. The proposed tertiary complex is shown in Figure 7B. Interestingly, the $tRNA^{Glu}$ binding sites were similar in isolated NisB dimer and the

maturation complex. Mapping residues, which resulted in impaired functionality, clustered around the $tRNA^{Glu}$-binding site (Figure 7C) suggesting that the model is of functional significance. Additional residues that were identified in mutational studies were also mapped on the proposed complex [64, 65, 93]. Noteworthy, the mutation of arginine and aspartate residues leading to a complete loss of dehydratase activity in NisB mapped in the close vicinity of the bound $tRNA^{Glu}$ (Figure 7C). Obviously, this *in silico* complex requires experimental verification. Nevertheless, it represents a starting point to design such experiments, which might help to understand the molecular mechanism by which the nisin maturation complex and eventually other maturation complexes of class I lanthipeptides operate.

6 Conclusions and Outlook

We have seen tremendous advances in our understanding of lanthipeptides, especially class I lanthipeptides (lantibiotics) on the genetic, functional and structural level. Here, we have focused mainly on the mechanistic and structural insights of the modification process. These findings have answered many questions, but some questions are still open and even new questions arose. Thus, it is for example important to understand the molecular coordination and timing of the maturation enzymes and its interplay with the exporter protein. Only then, the fundamental question of why maturations intermediates of the substrates are not secreted can be answered. Only a very detailed mechanistic understanding will allow to use these bacterial machineries for engineering and fine-tuning not only lanthipeptides, but also hybrid peptides on demand and most importantly on a rational basis.

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3.2 Chapter II – Structural investigations on the ABC transporter NisT

Title Initial crystallization trails prior to structure determination of NisT

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Further proportions:

Design of experiments, bioinformatics analysis, data analysis, writing the manuscript

Initial crystallization trails prior to structure determination of NisT

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Abstract

Lanthipeptides are ribosomally synthesized and post-translationally modified peptides containing the unusual amino acids dehydroalanine, dehydrobutyrine, lanthionine and methyl-lanthionine. One of the best-studied lanthipeptides is nisin, which is produced by the Gram-positive bacterium *Lactococcus lactis*. Nisin is synthesized as a precursor peptide (NisA) comprising of an N-terminal leader peptide and a C-terminal core peptide. The leader peptide is the enzyme recognition site for the modification enzymes NisB/NisC and acts as a secretion signal for the ABC transporter NisT. NisA is secreted by NisT in a proposed channelling mechanism via the modification complex of NisB and NisC. Although, the crystal structures of NisB and NisC are available, little is known about the exporter. Thus, a structural characterization of NisT would elucidate the mechanisms of NisA translocation via the modification complex.

In this study, a purification protocol for NisT was established and initial crystallization trails of detergent purified NisT were performed. Additionally, the bicelle approach was used to mimic a lipid-like environment to optimize the stability of NisT during crystallization trails. Finally, needle-shaped crystals of NisT were obtained and optimized to obtain diffracting crystals.

Introduction

The lanthipeptide nisin is a ribosomally synthesized and post-translationally modified peptide, which is produced as a precursor peptide (NisA) with an N-terminal leader peptide (LP) and a C-terminal core peptide (CP) (Kuipers, Beerthuyzen et al. 1993, Arnison, Bibb et al. 2013). Here, the LP is a signal sequence and recognition site for the modification enzymes and the exporter (Kuipers, Beerthuyzen et al. 1993, Kuipers, de Boef et al. 2004, Mavaro, Abts et al. 2011, Abts, Montalban-Lopez et al. 2013)). Additionally, the LP keeps the NisA inactive (van der Meer, Rollema et al. 1994). Within the CP the post-translational modifications (PTM) are installed, which are unusual amino acids (aa) such as didehydroalanine (Dha), didehydrobutyrine (Dhb) or (methyl-)lanthionine ((Me)Lan) (Newton, Abraham et al. 1953, Gross and Morell 1968, van der Meer, Polman et al. 1993).

Nisin is produced by the Gram-positive bacterium *Lactococcus lactis* (*L. lactis*), which harbours a biosynthetic gene cluster for the modification, secretion and maturation enzymes (Kuipers, Beerthuyzen et al. 1993). The ribosomal synthesized, unmodified NisA (uNisA) is first dehydrated by the dehydratase NisB. There, serine and threonine residues are modified to Dha and Dhb residues in a tRNA-depended glutamylation and elimination reaction (Karakas Sen, Narbad et al. 1999, Garg, Salazar-Ocampo et al. 2013, Ortega, Hao et al. 2015). Next, the cyclase NisC is catalysing the region- and stereospecific coupling of neighbouring cysteine residues to the dehydrated aa via an Michael-like addition (Koponen, Tolonen et al. 2002, Okeley, Paul et al. 2003, Li and van der Donk 2007). The enzymes catalyse the reaction in an alternating manner with a N- to C-terminus directionality yielding (Me-)Lan residues in NisA CP (Lubelski, Khusainov et al. 2009, Repka, Hetrick et al. 2018). The modified NisA (mNisA) is exported to the exterior by the ABC transporter NisT and the extracellular located serine protease NisP cleaves the LP off to release mature, active nisin (van der Meer, Polman et al. 1993, Quiao and Saris 1996).

All lanthipeptide exporters belong to the superfamily of ABC proteins, which can be found in all domains of life (Higgins 1992). Here, the bacterial ABC transporter comprise mainly of two domains: a transmembrane domain (TMD), which is the translocation pathway for the substrate and the nucleotide-binding domain (NBD), which binds and hydrolyses ATP to provide the energy for substrate translocation (Fath and Kolter 1993). One class of lanthipeptide exporter have a two-domain organization (TMD-NBD) and are termed LanT-type transporters (Figure 1A). Examples are the exporters of nisin (NisT), subtilin (SpaT) or

epidermin (EpiT) (Allgaier, Jung et al. 1986, Kaletta and Entian 1989, Klein, Schnell et al. 1992). The second class of lanthipeptide exporters have an additional N-terminal C39 peptidase (C39P) domain and belong to the group of ABC transporter of maturation and secretion (AMS) (e.g. NukT, LctT, or BovT) (Rince, Dufour et al. 1994, Sashihara, Kimura et al. 2000, Xiao, Chen et al. 2004).

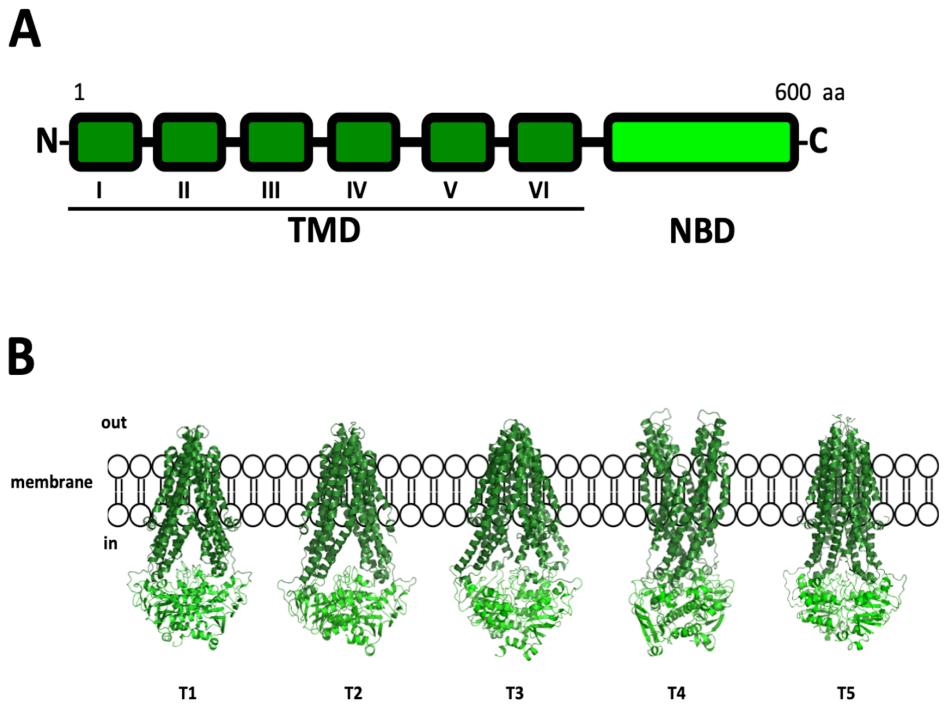

Figure 1: Model of lanthipeptide nisin ABC transporter NisT.
A) The two-domain organization of NisT (TMD-NBD). **B**) Homology model of NisT generated with Phyre2 (Kelley, Mezulis et al. 2015) based on six templates (T1) or on template TM287 (T2; PDB ID:3QF4; sequence identity 20%), PCAT1 (T3; PDB ID: 4RY2; sequence identity 21%), Sav1866 (T4; PDB ID:2HYD; sequence identity 22%) and McjD (T5; PDB ID: 4PI0 sequence identity 20%). Colour code: TMDs are displayed in dark green and NBDs in light green. The program PyMOL 2.1.1 was used to display the homology models in a cartoon representation.

All known lanthipeptide exporters function as dimers and show specificity towards its substrates, especially to the LP, as the translocation is LP-dependent (Klein, Kaletta et al. 1992, Schnell, Engelke et al. 1992, Bierbaum, Brotz et al. 1995, Kuipers, de Boef et al. 2004). Furthermore, for some exporters a membrane-localized, multimeric enzyme complex consisting of the modification and the exporter proteins is proposed (e.g. NisT, SpaT and NukT) (Siegers, Heinzmann et al. 1996, Kiesau, Eikmanns et al. 1997, Nagao, Aso et al. 2005). In general, little is known about the *in vitro* activity and the secretion process of lanthipeptide exporters. Only one *in vitro* study about the ATPase activity of NukT is available, which also investigated the role of the additional C39P domain during nukacin ISK-1 translocation (Zheng, Nagao et al. 2017). Moreover, there are none structural

information's of full-length lanthipeptide exporter available, which would allow an insight into the molecular mechanism of peptide export.

Some crystal structures of ABC transporter of the exporter type have been published and provided a deeper understanding of the substrate translocation process in these transporters. Interestingly, there are to our knowledge only two crystal structures of peptide exporter available. One crystal structure is from the microcin J25 exporter McjD from *Escherichia coli* (Choudhury, Tong et al. 2014) and the other from the AMS transporter PCAT1 from *Clostridium thermocellum* (Lin, Huang et al. 2015). Both ABC transporter are type I exporter and display a similar fold like other bacterial ABC transporters such as Sav1866 (Dawson and Locher 2006) or TM287/288 (Hohl, Briand et al. 2012). They share the same domain organization of TMD-NBD, except PCAT1 that has an additional N-terminal C39 peptidase domain. The TMD consists of six TM helices, which makes 12 TM helices for a functional dimer. The NBD are in both crystal structures in close contact, though in the crystal structure of PCAT1 the nucleotide is absent (Lin, Huang et al. 2015). In the crystal structure of McjD two AMP-PNP molecules are bound and mimic the ATP-bound state of the transporter (Choudhury, Tong et al. 2014). Although, a general alternating access model is proposed for the transport cycle of ABC transporters, some crystal structures like the outward-occluded from McjD hint to an intermediate state of the transport cycle (Holland 2011, Choudhury, Tong et al. 2014). These intermediate states give insights into the transition between the inward-facing and outward-facing conformation of ABC transporters.

The aim of the study was to get structural insights into the molecular mechanism of lanthipeptide export by determining the crystal structure of the ABC transporter NisT. Therefore, an expression and purification protocol was established to obtain a stable and homogeneous membrane protein sample for initial crystallization trails. A further optimization of the purification protocol and the usages of bicelle reconstitution of the membrane protein led to NisT crystals.

Results

Membrane protein purification

The lanthipeptide exporter NisT was purified by a two step purification protocol via affinity chromatography (AC) and size exclusion chromatography. For the AC purification a deca-histidine-tag (10H) with a putative TEV protease cleavage site (TEV) was cloned N-terminal of the *nisT* gene to purify the ABC transporter via immobilised metal ion chromatography (IMAC). NisT was homologous overexpressed in *Lactococcus lactis* (*L. lactis*) strain NZ9000, which was shown to be good overexpression host for homologous and heterologous overexpressed membrane proteins (Kunji, Slotboom et al. 2003).

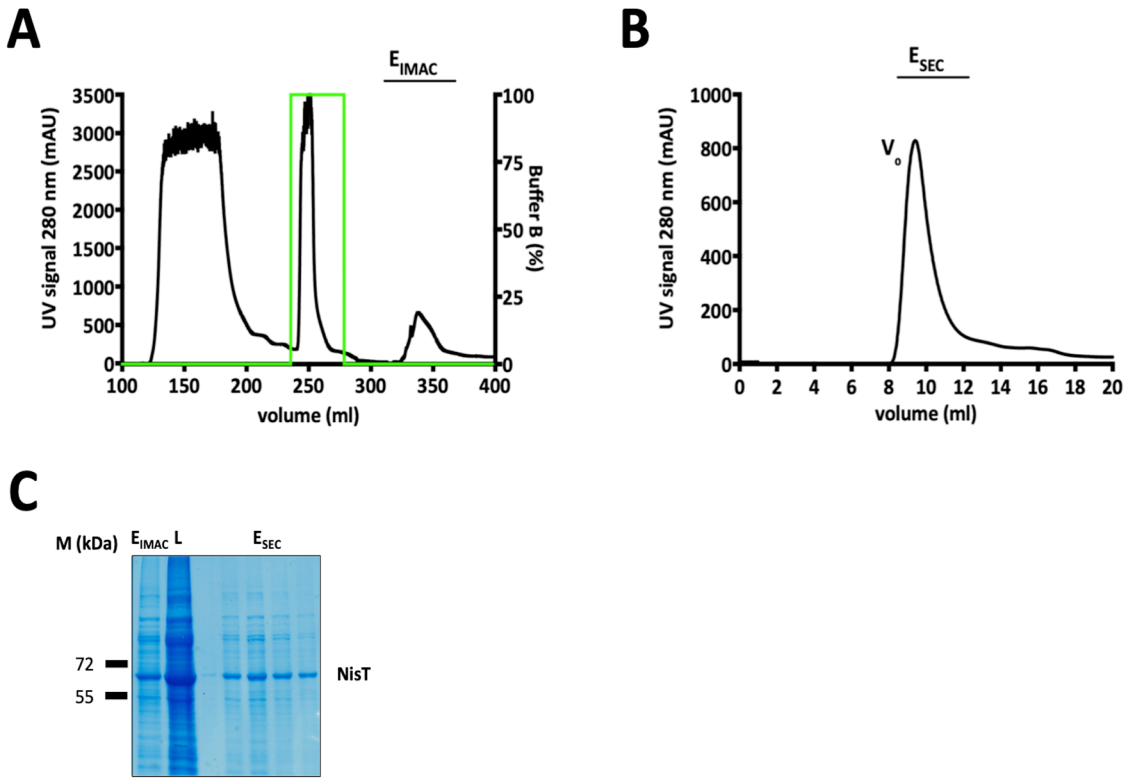

Figure 2: Purification of 10HTEVNisT.
A, **B**) Initial NisT was solubilized with 1% DDM and purified via IMAC and SEC. **B**) For SEC concentrated 10HTEVNisT (L) was applied onto a Superdex 200 10/300 column (25 mM HEPES pH 8, 100 mM NaCl, 10% glycerol, 0.015% DDM) and purification was monitored by UV absorption at 280 nm. The void volume of the column is indicated by V_0. **C**) The purity of main IMAC fractions (E_{IMAC}) and SEC fraction (E_{SEC}) was controlled by SDS-PAGE.

To overexpression NisT the NICE system (nisin-controlled gene expression) was used and gave sufficient amount of protein in the membrane fraction to perform initial solubilisation screens with the mild detergent n-dodecyl-β-D-maltopyranoside (DDM). There, an optimal total membrane protein concentration of 5-8 mg/ml was determined to obtain the best solubilisation results for 10HTEVNisT from homogenised membranes. For all screen

a solubilisation buffer of 100 mM HEPES pH8, 300 mM NaCl was used, where the amount of glycerol (5, 10, 20%), DDM (0.5, 1, 1.5%) and the time (0.5, 1, 2 h and over night) was varied.

Initial the IMAC purifications were performed with a HEPES buffer system (pH 8) supplemented with NaCl (50-250 mM), glycerol (5-20%) and DDM (0.03-0.1%). Additionally, the usage of HiTrap chelating columns (GE Healthcare) was superior to HiTrap IMAC columns (GE Healthcare) and was therefrom used for IMAC purifications. The best results regarding the yield were obtained, when 10HTEVNisT was eluted by one step with 250 mM imidazole or 50 mM histidine (Figure 2A; E_{IMAC}). Subsequently, the eluted membrane protein was desalted either by PD-10 desalting columns, anion exchange chromatography (aIEX) or SEC. There, the direct injection of the concentrated protein onto a SEC column gave the best results. A Superdex 200 10/300 GL column (GE Healthcare) was used, which was at least equilibrated with two times the column volume (CV). Here, a HEPES buffer system with lower ionic strength (10-150 mM) supplemented with 0.015-0.05% DDM or 0.01 LMNG was used (Figure 2B). The progress of purification was controlled by SDS-PAGE (Figure 2C). After SEC the detergent purified 10HTEVNisT was concentrated with Amicon ultrafiltration units (100 kDa MWCO; Millipore) to the highest possible concentration, under which the protein was stable for at least one day. Protein samples with concentrations ranging from 2.5 till 3.8 mg/ml were directly used for initial crystallization trails.

Initial crystallization trials of 10HTEVNisT

In the initial crystallization trails of 10HTEVNisT various purification attempts with different HEPES buffer systems were used. Some of these crystallization trials and the corresponding protein samples are presented in table 1. Unfortunately, the purification protocol did not provide a stable, sufficient concentrated protein sample to obtain conditions for crystal growths. Therefore, the purification protocol was further optimized.

Table1: Initial crystallization trails of detergent purified NisT.

The detergent purified protein from different purifications was used in initial crystallization screens provided by Qiagen or Hampton research. Some trails are exemplary shown in the following table.

screens	buffer composition			protein conc.	drop size	temp.	time	hits
	buffer	detergent	additive	mg/ml	µl	°C	days	
Additive screen	10 mM HEPES pH 8, 150 mM NaCl	0.01% LMNG	5% glycerol	3.8	0.2	12	>360	0
Classic I	10 mM HEPES pH 8, 150 mM NaCl	0.03% DDM	5% glycerol	2.5	0.2	12	>360	0
Classic II	10 mM HEPES pH 8, 150 mM NaCl	0.03% DDM	5% glycerol	2.5	0.2	12	>360	0
Core I	10 mM HEPES pH 8, 150 mM NaCl	0.03% DDM	5% glycerol	2.5	0.2	12	>360	0
Core II	10 mM HEPES pH 8, 150 mM NaCl	0.03% DDM	5% glycerol	2.5	0.2	12	>360	0
HR I	10 mM HEPES pH 8, 150 mM NaCl	0.03% DDM	5% glycerol	3.0	0.2	12	>360	0
HR II	10 mM HEPES pH 8, 150 mM NaCl	0.03% DDM	5% glycerol	3.0	0.2	12	>360	0
HR I	10 mM HEPES pH 8, 150 mM NaCl	0.01% LMNG	5% glycerol	3.8	0.2	12	>360	0
HR II	10 mM HEPES pH 8, 150 mM NaCl	0.01% LMNG	5% glycerol	3.8	0.2	12	>360	0
MbClass I	10 mM HEPES pH 8, 150 mM NaCl	0.01% LMNG	5% glycerol	3.8	0.2	12	>360	0
MbClass II	10 mM HEPES pH 8, 150 mM NaCl	0.01% LMNG	5% glycerol	3.8	0.2	12	>360	0
MemGold I	10 mM HEPES pH 8, 150 mM NaCl	0.01% LMNG	5% glycerol	3.8	0.2	12	>360	0
MemGold II	10 mM HEPES pH 8, 150 mM NaCl	0.01% LMNG	5% glycerol	3.8	0.2	12	>360	0
MemPlusMemSys	10 mM HEPES pH 8, 150 mM NaCl	0.01% LMNG	5% glycerol	3.8	0.2	12	>360	0
MemPlusMemSys	10 mM HEPES pH 8, 150 mM NaCl	0.03% DDM	5% glycerol	2.5	0.2	12	>360	0
Midas	10 mM HEPES pH 8, 150 mM NaCl	0.03% DDM	5% glycerol	2.5	0.2	12	>360	0
PEG I	10 mM HEPES pH 8, 150 mM NaCl	0.03% DDM	5% glycerol	2.5	0.2	12	>360	0
PEG II	10 mM HEPES pH 8, 150 mM NaCl	0.03% DDM	5% glycerol	2.5	0.2	12	>360	0
PGA	10 mM HEPES pH 8, 150 mM NaCl	0.03% DDM	5% glycerol	3.0	0.2	12	>360	0
pH clear	10 mM HEPES pH 8, 150 mM NaCl	0.01% LMNG	5% glycerol	3.8	0.2	12	>360	0

Optimized purification

In order to further optimize the purification protocol, a detergent solubilisation screen with various detergents was performed and analysed via dot blot (Figure 3A) (Ellinger, Kluth et al. 2013). Additionally, the buffer system was changed and a phosphate buffer system (pH 8) was used for solubilisation and IMAC. In the solubilisation screen the detergents LMNG (B4), FC-14 (C2) and FC-16 (C3) gave the best results. The aforementioned mild detergent LMNG was used in a phosphate buffer system (50 mM Na-phosphate buffer, pH 8, 100 mM KCl, 20% glycerol).

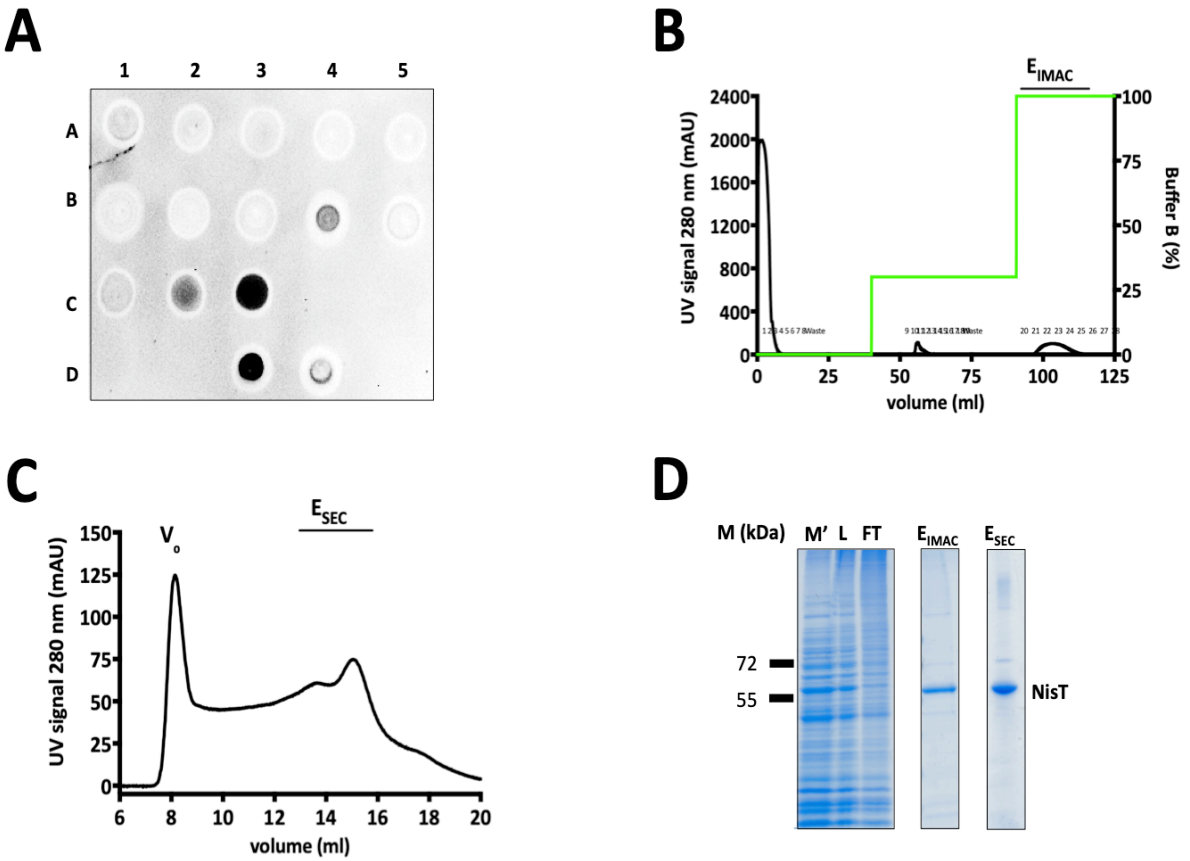

Figure 3: Optimized purification of 10HTEVNisT.
A) A small detergent solubilisation screen with 13 different detergents classes was performed (D3: 1% SDS). The supernatants were analysed via dot blot technique with an antibody against the $NisT_{NBD}$. After solubilisation screen 10HTEVNisT was solubilized with 1% LMNG (B4) and purified via **B**) IMAC and **C**) SEC. For SEC, 10HTEVNisT was applied onto a Superose6 10/300 column and purification was monitored by UV absorption at 280 nm. The void volume of the column is indicated by V_0. **D**) The purity of main IMAC fraction (E_{IMAC}) and SEC fraction (E_{SEC}) was controlled by SDS-PAGE.

The IMAC purification was performed in a phosphate buffer system (50 mM Na-phosphate buffer, pH 8, 100 mM KCl, 20% glycerol) supplemented with 0.01% LMNG. The solubilized membranes (conc. 5 mg/ml) were loaded over night onto a HiTrap chelating column (GE Healthcare) preloaded with zinc ions. The protein was eluted with 150 mM

histidine after an additional washing step (50 mM histidine) (Figure 3B). The main elution fractions containing 10HTEVNisT were pooled and concentrated with a Vivaspin20 (100 kDa MWCO) centrifugal concentrator (Sartorius AG). The concentrated protein samples was injected onto a Superose 6 10/300 GL column (GE Healthcare), which was equilibrated with 2xCV CAPS buffer system (25 mM CAPS pH 10, 100 mM KCl, 20% glycerol) supplemented with 0.01% LMNG (optional 2 mM DTT) (Figure 3C). The progress of purification was controlled by SDS-PAGE (Figure 3D). After SEC the detergent purified 10HTEVNisT was concentrated with a Vivaspin6 centrifugal concentrator (100 kDa MWCO; Sartorius AG) to 5-12 mg/ml. The protein purified with this optimized purification protocol showed increased stability over > four weeks at 8°C. The oligomeric state of the detergent purified 10HTEVNisT was analysed by blue native PAGE (BN-PAGE, Figure S1). There, in buffer supplemented with detergents such as DDM, LMNG and $C_{12}E_8$ 10HTEVNisT showed higher molecular weight species (> dimers). Only with the detergent FC-16 dimers of 10HTEVNisT with (ATP, ADP and AMP-PNP) and without nucleotides were observed. Nevertheless**,** protein sample purified with LMNG in CAPS buffer system were used for initial crystallization trails.

Optimized crystallization trials

The detergent purified NisT was used for two crystallization trails (screens MemGold I and MemGold II) and in parallel the same samples was reconstituted into bicelles. The bicelles mimic a lipidic environment for the membrane protein, which is forming a bilayer dics from the mixtures of a phospholipid (e.g. DMPC) and an amphiphile (e.g. the detergent CHAPSO) (Ujwal and Bowie 2011) (Figure 4A). To reconstitute 10HTEVNisT into bicelles two ratios of DMPC and CHAPSO were used (2.8:1 and 2.6:1). Prior, to the crystallization trail the membrane protein was premixed with the bicelle stock solution and incubated on ice. Subsequently, the sample was mixed with the reservoir solution of commercial available crystallization screens (Table 2) and incubated at RT (23°C) instead of 12°C. Interestingly, the protein/bicelle mixture showed an increased stability over one month at RT without aggregation and was used for further crystallization trails.

The initial screen MemGold II gave two hits (A3 and A4). There, needle shaped protein crystals appeared after 23 days (conditions A3), which showed UV signal. These conditions were used for a fine screening, where the parameter of the initial conditions were varied (e.g. the concentration of precipitant, salt and additives). Furthermore, the

concentration of detergent purified protein for bicelle reconstitution was reduced (4.0 to 5.6 mg/ml finial concentration in the protein/bicelle mixture). Additionally, additives such as the nucleotides ATP, ADP or AMP-PNP were added to the protein/bicelle mixture.

For the screen MemGold II A3 (PEG 1450 10%; 80 mM $MgSO_4$, 20 mM NaCl, 20 mM MES pH 6) the following optimized crystallization trials were prepared (Table 3). In the fine screen no. 1) and 2) the concentration of magnesium sulphate ($MgSO_4$) was varied (line A-D variation $MgSO_4$ 20/40/80/120 mM) as well as the PEG 1450 amount (row 1-6 variation of PEG 6/8/9/10/11/12%). In the fine screens no. 3) /4) and screens no 5.0) /5.1) have the same condition as screen no. 1)/ 2), but other purifications of 10HTEVNisT were used for bicelle reconstitution. In screen no. 6 the pH of the MES buffer (line A-D variation of pH 5/5.5/6/6.5 20 mM MES) and the salt concentration (row 1-6 variation of salt NaCl 0/10/20/40/80/160 mM) was changed. The amount of PEG1450 (11%) and the concentration of $MgSO_4$ (80 mM) were kept constant. Finally, in screen no. 7) conditions of fine screen no. 1 (lines A and B: A5/A6; B5/B6; C5/C6) and no. 6 (lines C and D: screen no. 6 C1-C6) were combined. All optimized fine screens were regularly monitored for crystal growth and the crystals were tested for diffraction at an X-ray source.

After 11 days needle shaped crystals of 10HTEVNisT were obtained from fine screen no. 1 and 6 (Figure 4B). Moreover, larger needle shaped crystals grown from screen no. 7 (condition C3-5), where 10 mM ADP was added to the 10HTEVNisT/bicelle mixture (Figure 4C). The diffraction of these crystals was tested at the in-house x-ray source. First, the crystals were tested to be salt crystals. Therefore, the following parameter were adjusted (detector distance 4 cm, angle Phi 0 and 90 °, duration 20 sec with 5° change). Next, the crystals were tested to be protein crystals with the following parameter (detector distance 4 cm, angle Phi 0 and 90 °, duration 20 sec with 5° change, after that detector distance 20 cm, angle Phi 0 and 90 °, duration 300 sec with 5° change). In the diffraction pattern no spots are visible (Figure 4D). Additionally, selected crystals of the same screen were tested at beamline of the European Synchrotron Radiation Facility (ESRF) in Grenoble (France) and resulted in non-diffracting crystals.

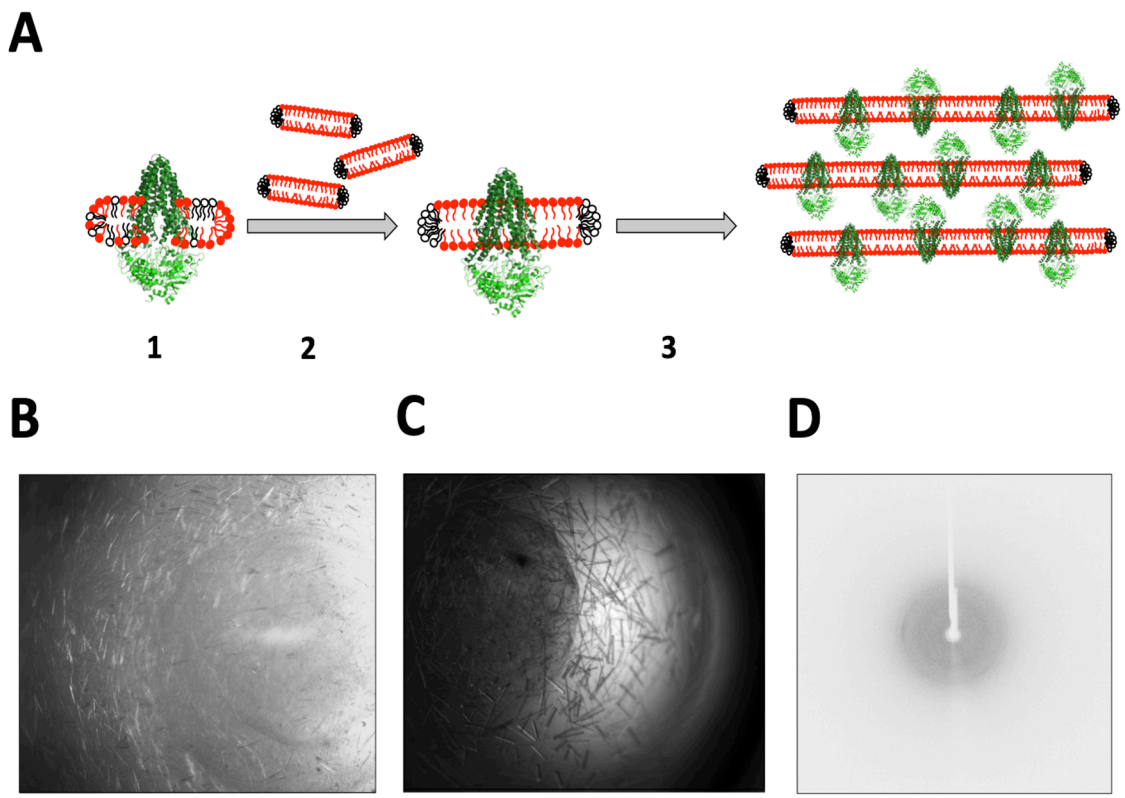

Figure 4: Crystals of NisT/bicelles.
A) Scheme of NisT/bicelle preparation. The detergents (red) purified NisT (**1**) was incubated with DMPC (white) /CHAPSO (red) mixture (2.6:1) in a ratio of 4:1 (**2**). NisT/bicelles under go phase transition with temperature change (**3**). Transition from dics-like to lamellar/multilamellar phases with possible protein/protein crystal contacts. **B**) Crystals (needles) of NisT/bicelles after 11 days. **C**) Crystals (needles) of NisT/bicelles and 10 mM ADP after 60 days. **D**) Diffraction pattern of needles from fine screen no. 7 C3.

Table2: **Initial crystallization trails of detergent purified NisT and NisT/bicelle mixture.**
The detergent purified protein from different purifications was used in initial crystallization screens provided by Qiagen or Hampton research.

screens	buffer composition			protein conc.	drop size	temp.	time	hits
	buffer	detergent	additive	mg/ml	µl	°C	days	
MemGold I	50 mM CAPS pH 10, 100 mM KCl	0.01% LMNG	20% glycerol	5.5; 11	0.2	12	>360	0
MemGold I	50 mM CAPS pH 10, 100 mM KC	0.01% LMNG	20% glycerol	7.8	0.2	RT	>360	0
MemGold I	50 mM CAPS pH 10, 100 mM KC	0.01% LMNG	20% glycerol	8.2	0.2	RT	>360	0
MemGold II	50 mM CAPS pH 10, 100 mM KC	0.01% LMNG	20% glycerol	5.5; 11	0.2	12	>360	0
MemGold II	50 mM CAPS pH 10, 100 mM KC	0.01% LMNG	20% glycerol	7.8	0.2	RT	>360	0
MemGold II	50 mM CAPS pH 10, 100 mM KC	0.01% LMNG	20% glycerol	8.2	0.2	RT	23;60	2
MemStartMemSys	50 mM CAPS pH 10, 100 mM KC	0.01% LMNG	20% glycerol	8.2	0.2	RT	>360	0
PEG I	50 mM CAPS pH 10, 100 mM KC	0.01% LMNG	20% glycerol	7.8	0.2	RT	>360	0
PEG I	50 mM CAPS pH 10, 100 mM KC	0.01% LMNG	20% glycerol	8.2	0.2	RT	>360	0
PEG II	50 mM CAPS pH 10, 100 mM KC	0.01% LMNG	20% glycerol	7.8	0.2	RT	>360	0
PEG II	50 mM CAPS pH 10, 100 mM KC	0.01% LMNG	20% glycerol	8.2	0.2	RT	>360	0
pH clear	50 mM CAPS pH 10, 100 mM KC	0.01% LMNG	20% glycerol	7.8	0.2	RT	>360	0
pH clear	50 mM CAPS pH 10, 100 mM KC	0.01% LMNG	20% glycerol	8.2	0.2	RT	>360	0

Table3: Optimized crystallization trails of NisT/bicelle mixtures.
The detergent purified protein from different purifications was used in optimized screens (24-well plates).

screens	buffer composition			protein conc.	drop size	temp.	time	hits
	buffer	detergent	additive	mg/ml	µl	°C	days	
No. 1	25 mM CAPS pH 10, 100 mM KCl	0.01% LMNG	20% glycerol bicelles DMPC/CHAPSO (2.6:1)	4.0	2	RT	10;60	2-6
No. 2	25 mM CAPS pH 10, 100 mM KCl	0.01% LMNG	20% glycerol bicelles DMPC/CHAPSO (2.6:1)	4.5	2	RT	10;60	2-6
No. 3	25 mM CAPS pH 10, 100 mM KCl	0.01% LMNG	20% glycerol bicelles DMPC/CHAPSO (2.6:1)	5.6	2	RT	8;60	-2;6
No. 4	25 mM CAPS pH 10, 100 mM KCl	0.01% LMNG	20% glycerol bicelles DMPC/CHAPSO (2.6:1)	5.0	2	RT	11	2
No. 5.0	25 mM CAPS pH 10, 100 mM KCl	0.01% LMNG	20% glycerol bicelles DMPC/CHAPSO (2.6:1)	4.6	2	RT	7	12
No. 5.1	25 mM CAPS pH 10, 100 mM KCl	0.01% LMNG	20% glycerol bicelles DMPC/CHAPSO (2.6:1) + 3.3 mM ATP	4.6	2	RT	7;10	12
No. 6.0	25 mM CAPS pH 10, 100 mM KCl	0.01% LMNG	20% glycerol bicelles DMPC/CHAPSO (2.6:1)	5.3	2	RT	7	8
No. 6.1	25 mM CAPS pH 10, 100 mM KCl	0.01% LMNG	20% glycerol bicelles DMPC/CHAPSO (2.6:1) + 3.3 mM ATP	5.3	2	RT	7	12
No. 7	25 mM CAPS pH 10, 100 mM KCl	0.05% DDM	20% glycerol bicelles DMPC/CHAPSO (2.6:1 and 3.0:1), 2 mM DTT, 10 mM ADP/ AMP-PNP	5.0	2	RT	>60	3

Discussion

In many biological processes, membrane proteins play an important role as transporter, signal transducer or channels to enable any cell the commutation with its surrounding environment. There, especially ABC transporters are important for the unidirectional transport of substrates such as nutrients, drugs or ions against a concentration gradient across a membrane (Higgins 1992). To study the molecular mechanism of an ABC transporter it is prerequisite to determine the 3D structure by using methods such as X-ray crystallography, NMR spectroscopy and electron microscopy (3DEM).

The X-ray crystallography was the method to elucidate the structure of the modification enzymes of the nisin system. In 2006, the crystal structure of the cyclase NisC gave the first insights into the modification mechanism of the precursor peptide NisA and how the formation of lanthionine and methyllanthionine rings is catalysed on a molecular level (Li, Yu et al. 2006). In 2016, a high resolution structure of the dehydratase NisB provide a novel mechanism of PTM based on the NisB co-crystal structure with its conjugated substrate NisA (Ortega, Hao et al. 2015). The two-domain organization of a glutamylation and elimination domain enabled to postulate a tRNA-depended dehydration mechanism for the class I lanthipeptide modification enzymes (LanB proteins), which differs from the nucleotide-depended mechanism of class II-IV modification enzymes (LanM, LanKC and LanL) (Goto, Li et al. 2010, Voller, Krawczyk et al. 2012, Dong, Tang et al. 2015). A SAXS analysis of the *in vitro* assembled modification complex of NisA, NisB and NisC allowed a structural view on a lanthipeptide synthetase complex, which was proposed in 1996 (Siegers, Heinzmann et al. 1996, Reiners, Abts et al. 2017). Nevertheless, we lack of structural information about the ABC transporter NisT, which is part of the multimeric lanthipeptide synthetase complex.

First insights into a the structure of the ABC transporter NisT are possible by using bioinformatics tools like SWISS-MODEL (Arnold, Bordoli et al. 2006), i-TASSER (Roy, Kucukural et al. 2010) or Phyre2 (Kelley, Mezulis et al. 2015) to compile homology models. In latter case a homology model is build based on the submitted amino acid sequence and its homologies to generate a validated evolutionary or statistically profile. This profile is compared to known structures in databases and a model is constructed based on the alignment of this templates. The web tool Phyre2 was used to build a homology model of NisT, which is based on six templates of known ABC transporter structures (Figure 1B; model T1 and Table S4). The confidence of the modelling was >90% over all 600 amino acid

residues of the aa sequence from NisT. All used templates share 18-21% sequence identity with NisT and belong to the superfamily of ABC transporters. The topology of NisT are six TM helices and a cytosolic NBD (Figure S1). Furthermore, they all belong to the type I exporter and share a similar fold as well as domain organisation. Additionally, homology models of NisT were created by using only one template (Table S4), which allowed to obtain different confirmations, that the original structures displayed (Figure 1B, model T2-T4). For example the inward-facing conformation of Tm287 (model T2), the occluded conformations of McjD (model T5) or the outward-facing conformation of Sav1866 (model T4) represent the possible conformational states of NisT during a transport cycle following the alternating access model (Holland 2011).

In order to gain a deeper understanding of the nisin modification and secretion system and further to obtain the 3D structure of NisT, we began a structural characterization of the nisin exporter by establishing a robust purification protocol. Here, the aim was to get high yield, purity, homogeneity and a long-term stable membrane protein. First, we choose to overexpress the membrane protein homologously with the established NICE system in the Gram-positive bacterium *L. lactis*, which is also used as a host for the overexpression of other homologous and heterologous membrane proteins (Kunji, Slotboom et al. 2003). It is commonly accepted, that the lipids around a membrane protein have a strong effect on its function, activity and stability (Seddon, Curnow et al. 2004, Gustot, Smriti et al. 2010, Yang, Wang et al. 2014, Martens, Stein et al. 2016, Mehmood, Corradi et al. 2016). Thus, the homologous expression in the host *L. lactis* would allow to obtain native lipids such as cardiolipin (CL) or phosphatidylcholine (PC) from the membrane, which may be solubilized and co-purified during the purification progress.

The initial purification of histidine-tagged NisT was successful (Figure 2) by purifying 10HTEVNisT via a two step purification protocol (IMAC and SEC). However, the yield: 24 µg membrane protein/ 1 l cell culture) and the long-term stability of the protein in the HEPES buffer system were not satisfying. There, the highest concentration of the protein sample was 3.8 mg/ml and its was only stable for three days at 8 °C without precipitating. The initial crystallization trails with various commercial crystallization screens from Qiagen and Hampton search were not successful and mostly resulted in precipitated protein after three days.

The optimisation of the purification protocol (Figure 3) by using an other buffer system (phosphate and CHAPS) lead to a more stable protein (> three weeks at 8 °C) with a higher yield (48 µg membrane protein/ 1 l cell culture). Additionally, the detergents was changed from DDM to LMNG, which further increased the stability of the protein. Subsequently, new crystallisation trials were set up with the detergents purified ABC transporter, but still no crystals were obtained (Table 2). Therefore, we chose the bicelle reconstitution approach to mimic a membrane bilayer during the crystallization approach, which might stabilize the membrane protein.

In general, five approaches can be chosen to crystalize a membrane protein. There are 1) the detergent based (Seddon, Curnow et al. 2004) and 2) the antibody based crystallization methods, which can also be used in combination, where the detergent purified membrane protein is stabilized by a bound antibody-fragment (e.g. as shown for P-gp) (Ward, Szewczyk et al. 2013). The next three approaches resides the membrane protein in a native-like environment by mimic the bilayer of a membrane: 3) lipidic cubic phase (LCP) (Landau and Rosenbusch 1996), 4) lipidic sponge phase (LSP) (Wadsten, Wohri et al. 2006) and 5) bicelle (bilayered micelles) (Faham and Bowie 2002). Other recently developed bilayer-like approaches such as SMALPs (styrene-maleic acid copolymers lipid particle) (Pollock, Lee et al. 2018), nanodiscs (Denisov and Sligar 2016), saposin lipid nanoparticle (Flayhan, Mertens et al. 2018) or peptidiscs (Carlson, Young et al. 2018) are also possible to use for structural determination. But the latter three examples are especially used in the 3DEM approach or SAXS for structure determination (Lyons, Boggild et al. 2017, Rouck, Krapf et al. 2017, Josts, Nitsche et al. 2018).

For X-ray crystallography, the bilayer mimic approaches are interesting, as the membrane protein tend to crystalize in type I crystals from 2D crystals or 2D layers, which have more crystal contacts in the transmembrane as well as in soluble region of a membrane protein (Russo Krauss, Merlino et al. 2013). The other type crystals (type II), which are usually obtained with detergent purified membrane proteins, have less lattice formation than type I crystals, as the detergent micelle reduces the surface especially of the hydrophobic transmembrane regions (Newstead, Ferrandon et al. 2008). Amongst, these the bicelle approach has the advantage over the LCP and LSP method, that at temperatures under the transition point, where the morphology is change from disc-like to lamellar and multilamellar phase, automated systems to set up crystallisation trails and standard

crystallizations techniques can be used. Moreover, the bicelle approach was a successful way to crystallize the ABC transporters PCAT1 (Lin, Huang et al. 2015) and ABCG5/G8 (Lee, Kinch et al. 2016). The AMS transporter PCAT1 was crystalized with a bicelle mixture (35% stock) of DMPC/CHAPSO (ratio 2.8:1), where in the finial crystallization condition 7% bicelles, 2 mM UDM and 1.4 mM deoxy Big CHAP were presented. The crystals of the human sterol transporter ABCG5/G8 were obtained in a bicelle mixture (10% stock) of DMPC/cholesterol/CHAPSO. The ration between the lipids and the detergent was 3:1, where the lipids contained 5 mol% cholesterol and 95 mol% of the phospholipid DMPC. The amount of bicelles in the finial crystallization condition was 8%.

In the optimized crystallization trials only the bicelle approach yielded crystals, where a similar amount of the bicelles like in the aforementioned examples was used to crystallize NisT. Many optimized conditions of the initial hit from the screen MemGold II A3 resulted in needle shaped crystals (Figure 4B , Table 2,3). The best condition, in which the larges crystals were received, was in 20 mM MES buffer (pH 6), 20 mM NaCl, 80 mM $MgSO_4$, PEG 1450 11%, 7% DMPC/CHAPSO bicelle mixture and 10 mM ADP (Figure 4C, Table 3). The diffraction pattern of the crystals gave no spots (Figure 4D), which would either point to non-protein crystals (salts was excluded) or that the size as well as the quality of the crystals was not optimal to obtain a diffracting crystals.

In summary, this study provide the successful purification of a lanthipeptide exporter and is the first *in vitro* characterization of NisT. We obtained first crystal hits from crystallization trials with the bicelle reconstituted NisT. There, the lipidic bilayer of DMPC and CHAPSO provided a more native membrane-like environment for the ABC transporter NisT and stabilized the membrane protein in comparison to the detergent based approach. Although, we received not diffracting crystals, the results are a starting point for further optimization of crystallization conditions.

Material and Methods

The detergents dodecyl octaglycol ($C_{12}E_8$), n-dodecyl-β-D-maltopyranoside (DDM), Fos-choline 14 (FC-14), Fos-choline 16 (FC-16) and lauryl maltose neopentyl glycol (LMNG) were obtained from Anatrace and 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) was purchased from Avanti Polar Lipids. The lyophilized nisin powder (2.5% nisin content) were obtained from Sigma-Aldrich. Antibody of $NisT_{NBD}$ was purchased from Davids Biotechnology (Germany) as a polyclonal antibody. All standard chemicals were purchased from Sigma-Aldrich or VWR.

Bacterial strains and growth conditions

Strains of *Escherichia coli* and *Lactococcus lactis* and plasmids used in this study are listed in Table S1. The strain *E. coli* DH5α were grown in LB medium at 37 °C under aerobic conditions with appropriate antibiotics (30 µg/ml kanamycin or 100 µg/ml ampicillin). The transformation of *E. coli* strains was performed following standard procedures.

The strain *L. lactis* NZ9000 (and its variants) was grown in M17 (Terzaghi and Sandine 1975) at 30 °C under semiaerobic conditions supplemented with 0.5% glucose (GM17) and the appropriate antibiotic (erythromycin at a final concentration of 5 µg/ml).

For transformation of *L. lactis* NZ9000 with the expression plasmids a standard procedure for preparation of competent cells and electroporation was used as described elsewhere (Holo and Nes 1989).

Cloning of *nisT* and *nisT* variants

A nucleotide sequence for a MCS with 10H nucleotide sequence and TEV protease cleavage site was ordered as a codon-optimized, synthetic gene fragment from Life Technologies to insert it into the pNZ-SV plasmid (AlKhatib, Lagedroste et al. 2014). The synthetic gene fragments was amplified by Phusion DNA polymerase (NEB) with the primer pair 10Hfor and 10Hrev (Table S2) for Gibson assembly. The plasmid pNZ-SV was amplified by Phusion DNA polymerase (NEB) with the primer pair infupNZ-SVfor and infupNZ-SVrev (Table S2) to linearize the vector. The gene fragment and the vector pNZ-SV were employed in the Gibson assembly by following the manufactures instructions (NEB). The Gibson assembly reactions

were transformed into *E. coli* DH5α. The sequence of the construct pNZ-SV10H was verified by DNA sequencing (Microsynth Seqlab).

The *nisT* gene (accession number: Q03203) was amplified using the genomic DNA from *L. lactis* NZ97000 (Kuipers, Beerthuyzen et al. 1993) as a template. There, the Phusion DNA polymerase (NEB) with the primer pair infunisTor and infunisTrev (Table S2) was used to create overhang sequences for Gibson assembly. The plasmid pNZ-SV10H was amplified by Phusion DNA polymerase (NEB) with the primer pair linpNZ-SVfor and linpNZ-SVrev (Table S2) to linearize the vector. Subsequently, the gene and the linearized vector pNZ-SV10H were employed in the Gibson assembly and the reactions were transformed into *E. coli* DH5α. The sequence of the construct pNZ-SV10HTEVnisT was verified by DNA sequencing (Microsynth Seqlab).

Expression and purification of NisT

L. lactis NZ9000 was transformed with pNZ-SV10HTEVnisT and placed on SMGG17 agar plates containing 5 µg/ml erythromycin. A GM17 (Erm) overnight culture was inoculated with one colony and incubated at 30°C. A GM17 (Erm) main culture was inoculated to an OD_{600} of 0.1 with the overnight culture. After 3 h incubation, expression was induced by adding 10 ng/ml nisin (powder from Sigma-Aldrich dissolved in 50 mM lactic acid) and further grown for additional 3 h. Cells were harvested by centrifugation at 4000xg for 20 min at 8°C and resuspended in R1-buffer (100 mM HEPES, pH 8, 300 mM NaCl, 20% glycerol) or R2-buffer (50 mM Na-phosphate buffer, pH 8, 100 mM KCl, 20% glycerol) to an OD_{600} of 200. To the resuspended cells 10 mg/ml lysozyme was added and incubated at 30°C for 30 min. Prior to cell disruption, cells were incubated on ice for 15min. The cell suspension was passed through a homogenizer (M-110P, Microfluidics System) at 1.5 kbar at least four times. The homogenized cell suspension was centrifuged at 12,000xg for 30 min at 8°C. Subsequently, the supernatant was centrifuged at 100,000xg for 120 min at 4°C to collect the membrane fraction. Membranes were resuspended with R-buffer containing 10 mM imidazole and 0.5 mM AEBSF. The total membrane protein concentration was measured by BCA assay (Thermo Fischer Scientific) and the concentration was adjusted to 5-7.5 mg/ml.

Purification of NisT

Homogenized membranes containing 10HTEVNisT were solubilized with 1% (w/v) of the detergents DDM or LMNG (Anatrace) for 1 h at 8°C. Insoluble material was removed by centrifugation at 100,000xg for 30 min at 4°C. The supernatant was applied to a 5 ml IMAC (Immobilized Metal-Ion-Affinity Chromatography) HiTrap chelating column (GE Healthcare) preloaded with 100 mM nickel chloride or 100 mM zinc sulphate and equilibrated with low IMAC1 buffer (100 mM HEPES buffer, pH 8, 300 mM NaCl, 10% glycerol and 10 mM imidazole) or to low IMAC2 buffer (50 mM Na-phosphate buffer, pH 8, 100 mM KCl, 20% glycerol and 10 mM imidazole containing 0.03-0.1% DDM or 0.01%-0.05% LMNG. Consecutively, non-bound protein was washed by increasing the amount of imidazole or histidine from high IMAC buffer. Finally, 10HTEVNisT was eluted with 100% high IMAC buffer. The main elution fractions were pooled and further concentrated with a Amicon or Vivaspin20 100 kDa molecular weight cut off (MWCO) centrifugal concentrator (Millipore/Sartorius AG). A size exclusion chromatography (SEC) was performed, where the concentrated protein sample was applied onto a Superdex 200 10/300 GL column (GE Healthcare) equilibrated or Superose 6 10/300 GL column (GE Healthcare) equilibrated with various SEC containing DDM or LMNG (Table S3). The main peak fractions were analysed via SDS-PAGE and further concentrated via Vivaspin6 100 kDa MWCO centrifugal concentrator (Sartorius AG) until a concentration of 5-10 mg/ml was reached. The protein concentration was determined by NanoDrop spectrophotometer (Thermo Fischer Scientific) using a molar extinction coefficient of 86,180 $M^{-1} \bullet cm^{-1}$ and the molecular mass of 72.6 kDa. Aliquots of 10HTEVNisT were directly used for crystallization trails and a portion (30 µl) was flash frozen in liquid N_2 and stored at −80 °C until further use.

Bicelle preparation

A bicelle stock solution was prepared as describe elsewhere (Ujwal and Bowie 2011, Ujwal and Abramson 2012, Poulos, Morgan et al. 2015). In short a mixture (e.g. 2.8:1 ratio) of 0.26 g DMPC and 0.09 g CHAPSO was prepared by dissolving them in chloroform. Evaporation was used to remove chloroform and the lipid/detergents film was dissolved in 600 µl of aqueous buffer (SEC3 buffer without detergents). Many cycles of heating to 42°C, cooling on ice and vortexing was used to obtain a homogeneous bicelle stock solution. The bicelles are

a liquid solution at temperature under 4°C and are kept on ice prior mixture with the membrane protein.

Bicelle reconstitution of 10HTEVNisT

The detergents purified 10HTEVNisT was mixed in a ration of 1:4 with the bicelle stock solution and incubated at least for 30 min on ice. After incubation the reconstituted membrane protein was directly used for initial and optimized crystallization trails.

Crystallization of 10HTEVNisT

Initial crystallization trails were performed with detergent purified 10HTEVNisT using the sitting drop vapor diffusion method in a 96-well plate. The initial temperature was 12 °C for the detergent purified protein and RT (23 °C) for the bicelle reconstituted membrane protein. In the crystallization trails the ration of sample and reservoir solution was 1:1, where 0.1 µl homogeneous protein solution (3-10 mg/ml) was mixed with 0.1 µl reservoir solution form commercial available crystallization screens (Table 1-2). The reservoir size was 50 µl. In terms of optimized crystallization trails a 24-well plate was used, with a reservoir size of 500 µl. There, 1 µl sample was mixed wit 1 µl of reservoir solution. The temperature was 4, 12 and 23 °C. The crystallization progress in initial crystallization trails was monitored automatically in a time interval over 21 days and then regularly over a time period of 360 days. Potential hits were checked via UV 280 nm signal.

Homology modelling of NisT

A homology model of NisT was generated by using the online tool Phyre2 web portal (Kelley, Mezulis et al. 2015). There, the NisT sequence (accession number: Q03203) was used and a final model (T1) was obtained based on six templates of available ABC transporter 3D structures (Table S4). The sequence identity to the NisT sequence was in the range of 18-21%. Here, the confidence of the model was 100% over all residues. Further models (T2-T5) were based on the templates TM287 (T2; PDB ID: 3QF4; sequence identity 20%), PCAT1 (T3; PDB ID: 2RY2; sequence identity 21%), Sav1866 (T4; PDB ID: 2HYD sequence identity 22%) and McjD (T5; PDB ID: 4PI0 sequence identity 20%). The confidence of the models was 100% over all residues.

SDS-PAGE and immunoblotting for protein analysis

In general the sodium dodecyl sulphate−polyacrylamide gel electrophoresis (SDS-PAGE) experiments were performed using standard procedures (Laemmli 1970). In the SDS-PAGE gels the acrylamide portion was 10% to have a separation range from 30 to 120 kDa). SDS−PAGE gels were stained with colloidal coomassie (cc) (Dyballa and Metzger 2009).

The analysis of detergent solubilisation screens were conducted via the dot blot technique, which is described elsewhere (Ellinger, Kluth et al. 2013).

All immunoblotting experiments were conducted following standard procedures.

Blue native PAGE

The blue native PAGE (BN-PAGE) was performed following the manufactures instructions (Invitrogen, Thermofischer Scientific). A sample (native: n) of detergents purified 10HTEVNisT (10 µM final conc.) was mixed with 4x BN PAGE loading dye, 2 mM DTT and the appropriate detergents (FC-16, LMNG, DDM, $C_{12}E_8$). As a control (denatured: dn) 0.3% SDS (final conc.) was added to the sample. Per lane 10 µl of the sample was loaded on the precast polyacrylamide mini-gel system (4-16%; Bis-Tris; Invitrogen, Thermofischer Scientific) without heating the sample (maximal 35 °C). BN-PAGE gels were stained with colloidal coomassie (cc) afterwards (Dyballa and Metzger 2009).

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Supplemental information

Figures supplemental information

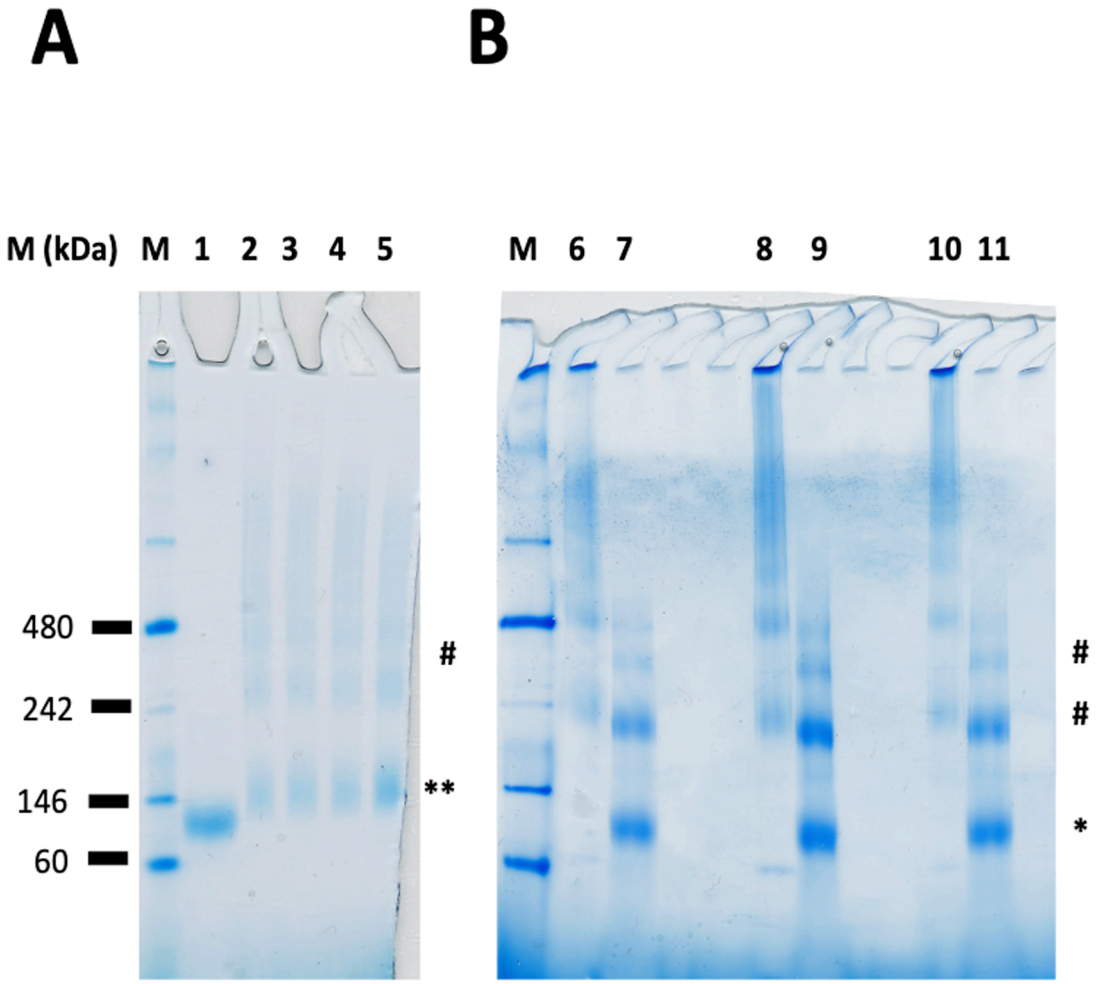

Figure S1: BN PAGE of 10HTEVNisT samples.
Different samples of 10HTEVNisT were subjected to BN PAGE. **A**) 10HTEVNisT in 0.015% FC-16. Control samples plus SDS (dn: 1), apo protein sample (n: 2), samples supplemented with 5 mM nucleotides and 10 mM $MgCl_2$ (3: AMP-PNP; 4: ADP; 5: ATP). **B**) 10HTEVNisT in 0.01% LMNG (dn: 6; n: 7), 0.1% DDM (dn: 8, n: 9) and in 0.05% $C_{12}E_8$ (dn: 10; n: 11). Monomer (*), dimer (**) and oligomer species (#).

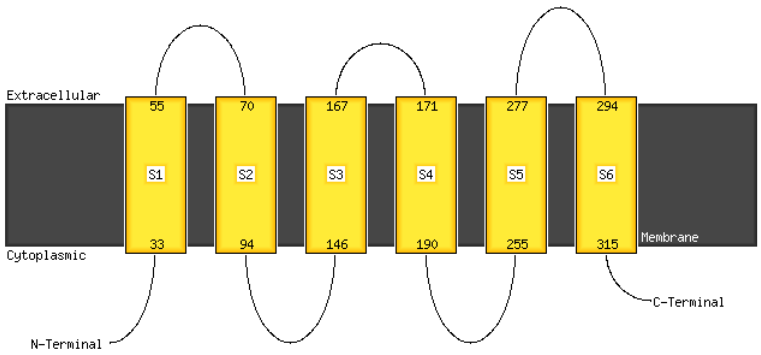

Figure S2: Topology model of NisT.
The topology of the transmembrane helices (TMH) within the NisT sequence was predicted with Phyre2 (Kelley, Mezulis et al. 2015). Colour code: the TMHs are displayed in yellow and numbered (S1-S6) from N- to C-terminus.

Tables supplemental information

Table S1: Strains used in this study.

strain	name	properties	reference
Escherichia coli DH5α	DH5α	cloning strain; F– Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK–, mK+) *pho*A *sup*E44 λ– *thi*-1 *gyr*A96 *rel*A1	(Grant, Jessee et al. 1990)
Lactoccocus lactis NZ9000	NZ9000	expression strain; *nisR* and *nisK* on the chromosome (*pepN*::*nisRnisK*)	(Kuipers, de Ruyter et al. 1997)

Table S2: Oligonucleotides used in this study.
The forward (for) and reverse (rev) oligonucleotides/primers used for the cloning are displayed in 5'-3' direction. Mutated codons are underlined.

name	sequence (5'-3')
EcoRIfor	GTTCGAAGGAATTCCAAAATAAATTATAAG
termlanArev	CTAATTTTGGTTCAAAGAAAGC
10Hfor	CAAAATAAATTATAAGGAGGCAC
10Hrev	CGGATCTCAGTGGTA
infupNZ-SVfor	CACCACCACCACCACTGAGATC
infupNZ-SVrev	TGAGTGCCTCCTTATAATTTATTTTGTAG
infunisTfor	TTTCAGGGCCCATGGATGGATGAAGTGAAAGAATTCA
infunisTrev	GTGGTGGTGTCTAGATTATTCATCATTATCCTCATATTGC
linpNZ-SVfor	TCTAGACACCACCACCACCACCACTG
linpNZ-SVrev	CCATGGGCCCTGAAAATACAGGTTTTCGG

Table S3: Overview of SEC buffer systems used for 10HTEVNisT purification.

name	buffer composition	additive
SEC1	10 mM HEPES pH 8, 150 mM NaCl	5% glycerol, X% DDM/ x% LMNG
SEC2	25 mM HEPES pH 8, 100 mM NaCl	10% glycerol, X% DDM
SEC3	25 mM CAPS pH 10, 100 mM KCl	20% glycerol, X% LMNG

Table S4: Finial homology model of NisT based on six ABC transporter crystal structures.

The homology models were generated with Phyre2 (Kelley, Mezulis et al. 2015) over all residues (600 aa residues) with a confidence of 100%. For the modelling six templates were used, which share a sequence identity of 18-21% with the NisT sequence.

ABC transporter	PDB ID (chain)	sequence identity (%)
Tm288	3QF4 (B)	20
Tm287	3QF4 (A)	20
PCAT1	4RY2 (A)	21
TmrA	5MKK (A)	21
ABCB8	5OCH (F)	20
TmrB	5MKK (B)	18

3.3 Chapter III – *In vitro* and *in vivo* characterization of a lanthipeptide exporter

Title Mechanism of lanthipeptide nisin secretion

Authors **Marcel Lagedroste**, Jens Reiners, Sander H. J. Smits, Lutz Schmitt

Published in manuscript in preparation

Impact factor -

Own proportion of this work 60%

Conduct following experiments:

Cloning of constructs, establishing and optimization of protein expression and purification protocols, *in vivo* secretion experiments, *in vitro* activity assay

Further proportions:

Design of experiments, data analysis, writing the manuscript.

Mechanism of lanthipeptide nisin secretion

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Keywords: Antimicrobial peptides, lanthipeptide, transport, ABC transporter, leader peptide, peptide secretion, *in vitro* activity, enzyme kinetic, RP-HPLC analysis, MS analysis

Abstract

Lanthipeptides are ribosomally synthesized and post-translationally modified peptides containing dehydrated amino acids and lanthionine rings. One of the best-studied examples is nisin produced by *Lactococcus lactis*. Nisin is synthesized as a precursor peptide comprising of an N-terminal leader peptide and a C-terminal core peptide. Amongst others, the leader peptide is crucial for enzyme recognition and acts as a secretion signal for the ABC transporter NisT. Nisin is secreted by NisT in a proposed channeling mechanism via the modification complex NisB/NisC, although the exact mechanism remains elusive.

Here, we present an *in vivo* secretion analysis of NisT in the presence and absence of the modification machinery. The apparent secretion rate gives insights how the modification enzymes enhance peptide secretion. Additionally, we determined for the first time the *in vitro* activity of a lanthipeptide transporter with and without the specific modification enzymes as well as the substrate. In summary, we unravel how the interaction partners influence the secretion activity of NisT in light of the proposed channeling mechanism.

Introduction

Many natural products (NP) produced as secondary metabolites by microorganisms can be used as pharmaceuticals (e.g. compounds as anticancer, antibacterial or antiviral drugs) (Newman and Cragg 2016). One class of these NPs are ribosomally synthesized and post-translationally modified peptides (RiPPs). The family of lanthipeptides, especially those with antimicrobial activity (lantibiotics), is gaining interest as a potential alternative for antibiotics to treat harmful multidrug resistance strains such as methicillin-resistant *Staphylococcus aureus* or vancomycin-resistant *Enterococci* (Dischinger, Basi Chipalu et al. 2014, Hudson and Mitchell 2018). Thus, it is important to gain further insights into lanthipeptide biosynthetic machineries to produce new peptides with novel properties.

Lanthipeptides are produced as precursor peptide (LanA) with an N-terminal leader peptide (LP) and a C-terminal core peptide (CP) (Arnison, Bibb et al. 2013). The LP serves as a signal sequence and recognition site for the modification enzymes and the export protein (Kuipers, Beerthuyzen et al. 1993, Kuipers, de Boef et al. 2004, Mavaro, Abts et al. 2011, Abts, Montalban-Lopez et al. 2013)). Furthermore, the LP keeps the modified peptide (mLanA) inactive (van der Meer, Rollema et al. 1994). Additionally, the post-translational modifications (PTM) are installed within the CP and not found in the LP (van der Meer, Polman et al. 1993). These PTMs are unusual amino acids (aa) such as didehydroalanine (Dha), didehydrobutyrine (Dhb) or (methyl-)lanthionine ((Me-)Lan) (Newton, Abraham et al. 1953, Gross and Morell 1968).

Lanthipeptides can be classified based on their modification enzymes into four classes (I-IV) (Repka, Chekan et al. 2017). There, LanA from class I is modified by two enzymes (LanB and LanC), whereas all other classes are modified by bifunctional enzymes (LanM, LanKC or LanL). One of the best-studied lanthipeptide is nisin (class I) and therefore its modification and secretion system is a model system for lanthipeptide production in general.

Nisin is produced by the Gram-positive bacterium *Lactococcus lactis* (*L. lactis*) as a precursor peptide (pre-NisA/NisA), where the genes for modification, secretion and maturation enzymes are located on one operon (Figure 1A) (Kuipers, Beerthuyzen et al. 1993). First, the ribosomal synthesized NisA is modified by the modification enzymes NisB and NisC (Figure 1B, I). Within the unmodified pre-NisA (uNisA) serine and threonine residues are dehydrated by the dehydratase NisB via a tRNA-depended glutamylation and elimination reaction to Dha and Dhb residues (Karakas Sen, Narbad et al. 1999, Garg,

Salazar-Ocampo et al. 2013, Ortega, Hao et al. 2015). Subsequently, the dehydrated aa are coupled to neighboring cysteine residues via an Michael-like addition catalyzed by the cyclase NisC in an region- and stereospecific manner (Koponen, Tolonen et al. 2002, Okeley, Paul et al. 2003, Li and van der Donk 2007). The reaction of both enzymes follows an alternating mode with a N- to C-terminus directionality yielding (Me-)Lan residues (Lubelski, Khusainov et al. 2009, Repka, Hetrick et al. 2018). Next, the exporter protein NisT secrets the modified NisA (mNisA) to the exterior (Figure 1B, II) (Quiao and Saris 1996). Finally, the LP is cleaved by the extracellular located serine protease NisP and active, mature nisin is released (Figure 1B, III) (van der Meer, Polman et al. 1993).

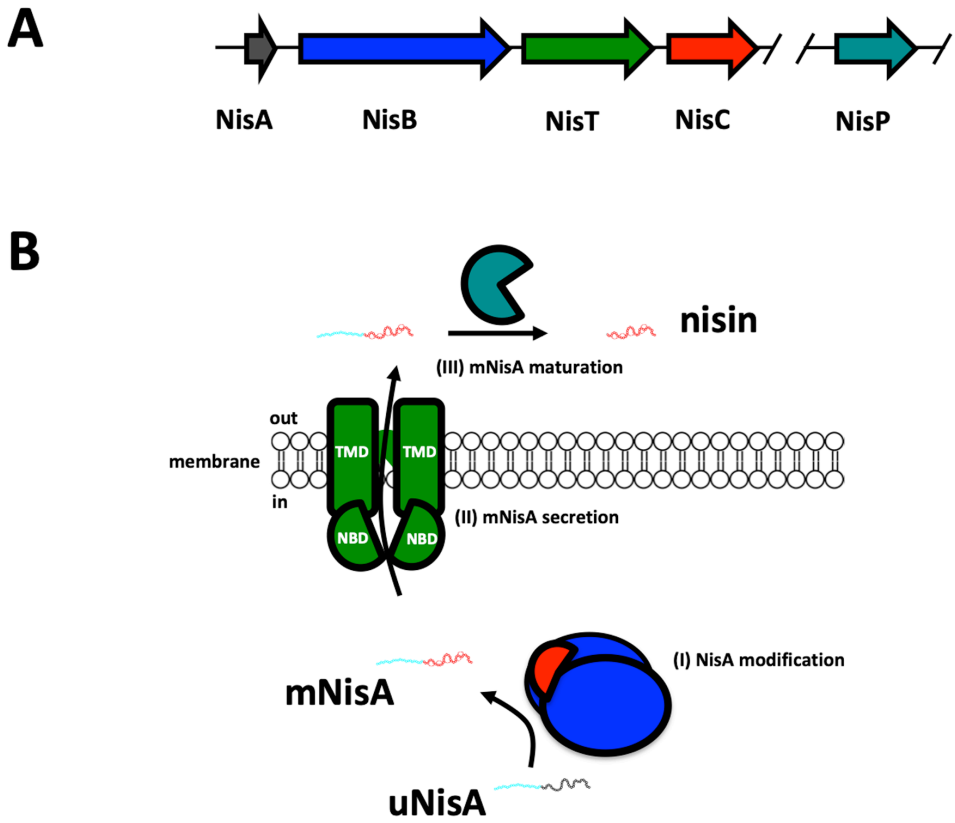


Figure 1: Scheme of nisin modification and secretion system.
(**A**) The lanthipeptide nisin (NisA, grey) operon encodes for the modification and secretion enzymes. (**B**) The enzymes NisB (blue) catalyze the dehydration reaction of unmodified NisA (uNisA), whereas NisC (red) catalyze the thioether ring formation resulting in modified NisA (mNisA). The ABC transporter NisT (green) translocates mNisA across the membrane to the exterior. Finally, the mature peptide is processed by the serine protease NisP (light grey) and active nisin is released. Please note, that the operon is partial represented and shows only proteins responsible for nisin maturation and secretion.

The lanthipeptide exporters belong to the superfamily of ABC proteins, which can be found in all domains of life (Higgins 1992). Bacterial ABC proteins function as transporter comprise mainly of two domains (Fath and Kolter 1993). One domain is the transmembrane domain (TMD) creating the translocation tunnel. The other domain is the nucleotide-binding

domain (NBD), which binds and hydrolyses ATP to energies conformational changes for substrate translocation.

Generally, the lanthipeptide exporters are classified into two classes. ABC transporters with a two-domain organization (TMD-NBD) are LanT proteins (e.g. NisT, SpaT or EpiT) (Allgaier, Jung et al. 1986, Kaletta and Entian 1989, Klein, Kaletta et al. 1992). Other transporters (e.g. NukT, LctT, or BovT) (Rince, Dufour et al. 1994, Sashihara, Kimura et al. 2000, Xiao, Chen et al. 2004), which have an additional N-terminal C39 peptidase (C39P) domain, are allocated to the ABC transporter of maturation and secretion (AMS transporter). This additional domain belongs to the cysteine protease superfamily, which is called C39 and classified as bacteriocin-processing endopeptidase from bacteria (Havarstein, Diep et al. 1995). We propose the nomenclature $Lan_{C39P}T$ for this class of lanthipeptide exporter (instead of $LanT_P$ (Repka, Chekan et al. 2017)) that will be used henceforth.

All known lanthipeptide exporters function as dimers and show specificity towards its substrates, especially towards the leader peptide, as the translocation is LP-dependent (Klein, Kaletta et al. 1992, Schnell, Engelke et al. 1992, Bierbaum, Brotz et al. 1995, Kuipers, de Boef et al. 2004). For some LanT/ $Lan_{C39P}T$ proteins it is proposed that the exporter and modification proteins assemble a multimeric enzyme complex at the membrane, where the LanA peptide is first modified by LanB and LanC (or LanM) proteins and the mLanA peptide is translocated to the exterior (e.g. nisin, subtilin and nukacin ISK-1) (Siegers, Heinzmann et al. 1996, Kiesau, Eikmanns et al. 1997, Nagao, Aso et al. 2005).

In 2004, the secretion of nisin was shown to be independent from NisB, NisC and NisP, which allows to study the translocation process independent of the modification and maturation step (Kuipers, de Boef et al. 2004). *In vivo* studies on the secretion process expanded the knowledge on the nisin modification and secretion system (Kluskens, Kuipers et al. 2005, Rink, Kuipers et al. 2005, Rink, Kluskens et al. 2007, Kuipers, Meijer-Wierenga et al. 2008). Accordingly, the observed high secretion efficiency of NisA by NisBTC was explained by a channeling mechanism (van den Berg van Saparoea, Bakkes et al. 2008, Lubelski, Khusainov et al. 2009). Other studies focused on the application of the nisin modification machinery to produce nisin variants or lantibiotics and secrete them by NisT (Zhou, van Heel et al. 2015, van Heel, Kloosterman et al. 2016, Zhou, van Heel et al. 2016, Lagedroste, Reiners et al. 2019). Despite the *in vivo* analysis of pre-NisA secretion, only a qualitative analysis of the supernatants was performed (van den Berg van Saparoea, Bakkes

et al. 2008). Thus, a systematic analysis of the secretion mechanism by determining kinetic parameter for pre-NisA translocation by NisT is still required.

For modification and secretion the LP is prerequisite and represents the main recognition site for NisB and NisC (Kuipers, de Boef et al. 2004, Khusainov, Heils et al. 2011, Mavaro, Abts et al. 2011, Abts, Montalban-Lopez et al. 2013, Khusainov, Moll et al. 2013). Consequently, mutations of the important FNLD-box and other mutations in the LP altered the modification and secretion of pre-NisA mutants (Plat, Kluskens et al. 2011, Plat, Kuipers et al. 2017). However, it is difficult to distinguish the effect of the LP mutants on the different enzymes. Hence, an investigation on the effect of certain mutants on NisT lacking NisB and NisC is required.

So far, we lack information´s about the multimeric NisT/NisBC complex. Only the modification complex ($NisB_2C$ + NisA) was reassembled and structurally investigated (Reiners, Abts et al. 2017). Furthermore, little is known about the *in vitro* activity and the secretion process of NisT and LanT proteins in general. Only one *in vitro* study on NukT is available. Here, the ATPase activity of the ABC transporter and the role of the additional C39P domain during nukacin ISK-1 translocation were analyzed (Zheng, Nagao et al. 2017).

In our study, we provide an *in vivo* and *in vitro* characterization of NisT to shed light on the secretion mechanism of pre-NisA. We determined the kinetic parameter for the pre-NisA secretion by analyzing the supernatant of pre-NisA secreting *L. lactis* strains via RP-HPLC. The resulting apparent secretion rate ($NisA \bullet NisT^{-1} \bullet min^{-1}$) of NisT is compared with the rate of the NisBTC system demonstrating a great enhancement in the presence of the modification machinery. Furthermore, we present a mutational analysis of the LP and LP hybrids to determine residues responsible for the interaction with NisT. The *in vitro* characterization of NisT is the first study revealing insight into the specific activity of a LanT lanthipeptide transporter and its modification enzymes as well as its substrate. In conclusion, our study demonstrates the influence of the modification enzymes NisB and NisC towards the activity of NisT and explains how pre-NisA is secreted by NisT based on the channeling mechanism of NisBTC.

Results

In vivo secretion assay of pre-NisA

To get further insights into the mechanism of lanthipeptide secretion, the pre-NisA secretion of the *L. lactis* strain NZ9000 was investigated using the presence and absence of the modification machinery. Here, the established nisin secretion and modification system (Rink, Kuipers et al. 2005, van den Berg van Saparoea, Bakkes et al. 2008, Lubelski, Khusainov et al. 2009, van Heel, Kloosterman et al. 2016) was used to establish an *in vivo* secretion assay, where the supernatants were employed to determine the secretion level of pre-NisA peptide via RP-HPLC analysis. In our study we used the strain L. *lactis* NZ9000BTC (Table S1) to modify pre-NisA by the modification enzymes NisB and NisC, thereupon the fully modified pre-NisA (mNisA) is secreted by NisT. The secretion of other modification states of pre-NisA can be analyzed by creating inactive mutants or deletions of the modification enzymes. The mutation $NisC_{H331A}$ (strain $NZ9000BTC_{H331A}$) or the deletion of NisC (strain NZ9000BT) results in secreted dehydrated NisA (dNisA). If NisB (strain NZ9000TC) or NisB and NisC (strain NZ9000T) are deleted unmodified NisA (uNisA) is secreted. The deletion of NisT (strain NZ9000BC) or the mutation of the histidine of the H-loop in the NBD domain (strain $NZ9000BT_{H551A}C$) totally hampers pre-NisA secretion (Figure 2). The last two strains are defined as the background for the secretion analysis. Thus, pre-NisA is expressed but not secreted. For the secretion analysis, all *L. lactis* strains were grown in minimal medium at 30°C and samples of supernatants were analyzed after induction every hour (0-6 h).

The peptides in the supernatants were separated by an acetonitrile/water gradient after a 20% washing step on a C-18 RP-HPLC column. The eluted peptides were monitored via UV signal at 205 nm and fractions of 1 ml were collected and used for MALDI-ToF-MS analysis. The MS analysis confirmed the correct mass for pre-NisA peptides (uNisA, dNisA or mNisA) in fractions 30-36 min of the chromatogram (Figure S1). The modified peptide mNisA with eight (-Met: 5689 Da) and seven (-Met: 5707 Da) dehydration from the strain NZ9000BTC was detected in fractions 34-36 min (Figure S1A, Table S2). Furthermore, the modified peptides (dNisA) from the strains $NZ9000BTC_{H331A}$ and NZ9000BT with eight dehydrations (-Met: 5689 Da) were found in the fractions 33-36 min (Figure S1B/D, Table S2). In the case of the peptide from strains NZ9000T and NZ9000TC the retention time is shifted and the unmodified peptide is eluted in fractions 30-32 min (Figure S1E/F). The corresponding molecular mass for unmodified pre-NisA (+Met: 5951 Da; -Met: 5834 Da) was

verified (Table S2). Subsequently, the peak area of pre-NisA peptides could be determined and the amount of nmol peptide of every time point after induction was calculated. The amount of secreted peptide was plotted against the time and a non-linear fitting was applied to determine V_{max} (maximal amount of peptide; nmol) and $K_{0.5}$ (time point of 50% secreted peptide: min). These kinetic parameter allowed a direct comparison of the secretion efficiency of the different *L. lactis* strains.

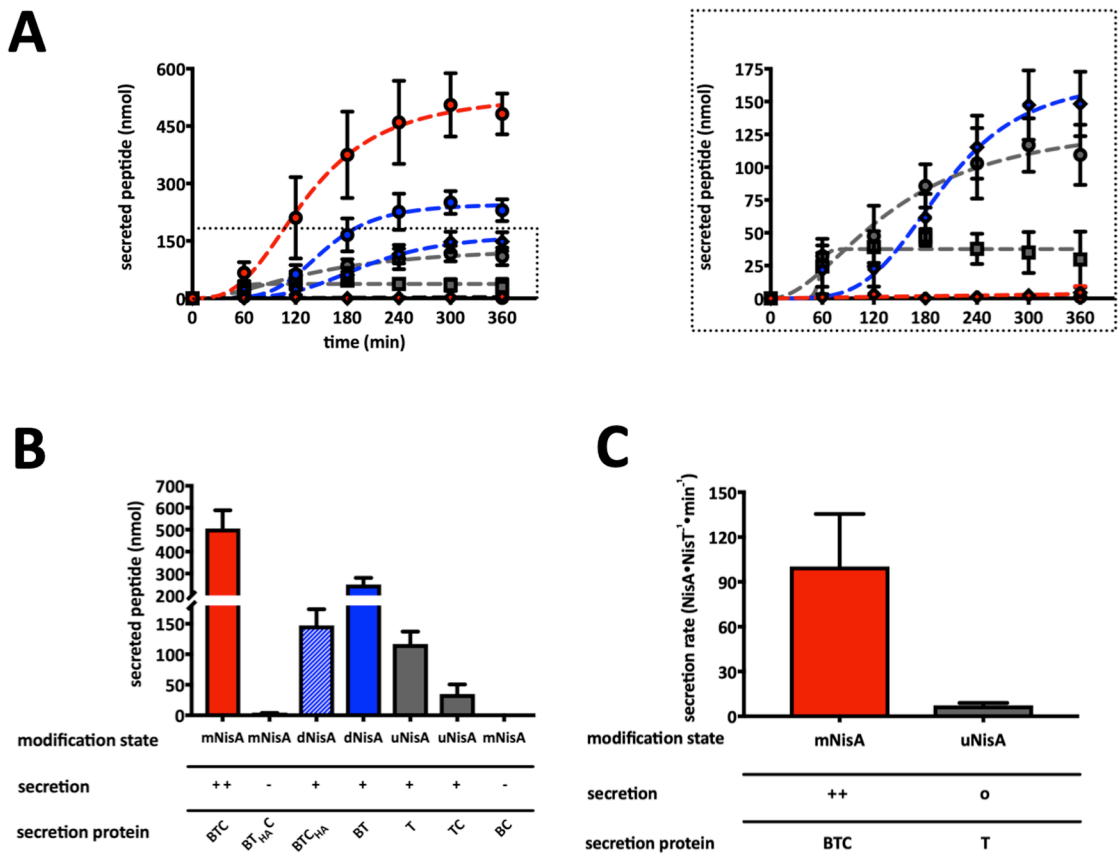


Figure 2: *In vivo* secretion assay of different *L. lactis* NZ9000 strains.
(**A**) The supernatants of pre-NisA secreting *L. lactis* NZ9000 strains was analyzed by RP-HPLC and the amount of pre-NisA was determined. Amounts of secreted pre-peptides (nmol) are plotted against the time (min) and the resulting curves were fitted by an allosteric sigmoidal fit. Modified NisA (mNisA, red) was secreted by the strains NZ9000BTC (red dots) and can be preclude by *nisT* deletion (strains NZ9000BC, clear dot) or an ATP-deficient mutant ($NZ9000BT_{H551A}C$, red rhomb). Dehydrated NisA (dNisA, blue) was secreted by the strains $NZ9000BTC_{H331A}$ (blue rhomb) and NZ9000BT (blue dot), whereas unmodified NisA (uNisA, grey) was secreted by the strains NZ9000T (grey dots) and NZ9000TC (grey square). Dashed square shows a zoom-in on strains with lower secretion level. (**B**) The kinetic parameter of V_{max} (nmol) of secreted peptides is plotted as bars against the various secretion systems. (**C**) The secretion rate of NisA molecules per NisT molecules was plotted against time (min) and fitted by linear regression. The slope represented the secretion rate of $NisA \bullet NisT^{-1} \bullet min^{-1}$ for the strains NZ9000BTC and NZ9000T, which is shown as bars. All data represent secretion experiments from at least five different transformants and are represented as means ± SD (n=5).
++: WT secretion; o: low secretion; -: no secretion

The strain NZ9000BTC has a V_{max} value of 534 ± 44 nmol and a $K_{0.5}$ of 134 ± 12 min, where it secrets the pre-NisA most efficiently in comparison to the other strains (Figure 2, Table S3). In the cytoplasmic fraction of this strain only a small amount of mNisA was detectable via WB in the first three hours after induction (Figure S2A). This finding is similar to the previously published data for the nisin secretion and modification system (van den Berg van Saparoea, Bakkes et al. 2008).

The mutation of the H-loop at position 551 to alanine in the NBD of the ABC transporter NisT abolished the secretion of mNisA and no peptide can be detected in the supernatant (Figure 2, Figure S2C). The same result was observed for the strain NZ9000BC (Figure S2G). In contrast to the strain NZ9000BTC high amounts of mNisA were found in the cytoplasmic fraction of the strains NZ9000BT$_{H551A}$C and NZ9000BC (Figure S1C/G). Thus, *nisT* deletion and H-loop mutation both hinders mNisA secretion.

The deletion of *nisC* (strain NZ9000BT) results in a lower secretion efficiency of pre-NisA and the V_{max} value of 247 ± 15 nmol and a $K_{0.5}$ of 152 ± 9 min (Figure 2, Table S3). The amount of secreted dNisA is reduced by a factor of 2.2 in comparison to mNisA (strain NZ9000BTC). Interestingly, the mutation of a histidine residue (H331) important for zinc ion binding to alanine (Li, Yu et al. 2006) reduces the V_{max} value (168 ± 16 nmol) by an additional factor of 3.2. The $K_{0.5}$ value increased to 200 ± 16 min (Figure 2). The analysis of the cytoplasmic fraction of the strain NZ9000BTC$_{H331A}$ shows that a higher amount of pre-NisA in detected inside the cell, which is lowly decreasing over the time (Figure S2B). Slightly lower amounts of pre-NisA are observed in the cytoplasmic fraction of NZ9000BT (Figure S2D).

Strain NZ9000T, which is obtained after the deletion of *nisB* and *nisC*, has a slightly reduced V_{max} value of 137 ± 30 nmol with a $K_{0.5}$ of 144 ± 41 min (Figure 2, Table S3). In comparison to the strain NZ9000BTC is the secretion of pre-NisA is reduced by a factor of 3.9. The lowest amount of secreted peptide was determined in the supernatant of strain NZ9000TC with a V_{max} value of 38 ± 8 nmol. Here, a higher amount of uNisA was detected in the cytoplasmic fraction, whereas no peptide was observed in the strain NZ9000T.

In all strains, NisB, NisC and NisT were detected in their corresponding fraction (cytoplasmic or membrane) (Figure S2A-G). The proteins NisB, NisC/NisC$_{H331A}$ and the pre-NisA were observed in the cytoplasmic fractions (except uNisA from NZ9000T). NisT was detected via WB in the membrane fraction of all strains (Figure S2A-G). In the case of mNisA

secreting strains (NZ9000BTC, NZ9000BT$_{H551A}$C, and NZ9000BC) all proteins were detected via WB even at time point zero.

Determine the apparent secretion rate of mNisA

The secretion rate of lanthipeptide secretion by a LanT ABC transporter is still unknown, although some lanthipeptide secreting systems have been studied (Meyer, Bierbaum et al. 1995, Izaguirre and Hansen 1997, Hille, Kies et al. 2001, van den Berg van Saparoea, Bakkes et al. 2008, Escano, Stauffer et al. 2015).

We determined the apparent secretion rate ($V_{S\ app.}$) by using the *in vivo* secretion assay. First, the amount of secreted mNisA at different time points was determined and plotted as nmol mNisA against time (Figure 2). Second the amount of the ABC transporter NisT was determined by analyzing the membrane fraction of strains NZ9000BTC and NZ9000T at each time point via WB (Figure S3A-B). Here, known concentrations of purified NisT$_{NBD}$ protein was used as a standard to determine the pmol amount of NisT (Figure S3C). Using these data, the secretion rate of NisA molecules per NisT molecules was calculated. The plot of the nmol NisA•nmol NisT^{-1} against time (min) made it possible to fit a linear regression (Figure S3D). The slope of the linear regression is $V_{S\ app.}$ of NisA•NisT^{-1}•min^{-1}, which is 100.3 ± 35.2 for the strain NZ9000BTC (Figure 2C). A strongly reduced secretion rate was determined for the strain NZ9000T (7.4 ± 1.6) as described qualitatively previously (van den Berg van Saparoea, Bakkes et al. 2008).

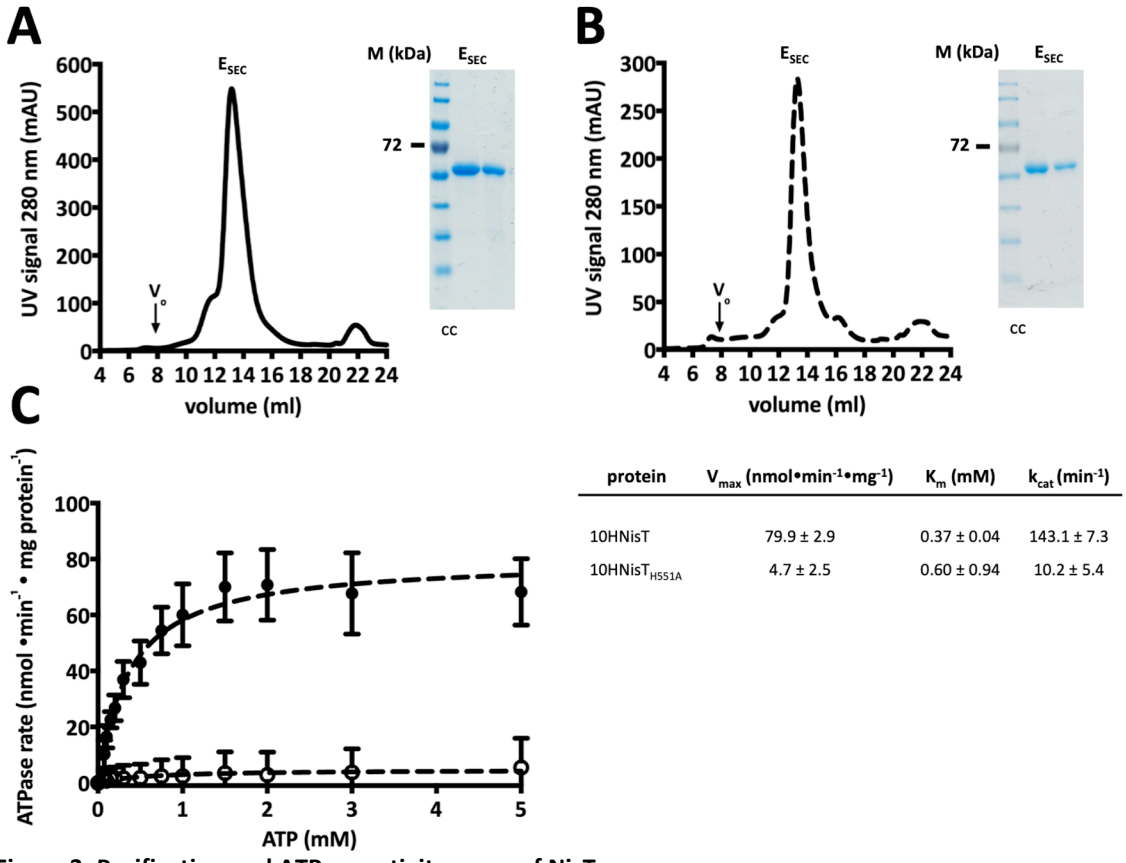
However, the functional unit of NisT is a dimer. Thus, the rate is twice as high as calculated. If one now considers, that one mNisA molecule consists of 57 amino acids, the $V_{S\ app.}$ can be further expressed as aa per NisT dimer per second. Here, the $V_{S\ app.}$ value of the nisin exporter NisT in the strain NZ9000BTC is 190.6 ± 66.8 aa•s^{-1}, where the secretion rate of the other strain NZ9000T is ~ 7% (13.9 ± 3.1 aa•s^{-1}). The determined secretion rate clearly demonstrated an enhancement in presence of the modification enzymes NisB and NisC.

Purification and basal ATPase activity of NisT, a class I lanthipeptide transporter

Little information is available about the *in vitro* activity of lanthipeptide ABC transporters and thereby about the functional characterization of these peptide exporters in general. One exception is a biochemical characterization of the AMS transporter NukT, which translocates

the class II lanthipeptide nukacin ISK-1 (Zheng, Nagao et al. 2017). But no *in vitro* data are available for ABC transporters of class I, III or IV lanthipeptides.

Therefore, we purified the ABC transporter NisT of the class I lanthipeptide nisin as a deca-histidine tag protein variant (10HNisT) and used it for further biochemical characterization. 10HNisT was homologously expressed in *L lactis* NZ9000 cells and purified to high purity (> 95% as estimated by SDS-PAGE, Figure 3A) in lipid-like surfactant Fos-choline-16 (FC-16, Anatrace) by immobilized metal ion affinity (IMAC) and size-exclusion chromatography (SEC). Similar to other membrane proteins, 10HNisT (72.5 kDa) showed a higher mobility during SDS-PAGE and migrated below the 72 kDa marker protein band. 10HNisT eluted as a homogeneous peak from SEC (Figure 3A). The main elution fractions were further concentrated and used for ATPase activity assay.



protein	V_{max} (nmol•min^{-1}•mg^{-1})	K_m (mM)	k_{cat} (min^{-1})
10HNisT	79.9 ± 2.9	0.37 ± 0.04	143.1 ± 7.3
$10HNisT_{H551A}$	4.7 ± 2.5	0.60 ± 0.94	10.2 ± 5.4

Figure 3: Purification and ATPase activity assay of NisT.
(**A**) SEC chromatogram of 10HNisT (WT, black line) displayed a homogeneous peak (E_{SEC}) at 13 ml on a Superose 6 10/300 GL column (V_0: void volume of the column). Inset: A typical colloidal coomassie (cc) stained SDS-PAGE gel shows a protein band between 55 and 72 kDa marker protein bands (M). (**B**) Purification of the H-loop mutant $10HNisT_{H551A}$ (HA, dashed line) showed comparable results for SEC profile and SDS-PAGE gel (inset). (**C**) The specific ATPase rate (nmol•min^{-1}•mg protein^{-1}) of purified WT (black dot) and HA mutant (unfilled circle) was plotted against the ATP concentration (mM) to determine kinetic parameter. The ATPase rate was fitted by Michaelis–Menten equation to determine V_{max} (nmol•min^{-1}•mg protein^{-1}), K_m (mM) and k_{cat} (min^{-1}). Activity assays were performed from five independent experiments with three replicates and are represented as means ± SD (n=5).

For the ATPase activity assay of NisT, the detergent was exchanged to CYMAL5 (Anatrace) and the ATPase rate was expressed as specific ATPase rate (nmol•min^{-1}•mg^{-1}). The kinetic parameters of 10HNisT in detergent solution were determined and resulted in V_{max}, K_m and k_{cat} values for the transporter without its substrate (basal ATPase rate). The concentration of 10HNisT was kept constant (1 µM), whereas the concentration of ATP was varied from 0 to 5 mM and the reaction was stopped after 30 min. The basal ATPase rate of 10HTNisT has a V_{max} value of 79.9 ± 2.9 nmol•min^{-1}•mg^{-1}, a Km value of 0.37 ± 0.04 mM and a k_{cat} value of 143.1 ± 7.3 min^{-1} (Figure 3C). As a control the H-loop mutant of 10HNisT (10HNisT$_{H551A}$; HA-mutant) was also purified following the same protocol and used for the ATPase activity assay (Figure3B). The ATPase rate of the HA-mutant is reduced by a factor of 17 (V_{max} value 4.7 ± 2.5 nmol•min^{-1}•mg^{-1}). The K_m value is increased by a factor 1.62 (0.60 ± 0.94 mM), whereas the k_{cat} value is decreased by a factor of 14 (3.8% relative to WT 10HNisT) (Figure 3C).

***In vitro* ATPase activity with pre-NisA variants**

To investigate the effect of substrate on the ATPase rate of 10HNisT we added different pre-NisA variants. First, the pre-NisA peptides in different modification states (uNisA, dNisA and mNisA) were purified via cIEX from culture supernatants (strains NZ9000T, NZ9000BT and strain NZ9000BTC) and used after buffer exchange (Figure S4). Additionally, the leader peptide of NisA (NisA$_{LP}$) was used in the ATPase assay to obtain insights into the recognition site, whether the LP only is sufficient for recognition by NisT. Here, the ATPase rate of the transporter might be influenced by the modification state of the peptide or by only NisA$_{LP}$.

For the activity assay the ATP concentration was kept constant at 5 mM, while the peptide concentration was varied from 0 to 40 µM. 10HNisT was pre-incubated with the peptides prior to the activity assay. The basal activity of 10HNisT was set to 100% and the ATPase rate with substrates was expressed as normalized ATPase rate. The ATPase rate of 10HNisT is slightly increased for all peptides. Some values like 20 µM uNisA showed a stronger stimulation of the transporter (140%, Figure 4B), while other (e.g. 20 µM mNisA) showed a lower stimulating effect (110%, Figure 4D). However, a concentration dependent stimulation of the transporter was not observed for the different tested peptides (Figure 4A-D).

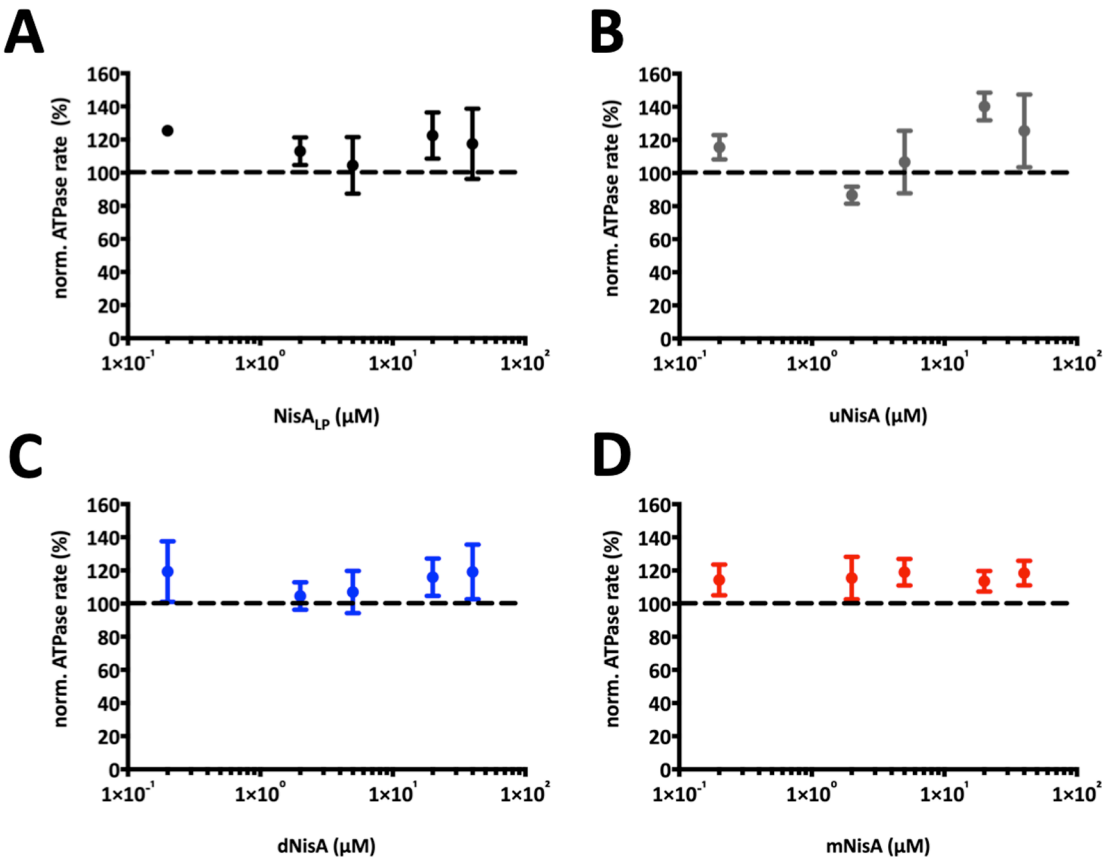


Figure 4: Dependence of NisT ATPase rate on different substrates.
The ATPase rate of purified 10HNisT was tested against different substrates. (**A**) The leader peptide of NisA (NisA$_{LP}$, black dots), (**B**) uNisA (grey dots), (**C**) dNisA (blue dots) and (**D**) mNisA (red dots) was used in various concentrations (µM) and the ATPase rate is shown as normalized ATPase rate (%). The basal ATPase rate is set as 100% (dashed line) and further values were normalized accordingly. The assays were performed in at least four independent experiments and are represented as means ± SD (n=4).

In vitro ATPase activity in presence of NisBC and mNisA

Previous studies (van den Berg van Saparoea, Bakkes et al. 2008) and our data showed, that the secretion of pre-NisA is strongly enhanced by the modification proteins NisB and NisC (see *in vivo* secretion assay) and therefore the ATPase rate of NisT might also be influenced by these interaction partner. To investigate the effect of NisB and NisC on the ATPase rate of 10HNisT the ATPase activity assay was repeated under the same conditions (ATP concentration constant, various concentration of interaction partner). We observed, that the ATPase rate of NisT is independent of the various concentrations of NisB or NisC. Thus, only fixed molar rations of 10HNisT to the interaction partner is used (NisB/NisC 1:2; in the case of NisBC 1:2:2) (Figure 5C). The basal ATPase rate of NisT was 62.5 ± 9.4 nmol•min^{-1}•mg^{-1} and it is slightly decreased in presence of NisB or NisC (54.7 ± 4.5 nmol•min^{-1}•mg^{-1}, 59.3 ± 4.9 nmol•min^{-1}•mg^{-1}). If both proteins were used in the assay (NisBC) the ATPase rate of

10HNisT was reduced by a factor of 1.3 (49.3 ± 4.4 nmol•min^{-1}•mg^{-1}), but the difference was not significant (Figure 5C).

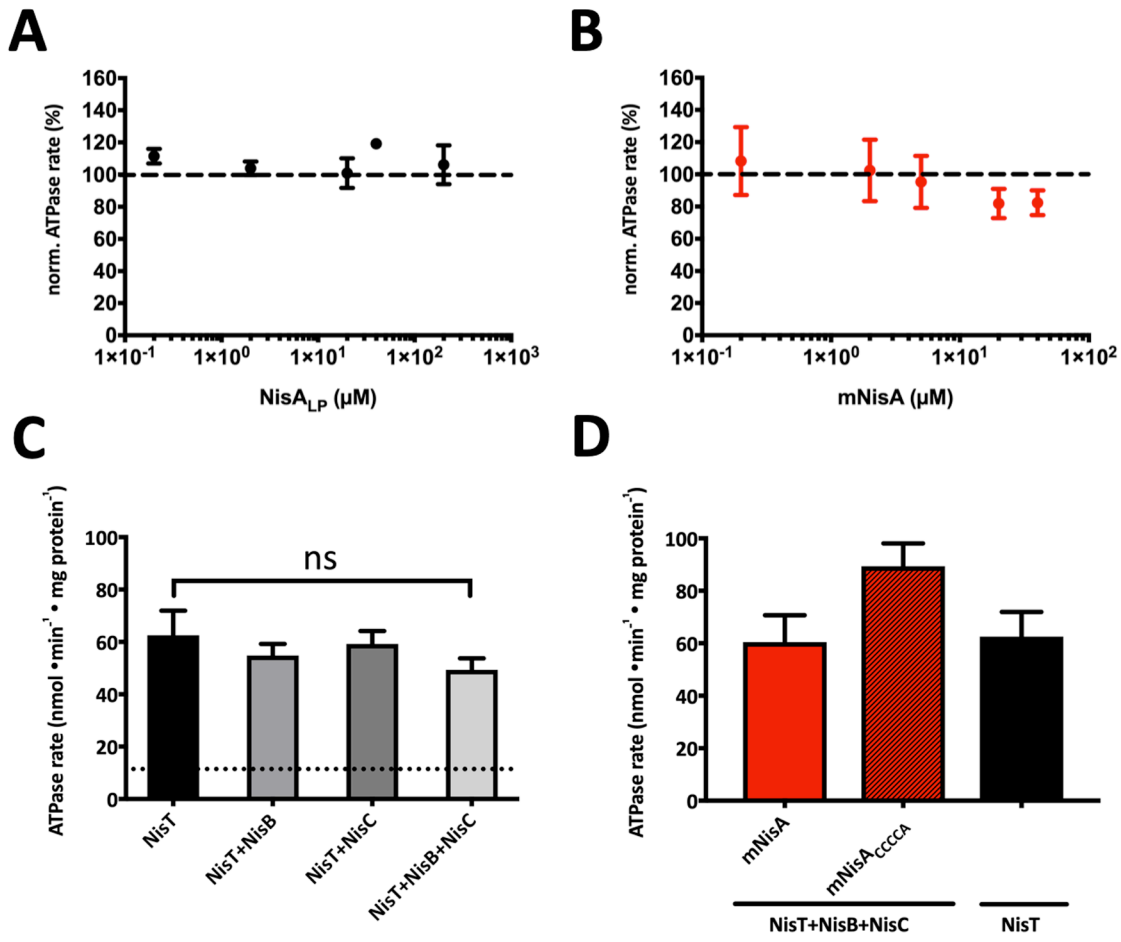


Figure 5: Influence of NisBC on the ATPase rate of NisT.
The ATPase rate of purified 10HNisT was tested against the modification enzymes NisB and NisC. (**A**) The substrate NisA$_{LP}$ (black dots) and (**B**) mNisA (red dots) were used in the assay with 10HNisT plus NisBC. The normalized ATPase rate was plotted against various concentrations (µM), where the ATPase rate of 10HNisT and NisBC is set to 100% (dashed line). (**C**) ATPase rate of 10HNisT is plotted against variation of 10HNisT and NisB, NisC and NisBC. (**D**) Comparison of ATPase rate from 10HNisT (black bar) plus NisBC with mNisA (red bar) and mNisA$_{CCCCA}$ (stripped red bar). All assay assays were performed in at least four independent experiments and are represented as means ± SD (n=4). The means were analyzed by a one-way ANOVA.
ns: not significant (p-value: ≥ 0.05)

Next, the ATPase rate of NisT with NisBC was investigated in presence of NisA$_{LP}$ and mNisA at concentrations ranging from 0 to 40 µM. The ATPase rate with the substrate NisA$_{LP}$ was slightly increased but not in a concentration-dependent manner (Figure 5A), while a decreasing effect on the ATPase rate was observed in presence of mNisA. Here, a concentration-dependent manner was observed with a lowest value of 82% at 40 µM (Figure 5B). Now, a variant of the mNisA was tested, where the last lanthionine-ring (ring E) was missing (mNisA$_{CCCCA}$). Here, an increase of the ATPase rate by a factor of 1.5 was determined (Figure5D). Thus, the interaction of NisT with NisB and NisC can be modulated by the last

ring in the pre-NisA LP, but not by the LP. Based on these results, a pull-down assay of NisT with its interaction partner and the substrate mNisA was conducted to obtain additional insights.

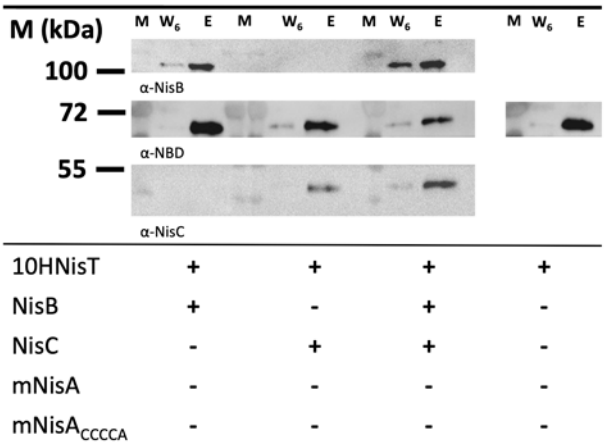
Interaction of NisT with NisBC

In 2017, the assembly of the nisin modification complex consisting of $NisB_2C$ and NisA was published and shed light on the stoichiometry and structure of the complex in solution (Reiners, Abts et al. 2017). Additionally, an influence of the last ring on complex disassembly was determined. The next step would be the interaction with NisT prior to secretion, as a proposed transient multimeric nisin modification/secretion complex (Siegers, Heinzmann et al. 1996), but detailed information´s about the interaction with the ABC transporter NisT are still missing.

Therefore, the interaction of NisT with NisB and NisC was investigated by a pull-down assay, where 10HNisT was immobilized on NTA-magnetic beads (Quiagen) before the interaction partner were added. Initially, the interaction of NisT was tested with 10 µM NisB, NisC or NisBC. In all case the interaction proteins of NisT could be observed in the elution fractions, which indicated a specific interaction of NisB and NisC with NisT (Figure 6A). The controls, where the NTA-magnetic beads were incubated with NisB and NisC without immobilized 10HNisT, showed no protein in the elution fractions (Figure S5).

The same set up was used in the presence of $mNisA_{CCCCA}$ and showed no additional effect on the interaction of NisB with NisT. However, the interaction of NisT and NisC was affected and the amount of co-eluted NisC is reduced (Figure 6B). If NisB was added additionally, the interaction of NisB and NisC with NisT was restored to the level without substrate in the assay (Figure 6B). The addition of mNisA instead of the ring-mutant shows the same result and NisB and NisC were detected in the elution fraction. Unfortunately, the analysis of all elution fractions with an antibody against the LP gave no signals for the substrates mNisA/$mNisA_{CCCCA}$. This might be due to low concentrations of the peptides in the elution fractions.

A



B

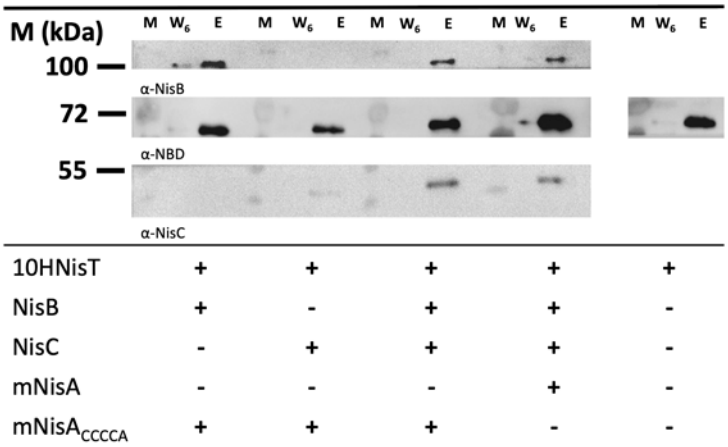


Figure 6: Pull-down assay of NisT with NisB and NisC.
The interaction of 10HNisT with NisB and NisC was studied by a pull-down assay. The ABC transporter was immobilized on NTA-magnetic beads, the specific interaction partner were added and incubated. After six washing steps, the last washing step (W_6) and the EDTA elution fraction (E) were analyzed by WB with the specific antibodies (α-NDB, α-NisB or α-NisC). Western blots display the eluted bands for NisB, NisT and NisC without substrate (**A**) and with substrates mNisA or $mNisA_{CCCCA}$ (**B**). The pull-down assay was repeated three time and showed similar results.
M: marker protein bands; +: protein was used in assay; -: protein was not used in assay

In summary, this is the first time that beside the interaction of NisT and NisC, an interaction of NisT with NisB was shown. Even the co-elution of NisB and NisC with NisT in the presence of the substrates mNisA and the ring-mutant $mNisA_{CCCCA}$ was observed, although no enhancement of the interaction between NisT and NisBC in presence of substrates mNisA/ $mNisA_{CCCCA}$ was detected.

Conserved amino acids within the leader peptides of LanAs

Next, the $NisA_{LP}$ was examined, which is not only important for the modification enzymes NisB and NisC, but also for the secretion by NisT. It was shown previously, that N-terminal fusions of $NisA_{LP}$ to different peptide variants resulted not only in secretion by NisT, but also in complete modification by NisBC (Kuipers, de Boef et al. 2004, Kluskens, Kuipers et al. 2005, Majchrzykiewicz, Lubelski et al. 2010, van Heel, Kloosterman et al. 2016). Thus, some parts within the LP sequence (Figure 7/8) are important for NisT recognition, which are independent from the CP. Other studies focused on mutations of the LP, where either aa of the FNLD-box or the C-terminal part were mutated to investigate the secretion by NisBTC (Plat, Kluskens et al. 2011, Plat, Kuipers et al. 2017). For example, the mutation of the FNLD-box hampers secretion, but it also influences the modification by NisBC (Khusainov, Heils et al. 2011, Mavaro, Abts et al. 2011, Plat, Kluskens et al. 2011, Abts, Montalban-Lopez et al. 2013). Although, NisB and NisC are prerequisite for a high secretion efficiency, the translocation of NisA mutants by NisT alone would reveal important residues.

Initially, selected LanA (Table S4) from various lanthipeptide class I and II ABC transporters (Table S5) were subject of a sequence alignment (Figure S6A) and furthermore, the relationship of these peptides was analyzed via a phylogenetic tree analysis (Figure S6B). The selected LanA peptides were divided into three main clades, which are named nisin-, Pep5- and epidermin-group. Interestingly, some class II lanthipeptides are located within the mentioned groups, except MrsA, MibA as well as PspA, which are dissect from the main root (Figure S6B). The same analysis was performed by using the corresponding known LP sequences of the selected LanA peptides (Figure S6C/D). This phylogenetic tree analysis showed the three different clades. The LPs from NisA and similar class I LanA peptide were classified as one group, the nisin-group. Within this group the C-terminus of the LP differs, where the peptidase cleavage site (CS; motif E/DφXXQ/N|p) for subtilin-like peptide differs from the LanP CS (motif GA/AGXXR/Q|φ), here X is any aa, φ a hydrophobic and p a polar residue. The other clade is the Pep5-group distinct from the nisin- and the epidermin-group, respectively. The LPs from PspA and MibA were allocated to the epidermin-group, whereas the LPs from the class II lanthipeptides (BovA, LasA, LctA, NukA and MrsA) are strongly unrelated and not included in the phylogenetic analysis. Although, the LP of SrtA1/2 belonged to the clade of the epidermin-group, they were assorted to the nisin-group due to a better matching of conserved aa in the sequence alignment.

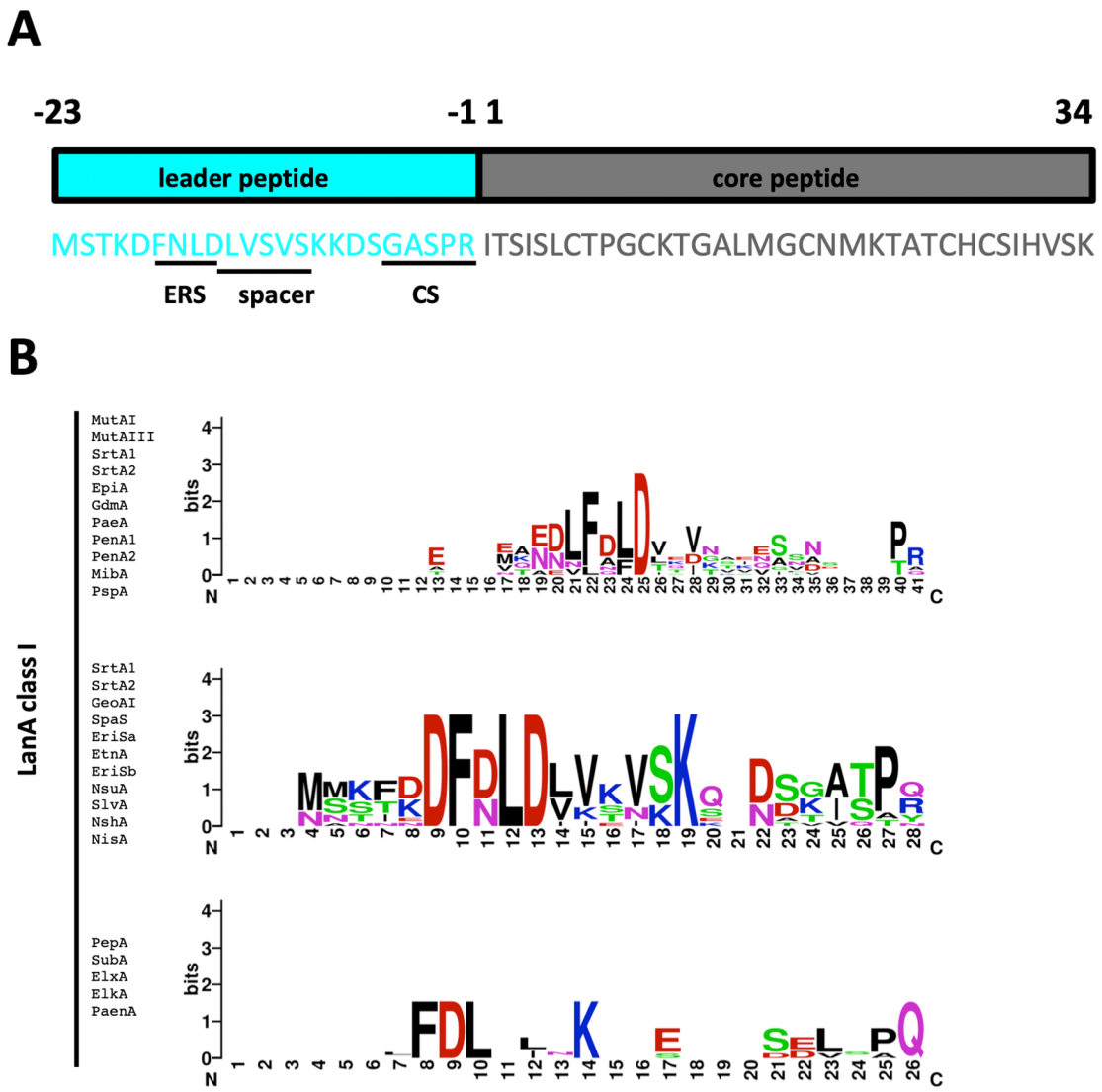


Figure 7: Bioinformatic analysis of NisA leader peptide mutants.
(**A**) Sematic representation of the NisA precursor peptide. The leader peptide from -23 to -1 (LP, cyan) and the core peptide from 1 to 34 (dark grey) are shown as amino acid sequence. Important parts of the LP are underlined. The enzyme recognition site (ERS) of the modification enzymes NisB/NisC follows a spacer region and then a cleavage site (CS) for the leader peptidase NisP. (**B**) The sequence alignment of selected LanA lanthipeptides from class I and II were performed by MUSCLE sequence alignment and the sequence conservation of class I $LanA_{LP}$ is illustrated by WebLogo.

Based on these results the sequence alignment was performed within the classified groups, while the class II lanthipeptides were analyzed independently (Figure 7B, Figure S6E). The class I LP of the nisin-group showed the conserved enzyme recognition site (ERS) with the sequence FD/NLD. Important to note are further conserved regions within the LP, which are marked with a red box (Figure 7B, Figure S6E). One part is in front of the ERS and consists of the charged aa lysine or aspartate (asparagine). A second region in located in the spacer region of the LP downstream of the ERS. Here, at least one lysine seems to be conserved, especially the lysine six aa downstream of the ERS (Figure 7B).

In the Pep5- and epidermin-group similar important residues, which seem to be highly conserved were identified upstream of the ERS (E or D). Other charged aa such as aspartate, glutamate, arginine or lysine were somehow distributed throughout the LP. In the group of the selected class II lanthipeptides only the C-terminally located recognition motif ELXXφX for the LanM enzyme was identified (Figure 7B, Figure S6E). Consecutively, to investigate the influence on uNisA secretion some mutants within the $NisA_{LP}$ sequence were introduced.

***In vivo* secretion assay of pre-NisA leader peptide mutants**

First, we choose the important recognition site for the enzymes NisB and NisC (FNLD-box) by creating four single mutants and one quadruple mutant. These mutants have been analyzed by Plat *et al.* in presence of the nisin modification and secretion system (NisBTC), but not in the background of NisT alone (Plat, Kluskens et al. 2011). Therefore, the *in vivo* secretion assay was used to investigate the supernatant of *L. lactis* NZ9000T cells, which secreted FNLD-box mutants (F-18A, N-17A, L-16A, D-15A and $_{F\text{-}18}AAAA_{D\text{-}15}$). The supernatants of five hour samples were analyzed via RP-HPLC and also the peptides were precipitated with TCA. The WB analysis of the TCA-pellet showed that the mutants F-18A and the quadruple-mutant ($_{F\text{-}18}AAAA_{D\text{-}15}$) were not detectable. Only the single mutants of N-17A, L-16A and D-15A mutants were detectable, however at lower levels compared to the wild-type (WT) (Figure 8A). The HPLC analysis of the mutants provided quantitative values for the secreted peptides (Figure 8A). The values of secreted peptides are expressed as concentration (µM), where the uNisA (WT) value is 0.73 ± 0.14 µM. The secretion of the mutants N-17A was reduced by a factor of 3 (0.25 ± 0.02 µM). The secretion of the mutant D-15A is further reduced to 0.21 ± 0.01 µM, the mutant F-18A has a value of 0.15 ± 0.03 µM and the mutant L-16A has a value of 0.13 ± 0.01 µM. If all aa in the ERS are mutated to the alanine residue ($_{F\text{-}18}AAAA_{D\text{-}15}$) the secretion has a value of 0.13 ± 0.02 µM, which is similar to the single mutants F-18A and L-16A. As a control the H-loop mutant $NisT_{H551A}$ was used to determine the background, which has a value of 0.09 ± 0.07 µM (Figure 8A). The substitution of the hydrophobic aa in the ERS (F-18 and L-16) to the alanine residue had the strongest effect on the secretion of uNisA.

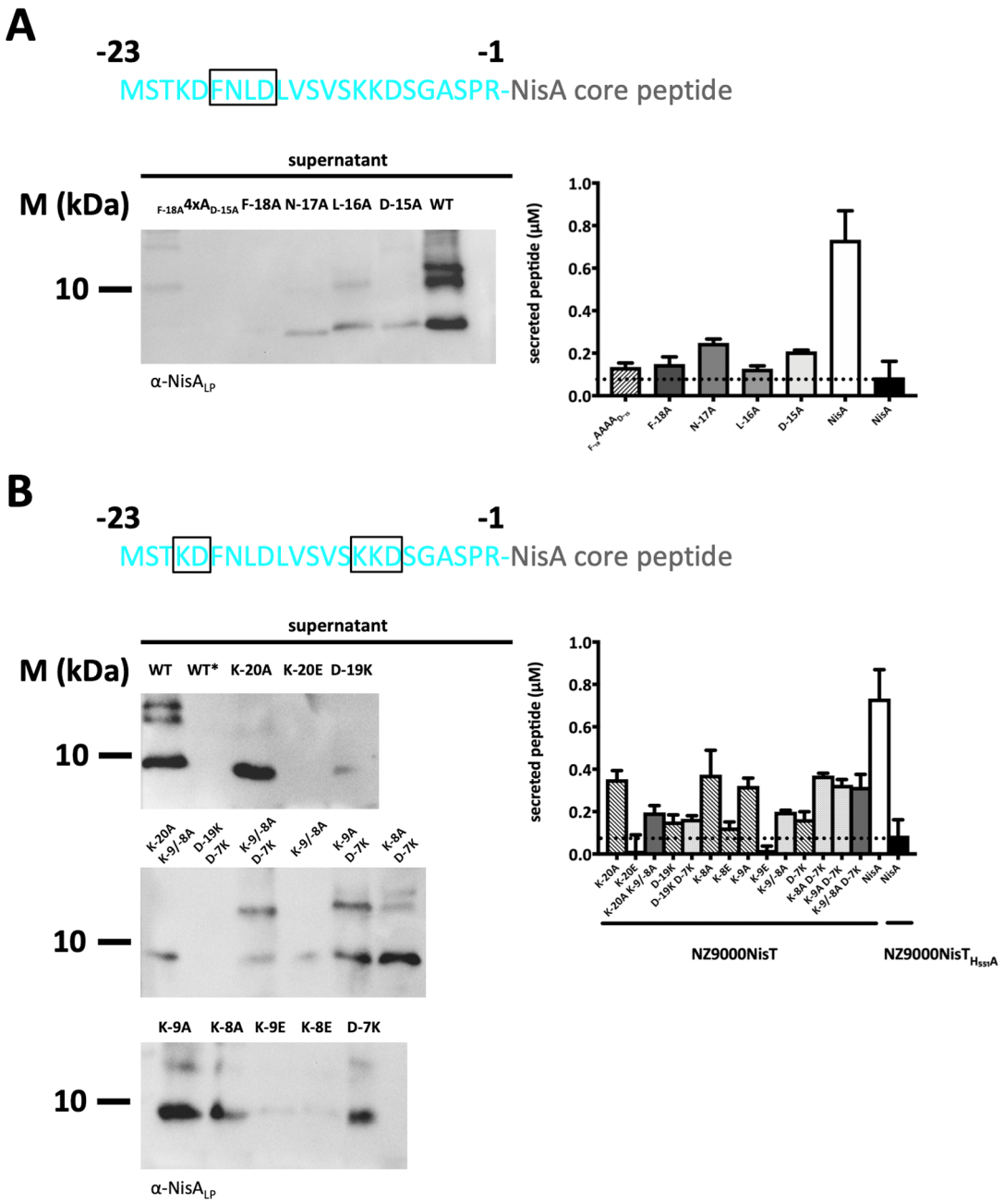


Figure 8: Secretion analysis of NisA leader peptide mutants.
(**A**) *In vivo* secretion analysis of FNLD-box mutants secreted by the strain NZ9000T. TCA-precipitated peptides from supernatants were analyzed by WB with a leader peptide antibody. The RP-HPLC determined concentrations of secreted peptide were plotted as bars. The FNLD-box mutants are shown as grey bars, the WT NisA as white bar and the NisA secreted from $NisT_{H551A}$ as black bar. (**B**) *In vivo* secretion analysis of leader mutants (single, double or triple mutants) secreted by the strain NZ9000T. TCA-precipitated peptides from 2 ml supernatants were analyzed by WB with a LP antibody. The RP-HPLC determined concentrations of secreted peptide were plotted as bars. The LP mutants are shown as grey bars, the WT NisA as white bar and the NisA secreted from $NisT_{H551A}$ as black bar. The background is represented by the HA mutant ($NZ9000T_{H551A}$) and illustrated as a dashed line. All data represent secretion experiments from at least three different transformants and are represented as means ± SD (n=3 for LP mutants; n=5 uNisA secretion by NZ9000T or $NZ9000T_{H551A}$). The means were analyzed by a one-way ANOVA.
M: marker protein bands; asterisks (*): highly conserved aa; double points (:): conserved aa; single point (.): less conserved aa
ns: not significant (p-value: ≥ 0.05)

To rule out that the expression level of the ABC transporter might influence the secretion of NisA, the membrane fraction of the cells was analyzed via WB against a specific NBD antibody. The WB demonstrated the equal expression of NisT in all samples (Figure S7A).

The importance of charged aa within the LP was subject of the next analysis. Especially, as Platt *et al.* showed that charged aa (P-2K or P-2D mutants) in the C-terminal part of $NisA_{LP}$ can enhance the secretion by NisBTC (Plat, Kuipers et al. 2017). Thus, the lysine and aspartate residue in front and after the FNLD-box were mutated to alanine or the opposite charged aa (D to K; K to E). First, the lysine residues (K-20, K-9 and K-8) were changed to alanine residues to study the effect of the positive charge in the LP. The concentration of secreted peptide in the supernatant was reduced to 0.35 ± 0.04 µM, 0.32 ± 0.04 µM and 0.37 ± 0.12 µM, respectively. These vales are around 40% of the WT level (0.73 ± 0.14 µM) (Figure 8B). If the lysine residues were exchanged to glutamate residues the effect is even more pronounced and the concentration of the peptide is below 10% of the WT level (K-20E: 0.01 ± 0.08 µM, K-9E: 0.02 ± 0.02 µM and K-8E: 0.13 ± 0.03 µM).

Next, the aspartate residues at position -19 and -7, which are located nearby the lysine residues, were mutated. The mutation D-19K reduced secretion to 0.15 ± 0.03 µM and the mutation D-7K to 0.16 ± 0.04 µM. By changing the negative charge or positive charge to the opposite lead to a strong reduction of the secretion level (Figure 8B).

Now, the double and triple mutants of the selected positions were investigated to find a difference between the mutated positions. The double mutant K-9/-8A reduced the secretion level to 0.2 ± 0.01 µM (additional 1.6 fold reduction in comparison to the single mutants). The other double mutants of this position (K-8A/D-7K and K-9A/D-7K) had a similar secretion levels as the single alanine mutants (K-9A and K-8A) with values of 0.33 ± 0.02 µM and 0.37 ± 0.01 µM. The double mutant D-19K/D-7K had a secretion level comparable to the single mutants D-19K or D-7K (0.17 ± 0.01 µM). The triple mutants K-20A/K-8/-9A and K-9/-8A/D-7K showed opposed effects. In the case of the triple mutant K-20A/K-8/-9A the secretion level of the double mutant K-8/-9A was alike with a value of 0.19 ± 0.03 µM. (0.2 ± 0.01 µM). Noteworthy, the secretion of the triple mutant K-9/-8A/D-7K was increased (0.32 ± 0.06 µM) and the secretion level of K-9/-8A was restored again by mutating the aspartate residue (D-7) to a lysine residue (Figure 8B). The membrane fraction of all samples were analyzed by WB and showed identical expression levels of NisT (Figure S7B).

The analysis of the LP mutants showed, that the charge at certain positions was important for the secretion by NisT. In front of the FNLD-box both charged aa (K-20 and D-19) were important as the change to the opposite charge has a drastically effect on the secretion. Equally important were positions -9 to -7, where the exchange to glutamate had a strong effect on secretion. A lower effect was observed for the double mutant K-9/-8A and the single mutant D-7K, but still the secretion level is below 20% of WT.

Correlation between leader peptides of LanA and the corresponding LanTs

Mutagenesis of $NisA_{LP}$ and the sequence alignment of LanA leader peptides, especially from class I lanthipeptide showed, that aa at certain positions are more conserved. Accordingly the position relative to the FNLD-box seems to be important and has an effect on NisT recognition (Figure 8A). Presumably, a correlation of LanA LPs and their corresponding LanT within one group of the phylogenetic tree exists. A similar correlation was observed for AMS transporter from Gram-positive bacteria, where especially the C-terminus of the LP was highly conserved (double Gly motif) (Havarstein, Holo et al. 1994).

In the case of $LanA_{LP}$, which showed a higher degree of identity (Figure S6D), it might be conjecture that these peptides are possible substrates of an adjoining LanT. Similar to LanA peptides, LanT transporters can be dissected in three groups (nisin-, epidemin- and Pep5-group) (Figure S6E). The clade of the nisin-group (e.g. NisT, SrtT and SpaT) separated early from the main root and from the other groups. The ABC transporters from the class II lanthipeptides are subdivided before the clade of the epidermin- /Pep5-group (e.g. GdmT or PepT), whereas LasT branches to the latter. A special case are the ABC proteins MibT/U and PspT/U, where the TMDs are located near the clade of the nisin-group but the NBDs (LanU) cannot be related to the other full-length ABC transporter.

Altogether, the analysis showed that the ABC transporters are likewise divided and can be classified into the same clades as the LPs. This reflects presumably the substrate specificity of the transporter towards its substrates and/or co-evolution. Nevertheless, if the LPs showed the same conserved aa within the LP it might be possible to substitute the native LP to become the substrate of another LanT.

***In vivo* secretion assay of $LanA_{LP}$-NisA hybrids**

This hypothesis was tested on the example of NisA and the corresponding exporter NisT. Of each LanA group (nisin-/ Pep5-/ epidermin- and lacticin-group; Table S4/S6) some LPs were selected and LP hybrids with the NisA CP were created ($LanA_{LP}$-NisA) (Figure 9). Sequence alignment of these selected LPs was performed and the sequence identity in comparison to $NisA_{LP}$ was determined (Figure 9A, Table S6). The nisin LP has a low sequence identity under 35% with the LPs of mutacin III (22.9%), Pep5 (29.4%) and lactocin S (33.3%). The identity is higher with the LP of gallidermin (44.1%). Within the nisin-group the LPs of subtilin and salivaricin D had a higher sequence identity (50% and 70.4%) (Table S6). Apparently, the charged aa up- and downstream of the FNLD-box are conserved within some LP (red boxes, Figure 9A). Interestingly, an FNLD-box within the $LasA_{LP}$ is absent and the peptide length of $MutIII_{LP}$ is increased to 41 aa (NisA 23 aa).

First the *in vivo* secretion assay was conducted with the strain NZ9000T to see, which LP hybrids were secreted by NisT. Therefore, the supernatants of the five hour samples were analyzed via RP-HPLC and Tricine-SDS-PAGE as TCA-precipitated peptides. The LP hybrids had the same secretion level as the $NZ9000NisT_{H551A}$ strain and displayed the background (0.09 ±0.08 µM) (Figure 9B). To test, if the peptides are secreted at all, the TCA-precipitated peptide pellets were obtained from 10 ml supernatant instead of 2 ml. Only the $SlvA_{LP}$-NisA peptide was visible on the Tricine-SDS-PAGE and showed a low secretion efficiency (Figure 9B). Thus, the expression of NisT was analyzed with an antibody against the NBD and confirmed the expression of NisT in the membrane fraction for all tested strains (Figure S8A). Next, the *in vivo* secretion assay was performed with the strain NZ9000BTC, as the NisA secretion efficiency by NisT is increased in presence of NisB and NisC.

The supernatants of the five hour samples from the different strains were used for RP-HPLC analysis (Figure S8C). In analogy to the secretion of native NisA the secretion of $LanA_{LP}$-NisA was increased for some peptides (Figure 9C). The peptide $SlvA_{LP}$-NisA had the highest value (0.98 ± 0.03 µM), which is ~35% of the WT (NisA: 2.83 ± 0.63 µM). For the peptide $SpaS_{LP}$-NisA the value was 0.26 ± 0.04 µM and is equal to ~10% of WT (Figure 9C).

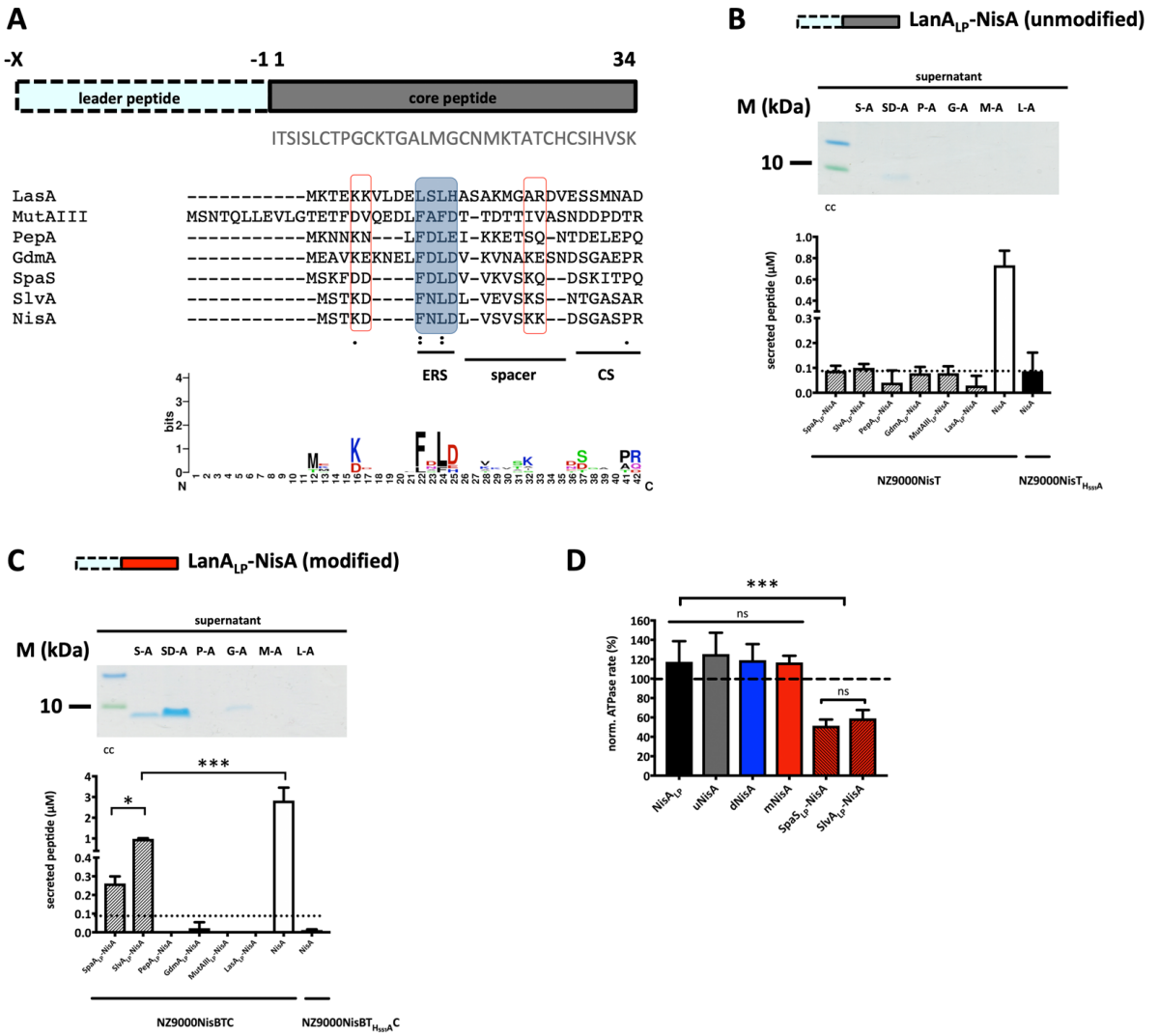


Figure 9: Secretion analysis of LanA$_{LP}$-NisA hybrids

(**A**) Sematic representation of the NisA peptide. The LP from -X to -1 (light cyan) and the CP from 1 to 34 (dark grey) are shown as amino acid sequence**.** Selected LP from class I lanthipeptide are analyzed by MUSCULE sequence alignment. Important parts of the leader peptide are highlighted by boxes or are underlined (ERS, spacer region and CS). Conserved aa within the LanA$_{LP}$ sequence are illustrate by WEBLogo. (**B**) *In vivo* secretion analysis of LanA$_{LP}$-NisA hybrids secreted by the strain NZ9000T or (**C**) by the strain NZ9000BTC. TCA-precipitated peptides from 10 ml supernatants were analyzed by SDS-PAGE and stained by colloidal coomassie (cc). The RP-HPLC determined concentrations of secreted peptide were plotted as bars. LanA$_{LP}$-NisA hybrids are shown as grey, stripped bars, the WT NisA as white bar and the NisA secreted from NisT$_{H551A}$ as black bar. (**D**) Comparison of normalized ATPase rate (%) from 10HNisT plus the substrates (at 40 µM) NisALP (black bar), uNisA (grey bar), dNisA (blue bar) mNisA (red bar), mSpaS$_{LP}$-NisA and mSlvA$_{LP}$-NisA (stripped red bar). All secretion experiments represent data from at least three different transformants and are represented as means ± SD (n=3 for LanA$_{LP}$-NisA hybrids; n=5 mNisA secretion by NZ9000BTC or NZ9000BT$_{H551A}$C). The background is represented by the HA mutant (NZ9000T$_{H551A}$) and illustrated as a dashed line. All activity assays were performed in at least four independent experiments and are represented as means ± SD (n=4). The means were analyzed by a one-way ANOVA.

M: marker protein bands; double points (:): conserved aa; single point (.): less conserved aa; asterisks (*): significant (p-value: 0.01-0.05); asterisks (***): significant (p-value: < 0.001); S-A: SpaS$_{LP}$-NisA; SD-A: SlvA$_{LP}$-NisA; P-A: PepA$_{LP}$-NisA; G-A: GdmA$_{LP}$-NisA; M-A: MutAIII$_{LP}$-NisA; L-A: LasA$_{LP}$-NisA

The other peptides like GdmA$_{LP}$-NisA had low values (0.02 ± 0.03 µM) near the background (0.01 ± 0.01 µM). Again the TCA-precipitated peptides from 10 ml supernatant were analyzed by Tricine-SDS-PAGE to further determine the secretion level. Peptides of

$SpaS_{LP}$-NisA, $SlvA_{LP}$-NisA and even of $GdmA_{LP}$-NisA (as a thin band) were visible (Figure 9C). The peptides of $MutIII_{LP}$-NisA, $Pep5_{LP}$-NisA and $LasA_{LP}$-NisA were not detected. For all strains the cytoplasmic fraction and the membrane fraction was analyzed via WB, where the expression of NisT, NisB and NisC was confirmed (Figure S8B).

The secreted peptides were further purified by cIEX purification to investigate the modification state (Figure S9). The cIEX and zip-tipped purified peptides were used for MALDI-ToF-MS analysis. In all peptides the dehydration of the $NisA_{CP}$ was not altered (Figure S10, Table S7). The CP carried eight, seven or six dehydrations (± start-Met), which is expected for the normal modification by NisB (Kuipers, de Boef et al. 2004, Lubelski, Overkamp et al. 2008). To further test the correct installation of the (methyl-)lanthionine-rings, free thiol-groups of the cysteine residues were cyanilated with CDAP. If a cysteine residue is not part of a thioether-ring, a mass shift of 25 Da would be observed. As a control for the coupling assay the unmodified NisA was used (Figure S10, Table S7). Here, a mass shift 125 Da was observed (5836 Da→ 5959 Da) demonstrating that all five cysteine residues were coupled. Next, the LP hybrids were subjected to the same assay. Again eight or seven dehydrations (+/- start-Met) were mainly observed within the CP. Only some spices inside the CP of $SpaS_{LP}$-NisA show one and two CDAP couplings (e.g. mass of 6003 Da (8xdh + 1xCDAP)). Thus, the activity of NisC seemed to be affected (Figure S10 and Table S7). The FNLD-box (FN/DLD) of the LP is the main recognition site for NisB as well as NisC. No differences are present in the LP of SpaS, SlvA and GdmA or the distance of the FNLD-box to the core peptide (15-17 aa) (Figure 9A). Therefore, the different secretion levels of the LP hybrids are connected to an altered activity of NisT.

Subsequently, the purified LP hybrids of $SpaS_{LP}$-NisA and $SlvA_{LP}$-NisA were used to test, if the ATPase activity of NisT was modulated. In comparison to the other tested substrates ($NisA_{LP}$, uNisA, dNisA and mNisA), where a slight stimulation effect on the ATPase activity of NisT was observed, is the ATPase rate reduced by a factor of 2 and 1.7. The normalized ATPase rate of $SpaS_{LP}$-NisA was 51.5 ± 6.3%, whereas the rate with $SlvA_{LP}$-NisA was 59.0 ± 8.5% (Figure 9D). Hence, the reduced secretion level of these LP hybrids seemed to be connected to a decreased ATPase activity of NisT.

Discussion

The mechanism of lanthipeptide modifications was subject of many studies, but only little is known about the secretion process of class I lanthipeptide ABC transporters (LanT). Only a few *in vivo* studies investigated the translocation of lanthipeptides nisin, subtilin, Pep5 or epidermin (Schnell, Engelke et al. 1992, Meyer, Bierbaum et al. 1995, Ra, Qiao et al. 1996, Izaguirre and Hansen 1997, Kuipers, de Boef et al. 2004). Amongst those the nisin modification and secretion system (NisBTC) is the best-studied one and is commonly employed to secrete nisin variants, lanthipeptides or non-lanthionine containing peptides (Kuipers, de Boef et al. 2004, Rink, Kuipers et al. 2005, van Heel, Kloosterman et al. 2016). It is commonly established, that nisin is ribosomally synthesized as a precursor peptide NisA and undergoes special post-translational modifications (e.g. Dha, Dhb, Lan and MeLan) (Newton, Abraham et al. 1953, Gross and Morell 1967, Ingram 1970). The PTM of the CP are installed in a coordinated manner by the nisin modification complex $NisB_2C$ (Khusainov, Heils et al. 2011, Reiners, Abts et al. 2017). There, NisA modification is following an N- to C-terminal directionally in alternating order of the dehydration and cyclisation reaction resulting in mNisA (Lubelski, Khusainov et al. 2009). For both modification enzymes the main interaction with the substrate is coordinated via the FNLD-box (Mavaro, Abts et al. 2011, Abts, Montalban-Lopez et al. 2013, Khusainov, Moll et al. 2013). The mutation of this recognition motif alters the modification pattern and leads to a lower production level (Plat, Kluskens et al. 2011).

Not only the modification is a tightly coupled process, but also the secretion of mNisA by NisT. A proposed channeling mechanism through interaction with NisB/NisC might explain the mNisA translocation. Here, the modification and secretion is a coupled process as mNisA can be detected in the medium within the first minute after induction (van den Berg van Saparoea, Bakkes et al. 2008). A deletion of *nisB* led to a drastically reduction of mNisA secretion, which was further reduced by a deletion of *nisC*. Thus, for optimal secretion efficiency NisT needs NisB and NisC. Interestingly, a localization at the cytoplasmic membrane was observed for both enzymes (Engelke, Gutowski-Eckel et al. 1992, Siegers, Heinzmann et al. 1996). Furthermore, results of co-immunoprecipitation experiments and yeast two-hybrid assay supported an interaction of NisC with NisT and NisC with NisB (additionally to the intramolecular interactions of each protein). Consequently, a multimeric enzyme complex of NisB/NisC and NisT was proposed. Similar results were obtained for the

subtilin modification and secretion system SpaTBC (Kiesau, Eikmanns et al. 1997). Unfortunately, *in vitro* activity of NisT as well as the mechanism of the secretion process remains elusive and to our knowledge no such data are available for LanT ABC transporter in general.

By expanding the search to other bacteriocin exporter some *in vitro* data can be gathered to get insight into peptide translocation. Examples are the exporter McjD (lasso peptide exporter), PCAT1 (peptidase-containing ABC transporter) and NukT (class II lanthipeptide exporter) (Choudhury, Tong et al. 2014, Lin, Huang et al. 2015, Zheng, Nagao et al. 2017). In the case of PCAT1 and McjD the crystal structure of these membrane proteins is available. Both proteins following the general fold of type I exporter of prokaryotic ABC transporters, whereas PCAT1 has an additional domain at the N-terminus.

In respect of the lasso peptide exporter McjD from *E. coli* even mechanistic aspects are available. This transporter translocates the substrate MccJ25 after modification by McjB and McjC via TolC across both membranes (Delgado, Solbiati et al. 1999). In general, the transporter is following the alternating access model, in which the inward-facing and outward-facing transition is induced after ligand binding, coupled to ATP binding and NBD dimerization (Jardetzky 1966, Chen, Sharma et al. 2001). These binding events are required for conformational changes in the TMDs. Additionally, for McjD an occluded (intermediate) conformation with closed periplasmic gate was observed (Choudhury, Tong et al. 2014). The TMDs form a cavity, in which the substrate binding site is located and after a transient opening (outward-facing) the substrate is released (Bountra, Hagelueken et al. 2017). The McjD transport cycle is reset after ATP hydrolysis and makes the transporter competent for another translocation event (Husada, Bountra et al. 2018). As MccJ25 has no LP after the modification process, the transport by McjD might have another mechanism as AMS transporter like PCAT1 or NukT.

The substrate of AMS proteins have an N-terminal LP, which contains a recognition motif ($_{-12}$LXXXEL$_{-7}$) and a double Gly motif (-2/-1): GG, GA or GS) (Havarstein, Diep et al. 1995, Ishii, Yano et al. 2010). Furthermore, the translocation of the substrate is coupled to ATP binding and hydrolysis as well as substrate maturation in a cooperative manner. In the case of PCAT1 proteolysis of the N-terminal LP is independent of ATP, where ATP binding enables conformation changes in the TMDs after cleavage and dissociation of the peptidase domain. The transporter also follows the alternating access model although an ATP-gated

channel model (like for CFTR (Anderson, Berger et al. 1991)) is discussed especially for large proteins (Lin, Huang et al. 2015). Similar to PCAT1 the bifunctional ABC transporter NukT shows a cooperative, interdomain interaction of the C39 peptidase domain and NBD prior to substrate translocation. Nishie *et al.* demonstrated that the cleavage reaction and the ATP hydrolysis are tightly coupled (Nishie, Shioya et al. 2009). Furthermore, the mature substrate (nukacin ISK-1) is secreted only to the exterior after LP cleavage from mNukA (Nishie, Sasaki et al. 2011, Zheng, Nagao et al. 2017). Thus, the translocation process does not require LP binding to the substrate binding site in the TMD cavity, but binding of nukacin ISK-1 as the unmodified peptide does not stimulate ATPase activity (Zheng, Nagao et al. 2017). Further *in vitro* studies are only available of isolated peptidase domains of AMS proteins. Examples are LctT involved in lacticin 481 secretion and maturation or LahT, where nine different precursor peptides are located inside the operon (Uguen, Hindre et al. 2005, Bobeica, Dong et al. 2019).

NukT is the only full-length lanthipeptide transporter, which is characterized *in vitro* but it belongs to the $Lan_{C39P}T$ family. Therefore, we characterized the LanT-type transporter NisT with respect to the secretion process and the specific activity. To study the mechanism of nisin secretion, we focused on three main topics: (I) *In vivo* secretion rate of NisA by NisT, (II) *in vitro* activity of NisT with and without substrate, (III) determination of important criteria within the leader peptide for secretion.

In 2008, van den Berg van Saparoea *et al.* conducted a kinetic analysis of nisin production with the strains NZ9700 and NZ9000 transformed with a two plasmid system (van den Berg van Saparoea, Bakkes et al. 2008). They demonstrated distinct contributions of the modification enzymes NisB and NisC towards the lanthipeptide secretion and proposed that the secretion process by NisT occurred via a channeling mechanism. This hypothesis was further supported by *in vivo* studies, in which some mechanistic aspects of NisB and NisC modification were investigated (Lubelski, Khusainov et al. 2009, Khusainov, Heils et al. 2011, Khusainov and Kuipers 2013).

Although, the early studies clearly demonstrated the dependence of pre-NisA secretion on the modification enzymes, it lacked a determination of kinetic parameter. To determine the kinetic parameter, we quantified the amount of secreted peptide via RP-HPLC from different time points of various NZ9000 strains (based on the established two plasmid system).

The first two kinetic parameter were V_{max} and $K_{0.5}$, which were obtained by an allosteric sigmoidal fit (Figure 2). In general, our results are consistent with the aforementioned studies, in which the strains NZ9000BTC had the highest V_{max} and the lowest $K_{0.5}$ value reflecting a high secretion efficiency. The strains, which secreted uNisA (NZ9000T and NZ9000TC), show lower V_{max} and higher $K_{0.5}$ values. Interestingly, we observed in our secretion assay some aberrations in respect to dNisA secretion. The expression of a catalytic-inactive NisC (H331A mutant) in the NisBTC system (strain NZ9000BTC$_{H331A}$) did not restore the secretion level of dNisA to the WT level. This is in contrast to Lubelski *et al.*, where a recovery of the pre-NisA secretion to WT level was observed (Lubelski, Khusainov et al. 2009). The secretion of dNisA by NZ9000BT has a higher V_{max} level and a lower $K_{0.5}$ value. However, one has to consider the time scale of the secretion assays, which might explain this difference. In our assay, the early kinetics of the secretion (also see (van den Berg van Saparoea, Bakkes et al. 2008)) might show the differences between the used strains more clearly as an end-point determination after over night secretion. The precise determination of secretion efficiency makes it possible to create a descending order of the pre-NisA secreting strains (NZ9000BTC > NZ9000BT > NZ9000BTC$_{H331A}$ > NZ9000T > NZ9000TC). The secretion efficiency clearly shows, that mNisA is secreted at high rates with the strain NZ9000BTC and every aberration of the secretion system reduces it at least by a factor of 2.2 (see strain NZ9000BT).

The third kinetic parameter was the apparent secretion rate $V_{S\,app.}$ (NisA•NisT^{-1}•min^{-1}), which we determined for the strains NZ9000BTC and NZ9000NisT, where the secretion efficiency was the highest for NisBTC system (Figure 2). These values are in general an approximation to receive a kinetic parameter, which can be related to other secretion system, e.g. the SecA translocon. In comparison to the nisin modification and secretion system ($V_{S\,app}$: 191 ± 67 aa•s^{-1}) the SecA translocon processes a secretion rate of 152-228 aa•s^{-1} per translocon (Robson, Gold et al. 2009). A lower secretion rate was determined for the hemolysin A type 1 secretion system (HlyA T1SS), which was ~16 aa•T1SS^{-1}•s^{-1} (Lenders, Beer et al. 2016). Similar to the latter, we observed that ATP hydrolysis is essential for the secretion process as the H551A mutant (strain NZ9000BT$_{H551A}$C) does not secrete mNisA. This mutant enables ATP binding and NBD dimerization but not ATP hydrolysis (Zaitseva, Jenewein et al. 2005).

As ATP hydrolysis is clearly important for ABC transporter mediated substrate translocation, we determined the *in vitro* activity of NisT in terms of ATPase rate without and with substrate. There, the basal ATPase activity has a V_{max} value of 79.9 ± 2.9 nmol•min^{-1}•mg^{-1} with a K_m value of 0.37 ± 0.04 mM, which is in the range of other ABC transporters (Choudhury, Tong et al. 2014, Lin, Huang et al. 2015, Reimann, Poschmann et al. 2016) (Figure 3). In comparison to NisT, the $Lan_{C39P}T$ transporter NukT has a low V_{max} value of 12.6 nmol•min^{-1}•mg^{-1}, but it is stimulated by mNuk to max. 500% (at 50 µM substrate). The cleaved substrate stimulates to maximal 200% (at 25 µM substrate) and the unmodified substrate does not stimulate at all (Zheng, Nagao et al. 2017). In the case of NisT, we observed a low stimulation in the presence of $NisA_{LP}$, uNisA, dNisA and mNisA, which is not depended on the substrate concentration (Figure 4). NisT is maximal stimulated to ~ 140%. We extended the ATPase activity assay by addition of NisB and NisC to emulate the WT system (NisBTC), in which the secretion of pre-NisA is the most efficient (Figure 5). The addition of Nis_{LP} had no effect on the ATPase rate and only a low stimulation, which is concentration independent, was observed. However, the addition of mNisA revealed an inhibiting effect on the ATPase rate with increasing substrate concentration (maximal ~80% of basal ATPase rate at 40 µM). Interestingly, for PCAT1 a similar behavior was observed (Lin, Huang et al. 2015). Another study of an ABC transporter homologue to PrtD from *Aquifex aeolicus* also showed an inhibition effect on ATPase activity after substrate addition (Morgan, Acheson et al. 2017). The open question is now, if NisT is also following an equivalent mechanism, in which the interaction of the modification/ secretion complex inhibits the ATPase rate prior to translocation.

In 1996, a multimeric enzyme complex of NisBTC was proposed, but the isolation of such a complex was not successful (Siegers, Heinzmann et al. 1996). Therefore, we choose to study the specific interaction of NisT with NisB and NisC via a pull-down assay. Such pull-down assay was performed with His-tagged pre-NisA, where NisB and NisC were co-eluted from cytoplasmic fraction (Khusainov, Heils et al. 2011, Khusainov, Moll et al. 2013). In our study, we expanded this set up and used purified His-tagged NisT, NisB, NisC and pre-NisA (Figure 6). We observed specific interaction of NisT with NisC, which is inline with previously observed interaction of NisT with NisC via co-immunoprecipitation and yeast two-hybrid assay (Siegers, Heinzmann et al. 1996). Another specific interaction of the modification enzyme with the ABC transporter was shown for NukM and NukT. There, the C-terminal

domain of LanM (LanC-like domain; aa 480-917) interacts with TMD and NBD of NukT, but not with the C39 peptidase domain (Nagao, Aso et al. 2005). Besides the interaction of NisT with NisC, we also noticed an interaction of NisT with NisB, which was not observed in the mentioned study but for SpaT and SpaB (Kiesau, Eikmanns et al. 1997). As secretion of dNisA by NZ900BT was observed (Kuipers, de Boef et al. 2004), an interaction of NisT and NisB was assumed. Furthermore, we observed for the first time the co-elution of NisBC with NisT. Remarkably, the co-elution of transporter with the modification enzymes is not increased by addition of the substrates mNisA or $mNisA_{CCCCA}$. Similar amounts of the enzymes are co-eluted and we conclude, that the interaction of NisT with NisB and NisC is independent of the substrate. One exception was the addition of $mNisA_{CCCCA}$ to NisT/NisC, where the amount of co-elute NisC was reduced. Only the addition of NisB to the sample increased the co-elution of NisC, whether this observation is artificial or part of the translocation mechanism we cannot answer, yet. However, it is commonly accepted that NisB represents the main component of the NisBTC modification/secretion complex (van den Berg van Saparoea, Bakkes et al. 2008, Lubelski, Khusainov et al. 2009).

Besides the modification enzymes NisB and NisC, the substrate pre-NisA is an important factor during the secretion process by NisT. The N-terminal located LP seems to be the most important part of the substrate for the translocation as even non-lanthipeptides are secreted by NisT, when fused to the LP (Kuipers, de Boef et al. 2004). In general, the LP of lanthipeptides pervade several functions e.g. secretion signal, a recognition motif for the PTM enzymes and a guardian that prevents activation of the precursor peptide prior to final maturation (Kuipers, Beerthuyzen et al. 1993, van der Meer, Rollema et al. 1994, Kuipers, de Boef et al. 2004, Li, Yu et al. 2006, Mavaro, Abts et al. 2011, Abts, Montalban-Lopez et al. 2013). Furthermore, no modification of the CP was observed, when the FNLD-box was mutated to alanine residues. But to abolish export by NisBTC totally, additional mutants within the LP had to be introduced (Plat, Kluskens et al. 2011). In good agreement with the described importance of the FNLD-box for the modification enzymes NisB and NisC, we also observed a dependence on the secretion by NisT (Figure 8). All NisA LP mutants display around 7-20% of the WT secretion level. In context of single alanine substitutions, the positions F-18 and L-16 showed the strongest reduction. Secretion of the quadruple mutant ($_{-18}AAAA_{-15}$) was at background level. Plat *et al.* observed similar results for the NisBTC secretion of $mNisA_{F-18A}$ and the other FNLD-box mutants (Plat, Kluskens et al. 2011).

Next, we mutated lysine and aspartate residues in regions, which seem to be conserved in class I lanthipeptides and impose hydrophobicity to the LP. In general, the LP of lanthipeptides have more hydrophilic aa than the CP (van den Hooven, Rollema et al. 1997). The decreased secretion levels of the K-20, D-19, K-9, K-8 and D-7 mutants in comparison to WT NisA supported this assumption (Figure 8). Additionally, mutations of the positions -9 (K-9L) and -7 (D-7A) in the LP of nisin Z also showed a reduced secretion level (van der Meer, Rollema et al. 1994). We assume, that the aa at positions K-20 or D-19 and also K-9, K-8 as well as D-7 are important for an electrostatic interaction with the binding site of NisT. The influence on the secretion of these LP mutants supported this hypothesis and especially the addition of opposite charges at these positions strongly reduced the secretion levels (e.g. mutation K-20E, K-9E and K-8E; Figure 8). The motif $_{-20}$KD$_{-19}$ seemed to be critical for NisT and the secretion process. The aa sequence $_{22}$STKD$_{-19}$ within the LP was shown to be important for NisB binding, but does not influence dehydration (Khusainov, Moll et al. 2013). Therefore, the authors speculate as this aa are important for the complex formation of NisT and NisB. We conclude that the observed reduction is mainly depended on alternate substrate recognition by NisT.

To further investigate the effect of NisA$_{LP}$ on the secretion, we created LanA$_{LP}$-NisA hybrids, in which the NisA$_{LP}$ was exchanged to six other LanA$_{LP}$. It was shown, that the peptide hybrids of SpaS$_{LP}$-NisA /SpaS$_{LP}$-NisZ were secreted and processed by the *L. lactis* and *B. subtilis* 6633 strains via the natural modification and secretion systems (NisBTC; SpaTBC) (Kuipers, Rollema et al. 1993, Rintala, Graeffe et al. 1993). Interestingly, an AMS transporter is also able to secret the Ent53C CP fused to the LP of enterocin A, pediocin PA-1, brochocins A and B as well as lactococcins Qα and Qβ. Here, the interaction of for EnkT from *Enterococcus faecium* NKR-5-3, which secretes Ent53C, with charge aa within the LP was shown to be important (Sushida, Ishibashi et al. 2018). Thus, one might assume that other LPs might be recognized to a certain extent, if their properties fit to the natural LP. We observed that even NisT is able to recognize the LP from other LanAs. Without the modification enzymes NisBC only the Salv$_{LP}$-NisA was detected in the supernatant (Figure 9). However, one has to stress out that Salv$_{LP}$ has the highest sequence identity to NisA$_{LP}$. The secretion efficiency was strongly increased in the NisBTC system and the LP hybrids SpaS$_{LP}$-/Salv$_{LP}$- and Gal$_{LP}$-NisA were also secreted. All LPs embody the FN/DLD-box, but show some diversity concerning the position of the Lys and Asp residues. The secretion level reaches

approximately 33% of WT level ($Salv_{LP}$-NisA hybrid, where the motifs matches the best to NisA). The $SpaS_{LP}$ has $_{-20}$DD$_{-19}$ in front of the FNLD-box, but lacks the Lys, which seemed to be the most critical. The insertion of four aa (KNEL) in the LP of GdmA at this position shifts the critical Lys residue and replaces it to a Glu residue. Furthermore, another insertion at position -9/-8 shifts the other Lys residues and the FNLD-box. All other selected LPs do not have the correct positioning of the Lys and Asp residues due to insertions (LasA and MutA III), aa substitutions or lack of the FNLD-box (e.g. LasA). We conclude, that the specific interaction by these motifs in the LP guide to the substrate binding site in NisT. Moreover, we hypothesize that electrostatic interaction of NisT with the LP and the hydrophobic interaction with the FNLD-box are the main contributions with the substrate binding site.

Based on these data, we propose a model for the entire modification and transport cycle of NisA (Figure 10). First, the ribosomally synthesized NisA is modified by NisB and NisC to mNisA. The modification enzymes are located at the cytoplasmic membrane with NisT to enable efficient translocation after modification. Interestingly, a transient complex of NisT with NisB and NisC might be even possible in the absence of substrate, as our results of the pull-down assay indicated. The transporter NisT is adopting an inward-facing conformation with closed extracellular gates similar to other type I exporters. As the intercellular ATP level in *L. lactis* is around 1 mM, the NBDs of NisT are constantly loaded with ATP (Palmfeldt, Paese et al. 2004). This induces the NBD dimerization following the ATP switch model and the substrate binding site at the TMDs is accessible for pre-NisA.

The complex of $NisB_2C$ delivers the substrate after complete modification, as the binding affinity of NisB to the mNisA (K_D: 10.5 µM) is lower as to dNisA (K_D: 0.31 µM) or uNisA (K_D: 1.05 µM) (Mavaro, Abts et al. 2011). The peptide release after modification of the last lanthionine-ring is initiating the complex release of $NisB_2CA$ (Reiners, Abts et al. 2017). The delivery of the substrate to the transporter NisT is less efficient, when NisC is affect through an inactive mutation or deletion (compare section about the *in vivo* secretion). Furthermore, a pull-down assay of pre-NisAHis with NisB and $NisC_{H331A}$ resulted in higher co-elution of the modification enzymes in comparison to WT enzyme. This implies a more stable complex between NisBC and pre-NisA, where the release of the substrate is hampered (Khusainov, Heils et al. 2011).

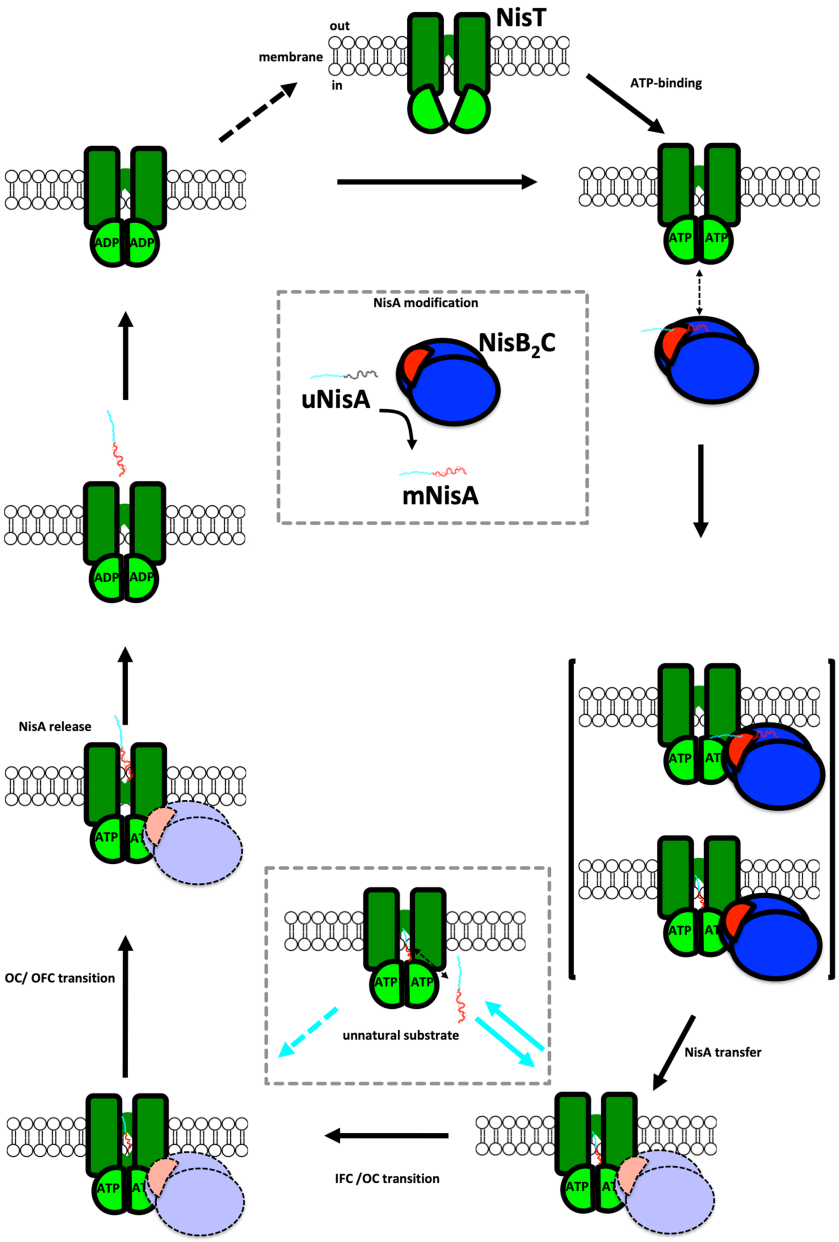


Figure 10: Model of NisT transport cycle.
The scheme illustrates the secretion of mNisA by the modification and secretion complex consisting of a NisB (blue) dimer, one NisC (red) and a NisT (TMD dark green, NBD light green) dimer at the cytoplasmic membrane. Steps during the transport cycle of NisT, where the proposed substrate loaded complex is assembled are illustrated by either brackets or by transparent and dashed line circled NisB and NisC molecules. The modification of unmodified NisA (uNisA) to modified NisA (mNisA) can be independent of the translocation, but it is assumed that the final step of modification and translocation of NisA are a tightly coupled processes. The overall mechanism is a channeling mechanism, in which the substrate is delivered by the modification complex and binding as well as translocation by NisT follows instantaneously.
IFC: inwards-facing conformation; OC: occluded state; OFC: outward-facing conformation

Additionally, an incomplete modification (e.g. dehydration) by introducing mutants in the PTM enzymes (e.g. NisB Y80F or H961A) led to non-secreted peptide, which might reflect an impaired delivery to NisT (Khusainov, van Heel et al. 2015).

As the translocation of mNisA follows a channeling mechanism, a proper function of the modification enzymes is necessary for a high secretion efficiency (van den Berg van

Saparoea, Bakkes et al. 2008). Binding of the substrate favors the transition to a transport competent conformation. Presumably, NisT undergoes the transition from the inward-facing conformation to an occulted conformation as observed for McjD, PtrD or PCAT1 (Choudhury, Tong et al. 2014, Lin, Huang et al. 2015, Morgan, Acheson et al. 2017). Now, the extracellular gate is opening after correct interaction of LP with the recognition site in the cavity resulting in a conformational change to the outward-facing conformation. If the substrate, especially the LP has less strong interactions with the binding site, which is in the case of FNLD-box mutant F-18A as well as the K-20E, D-19K, K-9E, K-8E and D-7K mutants, the transition is less likely. A false interaction such as with other $LanA_{LP}$ hinders or reduces secretion by NisT/NisBTC (compare *in vivo* secretion of $LanA_{LP}$-NisA hybrids).

Upon peptide release ATP hydrolysis resets the transporter and facilitates a new transport cycle. In our study, we observe that the ATPase rate of NisT is not strongly stimulated by the substrate or the interaction partner. In contrast, the ATPase rate was inhibited in the NisBTC system (Figure 5). Thus, the secretion rate is not coupled to the ATPase rate but to the interaction with the lanthipeptide modification complex of $NisB_2C$ and the substrate mNisA. In the channeling mechanism ATP hydrolysis might be needed for the reset of the transporter and does not initiate the translocation itself. But a false initiation hamper the reset, as we observed a reduced ATPase activity of 51-59% with the $LanA_{LP}$-NisA hybrids from subtilin and salivaricin D (Figure 9). This leads to a lower secretion level, as it takes more time for NisT to be transport competent again.

In summary, we have determined the kinetic parameter for the *in vivo* secretion of the nisin modification and secretion complex NisBTC. We show that alternations in the NisBTC system lead to impaired secretion of pre-NisA or even to none secretion, when an ATP hydrolysis deficient mutant of NisT was used. For an efficient secretion by NisT the modification enzymes NisB and NisC are prerequisite and their interaction with NisT enhances the secretion process by the proposed channeling mechanism (van den Berg van Saparoea, Bakkes et al. 2008). We proposed a model of the transport cycle taking into account the properties of the LP in respect to recognition motifs within NisT. Similar to mutacin 1140 the PTM enzyme activity and the transport are tightly coupled processes (Escano, Stauffer et al. 2015), where the different recognition sites within the LP coordinate the binding of the PTM enzymes and transporter to the LP itself or to the CP.

Material and Methods

Chemicals and Antibodies

Fos-choline 16 (FC-16) and CYMAL5 (C5) were obtained from Anatrace. The lyophilized nisin powder (2.5% nisin content) and insulin chain B were obtained from Sigma-Aldrich. The leader peptide of NisA was synthesized and obtained from JPT peptide technologies. Antibodies for $NisT_{NBD}$, NisC and $NisA_{LP}$ peptide were purchased from Davids Biotechnology (Germany) as polyclonal antibodies. NisB antibody was kindly provided by Dr. G. Moll (Lanthio Pharma; Groningen, Netherlands). All standard chemicals were purchased from Sigma-Aldrich or VWR.

Bacterial strains and growth conditions

Strains of *Escherichia coli* and *Lactococcus lactis* and plasmids used in this study are listed in table S1. The strains *E. coli* DH5α or BL21 were grown in LB medium at 37 °C under aerobic conditions with appropriate antibiotics (30 µg/ml kanamycin or 100 µg/ml ampicillin). The transformation of *E. coli* strains was performed following standard procedures.

The strain *L. lactis* NZ9000 (and its variants) was grown in M17 (Terzaghi and Sandine 1975) or minimal medium (MM) (Jensen and Hammer 1993, Rink, Kuipers et al. 2005) at 30 °C under semiaerobic conditions supplemented with 0.5% glucose (GM17/ GMM) and appropriate antibiotics (erythromycin or/and chloramphenicol at a final concentration of 5 µg/ml). To MM a vitamin mix (100x stock solution, 1x final) was added.

For transformation of *L. lactis* NZ9000 with the expression plasmids a standard procedure for preparation of competent cells and electroporation was used as described elsewhere (Holo and Nes 1989).

Cloning of *nisA* mutants

The cloning of *nisA* leader peptide mutants was performed by the substitution of selected residues (positions -20 to -15, -9, -8 and -7) with alanine, lysine or aspartate residues by site-directed mutagenesis. A polymerase chain reaction using Pfu DNA polymerase (Thermo Fischer Scientific or Promega), the template pNZ-SVnisA (or the appropriate derivatives for the double, triple or quadruple mutants), and the matching pair of oligonucleotides (Table S8) was performed according to standard procedures. The sequence of the constructs (Table S9) was verified by DNA sequencing (Microsynth Seqlab).

Cloning of *lanA$_{LP}$-nisA* hybrids

The nucleotide sequence of selected LanA was used to order codon-optimized, synthetic gene fragments (200-300 bp) of *lanA$_{LP}$-nisA* hybrids from Eurofins Genomics. The fragments were amplified by Phusion DNA polymerase (NEB) with the primer pair EcoRIfor and termlanArev (Table S8) to receive overlaps with restriction enzymes site for EcoRI and HindIII. The fragments and the vector pNZ-SVnisA were digested with EcoRI (NEB) and HindIII (NEB). Subsequently, the fragments were ligated into the vector with T4-ligase (NEB) and transformed into *E. coli* DH5α. The sequence of the constructs pNZ-SVlanA$_{LP}$-nisA (Table S9) was verified by DNA sequencing (Microsynth Seqlab).

Cloning of *nisT* and *nisT* variants

A nucleotide sequence for a MCS with 10H nucleotide sequence was ordered as a codon-optimized, synthetic gene fragment from Life Technologies to insert it into the pNZ-SV plasmid (AlKhatib, Lagedroste et al. 2014). The synthetic gene fragments was amplified by Phusion DNA polymerase (NEB) with the primer pair 10Hfor and 10Hrev (Table S8) for Gibson assembly. The plasmid pNZ-SV was amplified by Phusion DNA polymerase (NEB) with the primer pair infupNZ-SVfor and infupNZ-SVrev (Table S8) to linearize the vector. The gene fragment and the vector pNZ-SV were employed in the Gibson assembly by following the manufactures instructions (NEB). The Gibson assembly reactions were transformed into *E. coli* DH5α. The sequence of the construct pNZ-SV10H (Table S9) was verified by DNA sequencing (Microsynth Seqlab).

The *nisT* gene (accession number: Q03203) was amplified using the genomic DNA from *L. lactis* NZ97000 (Kuipers, Beerthuyzen et al. 1993) as a template. There, the Phusion DNA polymerase (NEB) with the primer pair infunisTor and infunisTrev (Table S8) was used to create overhang sequences for Gibson assembly. The plasmid pNZ-SV10H was amplified by Phusion DNA polymerase (NEB) with the primer pair linpNZ-SVfor and linpNZ-SVrev (Table S8) to linearize the vector. Subsequently, the gene and the linearized vector pNZ-SV10H were employed in the Gibson assembly and the reactions were transformed into *E. coli* DH5α. The sequence of the construct pNZ-SV10HnisT (Table S9) was verified by DNA sequencing (Microsynth Seqlab).

To generate the plasmid pIL-SVnisT the *nisT* gene from pNZ-SV10HnisT was amplified by Phusion DNA polymerase (NEB) with the primer pair infupIL-SVfor and infupIL-SVrev (Table

S8) to create overhanging sequences. The plasmid pIL-SV (AlKhatib, Lagedroste et al. 2014) was linearized by Phusion DNA polymerase (NEB) with the primer pair termpNZfor and pnisArev (Table S8). The gene with overhang sequences and the vector was employed in Gibson assembly. The Gibson assembly reactions were transformed into *E. coli* DH5α. Additionally, the 10H nucleotide sequence was deleted by Phusion DNA polymerase (NEB) with the primer pair 10Hfor and infupNZ-SVrev (Table S8). The sequence of the construct pIL-SVnisT (Table S9) was verified by DNA sequencing (Microsynth Seqlab).

To generate *nisT*$_{H551A}$ mutant, a polymerase chain reaction using Pfu DNA polymerase (Thermo Fischer Scientific) or Pfu DNA polymerase (Promega), the template pNZ-SV10HnisT or pIL-SVnisT and the primer pair nisT$_{H551A}$for and nisT$_{H551A}$rev (Table S8) was performed according to standard procedures. The sequence of the constructs (Table S9) was verified by DNA sequencing (Microsynth Seqlab).

The plasmid pNZ-SVnisTNBD$_{H348}$ was obtained by the deletion of the TMD sequence (1-347) from the plasmid pNV-SV10HnisT. The plasmid was amplified with Phusion DNA polymerase (NEB) with the primer pair ΔnisT$_{TMD}$for and ΔnisT$_{TMD}$rev (Table S8). The linear vector was ligated with T4-ligase (NEB) and transformed into *E. coli* DH5α. The sequence of the construct pNZ-SVnisTNBD$_{H348}$ (Table S9) was verified by DNA sequencing (Microsynth Seqlab).

Cloning of nisBTC and *nisBTC* variants

The plasmid pIL-SVnisBTC was generated from pIL-SV and pIL3BTC (Rink, Kuipers et al. 2005). The plasmid pIL3BTC was digested with the restriction enzymes NotI (NEB) and BstXI (NEB) to receive a fragment BTC containing the genes *nisB*, *nisT* and *nisC*. Next, pIL-SV (AlKhatib, Lagedroste et al. 2014) was also digested with NotI and BstXI (pIL-SV**). The fragment BTC and pIL-SV** were ligated with T4-ligase (NEB) and transformed into *E. coli* DH5α. The sequence of the construct pIL-SVnisBTC (Table S9) was verified by DNA sequencing (Microsynth Seqlab).

By using Phusion DNA polymerase (NEB) with the appropriate primer pairs (Table S8) the gene deletions of *nisB*, *nisC* or *nisT* were performed to generate pIL-SVnisBTC derivatives. Subsequently, the linear vectors were ligated with T4-ligase (NEB) and transformed into *E. coli* DH5α. The sequence of the constructs (Table S9) were verified by DNA sequencing (Microsynth Seqlab).

To generate *nisT*$_{H551A}$ and nisC$_{H331A}$ mutants, a polymerase chain reaction using PfuUltra II Fusion DNA polymerase (Agilent Technologies), the template pIL-SVnisBTC and the appropriate pair of oligonucleotides (Table S8) was performed according to standard procedures. The sequence of the new constructs (Table S9) were verified by DNA sequencing (Microsynth Seqlab).

***In vivo* secretion assay: Expression and secretion of pre-NisA**

Strain *L. lactis* NZ9000 harboring the plasmids pIL-SVnisBTC and pNZ-SVnisA hereupon termed NZ9000BTC (Table S1) was used to investigate the *in vivo* secretion activity of the nisin modification and secretion system (NisBTC). The use of pIL-SVnisBTC derivatives and pNZ-SVnisA for transformation into *L. lactis* NZ9000 led to strains described in detail in Table. S1. For each secretion experiment new transformants were prepared and used to inoculate GM17 (Erm+Cm) with one colony. The overnight culture was centrifuged at 4000xg for 20 min and cells were resuspended in GMM. Then, 0.5 l GMM (Erm+Cm) were inoculated to OD_{600} of 0.3 and incubated at 30°C. After 60-90 min the culture (OD_{600} of 0.4-0.5) was induced with 10ng/ml nisin (powder from Sigma-Aldrich dissolved in 50 mM lactic acid). A 50 ml sample before induction (0 hour) and every other hour (1-6h) was taken. For each sample the cell were harvested by centrifugation at 4000xg for 20 min. Subsequently, the cells were resuspended in R-buffer (50 mM Na-phosphate buffer, pH 8, 100 mM KCl, 20% glycerol) to an OD_{600} of 200 and were flash frozen in liquid nitrogen (N_2) and stored at 80 °C until further use. The supernatant was additionally centrifuged at 17,000xg for 20 min at 8°C. Supernatants were kept on ice before the RP-HPLC analysis. Furthermore, 2 ml or 10 ml of the supernatant were precipitated by 1/10 volume (10%) TCA. TCA samples were incubated at 8°C over night. The TCA-precipitated peptide were centrifuged at 17,000xg for 20 min at 8°C and consecutively washed three-times with ice-cold acetone. The pellets were vacuum-dried and resuspended in 60 µl per OD_{600} 1 of 1x SDS-PAGE loading dye containing 5 mM β-mercaptoethanol (β-ME). These resuspended TCA-pellets were analyzed by Tricine-SDS-PAGE and Western blot.

***In vivo* secretion assay: Analysis of cell pellets**

The resuspened cell pellets were thawed on ice and 1/3 (w/v) glass beads (0.3 mm diameter) were added. Cells were disrupted on a vortex-shaker (Disrutor Genie, Scientific Industries). A

cycle of 2 min disruption and 1 min incubation on ice was repeated five times. A low spin step at 17,000xg for 30 min at 8°C and subsequently a high spin step at 100,000xg for 120 min at 8°C was performed. The supernatant of the latter centrifugation step represents the cytoplasmic fraction and the pellet corresponds to the membrane fraction. The SDS-PAGE samples of cytoplasmic and membrane fractions were prepared by adding 4x SDS-PAGE loading dye containing 5 mM β-ME and used for SDS-PAGE as well as Western blot analysis.

***In vivo* secretion assay: Analysis of culture supernatant**

The culture supernatants containing pre-NisA variants were analyzed by RP-HPLC (Agilent Technologies 1260 Infinity II). A LiChrospher WP 300 RP-18 end-capped column and an acetonitrile/water solvent system were used as described previously (Abts, Montalban-Lopez et al. 2013). In the case of modified pre-NisA 300 µl and for all other variants (dehydrated/unmodified) 500 µl sample were injected. Prior to the gradient (20-50% acetonitrile) a washing step of 20% acetonitrile was used to remove most of the casein peptides. The peptide amount or pre-NisA in the supernatant was determined using the peak area integration analyzed with the Agilent Lab Advisor software. A calibration with known amounts of nisin or insulin chain B was used to obtain a linear regression line. Unknown amounts in the *in vivo* secretion assay samples were calculated as nmol or µM based on this linear regression line.

***In vivo* secretion assay: Determination of kinetic parameter**

The secreted pre-NisA amount of the different *L. lactis* NZ9000 strains were plotted against time and fitted using an allosteric sigmoidal fit (1). Note that y is the amount secreted peptide (nmol), V_{max} the maximal secreted amount, x is time (min), $K_{0.5}$ the time point at which 50% of V_{max} is present and h is the Hill slope indicating cooperatively. The analysis was performed using Prism 7.0c (GraphPad).

(1) $$y = V_{max} \frac{X^h}{K_{0.5}{}^h + X^h}$$

The apparent secretion rate ($V_{s\ app}$) was determined by plotting the amount of NisA and NisT against time (min). The values were fitted using a linear regression (2). Note that y is the amount of NisA molecules per NisT molecules ($NisA \bullet NisT^{-1}$), m is the slope $V_{s\ app}$ ($NisA \bullet NisT^{-}$

^{1}•min^{-1}), x is the time (min), and b the y –axis interception. The analysis was performed using Prism 7.0c (GraphPad).

(2) $$y = mx + b$$

Expression and purification of NisT

L. lactis NZ9000 strain was transformed with pNZ-SV10HnisT and placed on SMGG17 agar plates containing 5 µg/ml erythromycin. A GM17 (Erm) overnight culture was inoculated with one colony and incubated at 30°C. A GM17 (Erm) main culture was inoculated to an OD_{600} of 0.1 with the overnight culture. After 3 h incubation, expression was induced by adding 10 ng/ml nisin (powder from Sigma-Aldrich dissolved in 50 mM lactic acid) and further grown for additional 3 h. Cells were harvested by centrifugation at 4000xg for 20 min at 8°C and resuspened in R-buffer (50 mM Na-phosphate buffer, pH 8, 100 mM KCl, 20% glycerol) to an OD_{600} of 200. To the resuspened cells 10 mg/ml lysozyme was added and incubated at 30°C for 30 min. Prior to cell disruption, cells were incubated on ice for 15min. The cell suspension was passed through a homogenizer (M-110P, Microfluidics System) at 1.5 kbar at least four times. The homogenized cell suspension was centrifuged at 12,000xg for 30 min at 8°C. Subsequently, the supernatant was centrifuged at 100,000xg for 120 min at 4°C to collect the membrane fraction. Membranes were resuspended with R-buffer containing 10 mM imidazole and 0.5 mM AEBSF. The total membrane protein concentration was measured by BCA assay (Thermo Fischer Scientific) and the concentration was adjusted to 5-7.5 mg/ml. Membranes were solubilized with 1% (w/v) of the lipid-like detergents FC-16 (Anatrace) for 1 h at 8°C. Insoluble material was removed by centrifugation at 100,000xg for 30 min at 4°C. The supernatant was applied to a 5ml IMAC (Immobilized Metal-Ion-Affinity Chromatography) HiTrap chelating column (GE Healthcare) preloaded with 100 mM zinc sulphate and equilibrated with low IMAC1 buffer (50 mM Na-phosphate buffer, pH 8, 100 mM KCl, 20% glycerol and 10 mM imidazole) containing 0.5 mM AEBSF and 0.005% FC-16. Consecutively, non-bound protein was washed and the buffer was exchanged to low IMAC2 buffer (50 mM Tris-HCl pH 8, 100 mM KCl, 10% glycerol, 10 mM imidazole, 0.5 mM AEBSF and 0.005% FC-16). After an additional washing step with 30% high IMAC buffer (50 mM Tris-HCl pH 8, 100 mM KCl, 10% glycerol, 150 mM histidine, 0.5 mM AEBSF and 0.005% FC-16), 10HNisT was eluted with 100% high IMAC buffer. The elution fractions containing NisT were pooled, 10 mM DTT was added and further concentrated with a Vivaspin20 100 kDa

molecular weight cut off (MWCO) centrifugal concentrator (Sartorius AG). Next, a size exclusion chromatography (SEC) was performed, where the concentrated protein sample was applied onto a Superose 6 10/300 GL column (GE Healthcare) equilibrated with SEC buffer (25 mM Tris-HCl pH 8, 50 mM KCl, 10% glycerol, 0.5 mM AEBSF, 2 mM DTT and 0.0015% FC-16). The main peak fractions were analyzed via SDS-PAGE and further concentrated via a Vivaspin6 100 kDa MWCO centrifugal concentrator (Sartorius AG) until a concentration of 50 µM was reached. The protein concentration was determined by NanoDrop spectrophotometer (Thermo Fischer Scientific) using a molar extinction coefficient of 86,180 $M^{-1} \bullet cm^{-1}$ and the molecular mass of 72.6 kDa. Aliquots of 50 µM 10HNisT were flash frozen in liquid N_2 and stored at 80 °C until further use. The NisT variants $10HNisT_{H551A}$ was expressed following the same protocol.

Expression and purification of $NisT_{NBD}$

L. lactis NZ9000 was transformed with pNZ-$SVnisTNBD_{H348}$ and placed on SMGG17 agar plates containing 5 µg/ml erythromycin. A GM17 (Erm) overnight culture was inoculated with one colony and incubated at 30°C. A GM17 (Erm) main culture was inoculated to an OD_{600} of 0.1 with the overnight culture. After 90 min incubation the culture was incubated on ice. Following, expression was induced by adding 10 ng/ml nisin (powder from Sigma-Aldrich dissolved in 50 mM lactic acid) and further grown for additional 3 h at 20 °C. Cells were harvested by centrifugation at 4000xg for 20 min at 8°C and resuspened in R-buffer (50 mM Na-phosphate buffer, pH 8, 100 mM KCl, 20% glycerol) to an OD_{600} of 200. Then, 10 mg/ml lysozyme was added and incubated at 30°C for 30 min. Prior to cell disruption, cells were incubated on ice for 15 min. Following, the cell suspension was passed through a homogenizer (M-110P, Microfluidics System) at 1.5 kbar at least four times. The homogenized cell suspension was centrifuged at 12,000xg for 30 min at 8°C. Subsequently, the supernatant was centrifuged at 100,000xg for 120 min at 4°C. The supernatant was applied to a 5ml IMAC HiTrap chelating column (GE Healthcare) preloaded with 100 mM zinc sulphate and equilibrated with low IMAC1 buffer (50 mM Na-phosphate buffer, pH 8, 100 mM KCl, 20% glycerol and 10 mM imidazole). Consecutively, non-bound protein was washed by a 30% step with high IMAC buffer (50 mM Na-phosphate buffer, pH 8, 100 mM KCl, 20% glycerol, 500 mM imidazole). The protein $10HNisT_{NBDH348}$ was eluted by a gradient of 30 to 100% high IMAC buffer. The elution fractions containing $NisT_{NBDH348}$ (T_{NBD}) were pooled, 10

mM DTT was added and further concentrated with an Amicon 10 kDa MWCO centrifugal concentrator (Millipore). Next, the concentrated protein sample was applied onto a HiLoad Superdex 200 16/60 prep grade column (GE Healthcare) equilibrated with SEC buffer (25 mM CAPS pH 10, 20% glycerol, 2 mM DTT). The main peak fractions are analyzed via SDS-PAGE and further concentrated with an Amicon 10 kDa MWCO centrifugal concentrator (Millipore). The protein concentration was determined by NanoDrop spectrophotometer (Thermo Fischer Scientific) using a molar extinction coefficient of 45,840 $M^{-1} \bullet cm^{-1}$ and the molecular mass of 32.2 kDa. Aliquots of 30 µM T_{NBD} were flash frozen in liquid N_2 and stored at 80 °C until further use.

Expression and purification of nisin modification enzymes NisB and NisC

NisB and NisC were expressed and purified as described previously (Mavaro, Abts et al. 2011, Abts, Montalban-Lopez et al. 2013, Reiners, Abts et al. 2017). Aliquots of concentrated proteins (90 µM, 110 µM) were flash frozen in liquid nitrogen N_2 and stored at 80 °C until further use. For the *in vitro* ATPase activity assay and pull-down assay the buffer of the proteins was exchanged via PD SpinTrap G-25 spin columns (GE Healthcare) to activity assay buffer (25mM Tris-HCl pH 7.5, 50 mM KCl) containing 400 mM glutamate and 0.4% CYMAL5 (3 x cmc). Proteins were stored on ice and directly used for the *in vitro* assays. The protein concentration was determined by NanoDrop spectrophotometer (Thermo Fischer Scientific) by using the theoretical molar extinction coefficient and the molecular mass of the proteins.

Expression and purification of NisA variants

All pre-NisA variants used in this study were expressed and purified as described previously with modifications (Lubelski, Khusainov et al. 2009, Mavaro, Abts et al. 2011). Briefly, pre-NisA variants were purified after 5 h (instead overnight) expression in MM from 2 l (0.5 l in the case of the secretion experiments) culture supernatant via cation-exchange chromatography (cIEX). The cell-free supernatant was diluted 1:1 with LA buffer (50 mM lactic acid, pH 3) and applied to a 5 ml HiTrap SP Sepharose column (GE Healthcare). The column was washed by applying a pH gradient from 100% LA buffer to 100% H buffer (50 mM HEPES-NaOH, pH 7). Finally, the peptides were eluted with H buffer containing 1 M NaCl. The purification of unknown peptides ($LanA_{LP}$-NisA hybrids) was performed by a two buffer system of LA buffer and LA buffer containing 1 M NaCl. The fractions containing the

peptides were pooled and concentrated with an Amicon (Millipore) centrifugal concentrator. First, the flow through (FT) fraction of a 30 kDa molecular weight cut off (MWCO) concentrator was collect. Then, a 3 kDa MWCO filter was used to concentrate the peptides in the FT fraction. Aliquots of concentrated peptides were flash frozen in liquid N_2 and stored at 80 °C until further use. For concentration determination the pre-NisA variants were analyzed by RP-HPLC (Agilent Technologies 1260 Infinity II) with a LiChrospher WP 300 RP-18 end-capped column and an acetonitrile/water solvent system as described previously (Abts, Montalban-Lopez et al. 2013).

***In vitro* ATPase activity assay**

The ATPase activity of NisT was determined with the malachite green assay as described previously with experimental alternations (Infed, Hanekop et al. 2011). In this assay the release of free inorganic orthophosphate after ATP hydrolysis was colorimetric quantified based on a Na_2HPO_4 standard curve.

All reactions were performed at 30°C in a total volume of 30 µl in activity assay buffer containing 0.4% CYMAL5 and 10 mM $MgCl_2$.

In each reaction ~2 µg of detergent-solubilized and purified NisT was used and the reaction was started by adding ATP (0-5 mM). The background of the reaction was a sample without the addition of 10 mM $MgCl_2$. After 30 min the reaction was stopped by transferring 25 µl of each reaction into a 96-well plate containing 175 µl stop-solution of 20 mM sulphuric acid. Consecutively, 50 µL of a staining solution (0.096% (w/v) malachite green, 1.48% (w/v) ammonium heptamolybdate and 0.173% (w/v) Tween-20 in 2.36 M sulphuric acid) was added. After 10 min the amount of free inorganic orthophosphate was quantified by measuring the absorption at 595 nm using an iMark microplate reader (Bio-Rad).

The specific ATPase activity of NisT was plotted against ATP concentrations and fitted using the Michaelis Menten equation (3). Note that y is the reaction velocity, V_{max} the maximal reaction velocity, x is the substrate concentration and K_m the Michalis-Menten constant. The analysis was performed using Prism 7.0c (GraphPad).

(3) $$y = V_{max}\frac{x}{K_m + x}$$

For the reactions with substrates (pre-NisA variants) or interaction partners (NisB and NisC) NisT was pre-incubated at 30°C for 10 min before ATP was added to start the reaction. All

reactions were performed at 30°C in a total volume of 30 µl in activity assay buffer containing 0.4% CYMAL5, 400 mM glutamate and 10 mM $MgCl_2$. In each reaction ~2 µg of detergent-solubilized and purified NisT was used and the reaction was started by adding 5 mM ATP and stopped after 15 min following the procedure described above. In this reaction the concentration of the different substrates (0-40 µM) and /or interaction partner was varied and the ATPase activity was normalized to the specific ATPase activity of NisT without substrate/interaction partner. In these cases, the background was subtracted prior to normalization.

***In vitro* pull-down assay**

The immobilization of 10HNisT to Ni-NTA magnetic beads (Quiagen) was performed as described in the manufactures manual. In brief, ~ 15 µg 10HNisT was incubated with Ni-NTA magnetic beads for 30 min at 30°C. Excess of protein was removed by three washing steps with activity assay buffer containing 0.4% CYMAL5 and 400 mM glutamate. Interaction partners NisC and NisB were incubated in 1:1 molar ratio (but > 10x molar excess to NisT) separately with or without mNisA/ $mNisA_{CCCCA}$ (20x molar excess to NisT) in activity assay buffer containing 0.4% CYMAL5, 400 mM glutamate and 5 mM MgATP for 15 min on ice. Next, interaction partner were added to 10HNisT immobilized to Ni-NTA magnetic beads and incubated for 1 h at 30°C. Positive control (only 10HNisT) and negative control (NisB, NisC) samples were prepared by incubating the proteins with Ni-NTA magnetic beads separately. After binding the Ni-NTA magnetic beads were washed six times with activity assay buffer. Finally, 10HNisT was eluted by adding activity assay buffer containing 50 mM EDTA. The SDS-PAGE samples of pull-down assay fractions were prepared by adding 4x SDS-PAGE loading dye containing 5 mM β-ME and used for Western blot analysis.

SDS-PAGE and immunoblotting for protein/ peptide analysis

In general, the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) experiments were performed using standard procedures (Laemmli 1970). In the SDS-PAGE gels the acrylamide portion was 10% to have a separation range from 30 to 120 kDa for the proteins NisC (~48 kDa), NisT (~69 kDa) and NisB (~117 kDa).

All peptides (e.g. pre-NisA variants) from secretion experiments or from cIEX purification were analyzed by Tricine-SDS PAGE (Schagger 2006). For Tricine-SDS-PAGE gels (12%) a

Mini-Protean system (Bio-Rad) was used. Tricine-SDS PAGE and SDS PAGE gels were stained with colloidal coomassie (cc) (Dyballa and Metzger 2009).

All immunoblotting experiments were conducted following standard procedures. For the quantification of NisT in the membrane fractions (*in vivo* secretion assay) various amounts of a T_{NBD} standard (stock solution 12.5 µg/ml) was added to create a calibration curve. The band intensities on the Western blots were processed and determined by ImageJ (Schneider, Rasband et al. 2012). The amount of NisT was determined as pmol protein of the different membrane fractions of the time points 2-6 h.

Mass spectrometry analysis

Pre-NisA variants were either desalted via ZipTip (C18 resin) purification accordingly to the manufacture manual (Merk-Millipore) or by RP-HPLC and vacuum dried. For the MALDI-ToF-MS analysis the vacuum dried pellet was dissolved in 50% acetonitrile solution containing 0.1% TFA and analyzed as described elsewhere (Lagedroste, Reiners et al. 2019).

Lan/MeLan analysis by CDAP coupling assay

To investigate the thioether ring formation, a coupling assay with CDAP was performed as described elsewhere (Lagedroste, Reiners et al. 2019). With this assay potential free cysteine residues within the core peptide are coupled resulting in a mass shift of 25 Da per CDAP addition.

Bioinformatic analysis of LanA and LanT

The sequences of class I and II lanthipeptide (LanA) were retrieved from Bagel4 database (van Heel, de Jong et al. 2018) and used for a sequence/phylogenetic analysis with Phylogeny.fr (Dereeper, Guignon et al. 2008). First a sequence alignment with MUSCULE was generated. Then, the phylogeny with PhyML was created to compute the maximum likelihood. The phylogenetic trees are rendered with TreeDyn. Finally, the phylogenetic trees are displayed with iTOL (Letunic and Bork 2016). The sequence identity of selected $LanA_{LP}$ was determined by using the pairwise sequence alignment tool SIM (www.expasy.org/sim/)(Huang, Hardison et al. 1990). Conserved residues in $LanA_{LP}$ are highlighted with the web tool WebLogo 3 (Crooks, Hon et al. 2004). The sequences of the

corresponding ABC transporter (LanT) were retrieved from Uniprot or the NCBI database and analyzed similarly to LanA sequences.

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Author contributions

M.L., S.H.J.S. and L.S. designed the experiments, M.L. performed the experiments, J.R. purified NisB and NisC, M.L., S.H.J.S. and L.S. analyzed and interpreted the data, M.L., S.H.J.S. and L.S. wrote and revised the manuscript.

Supplemental information

Mechanism of lanthipeptide nisin secretion

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Figures supplemental information

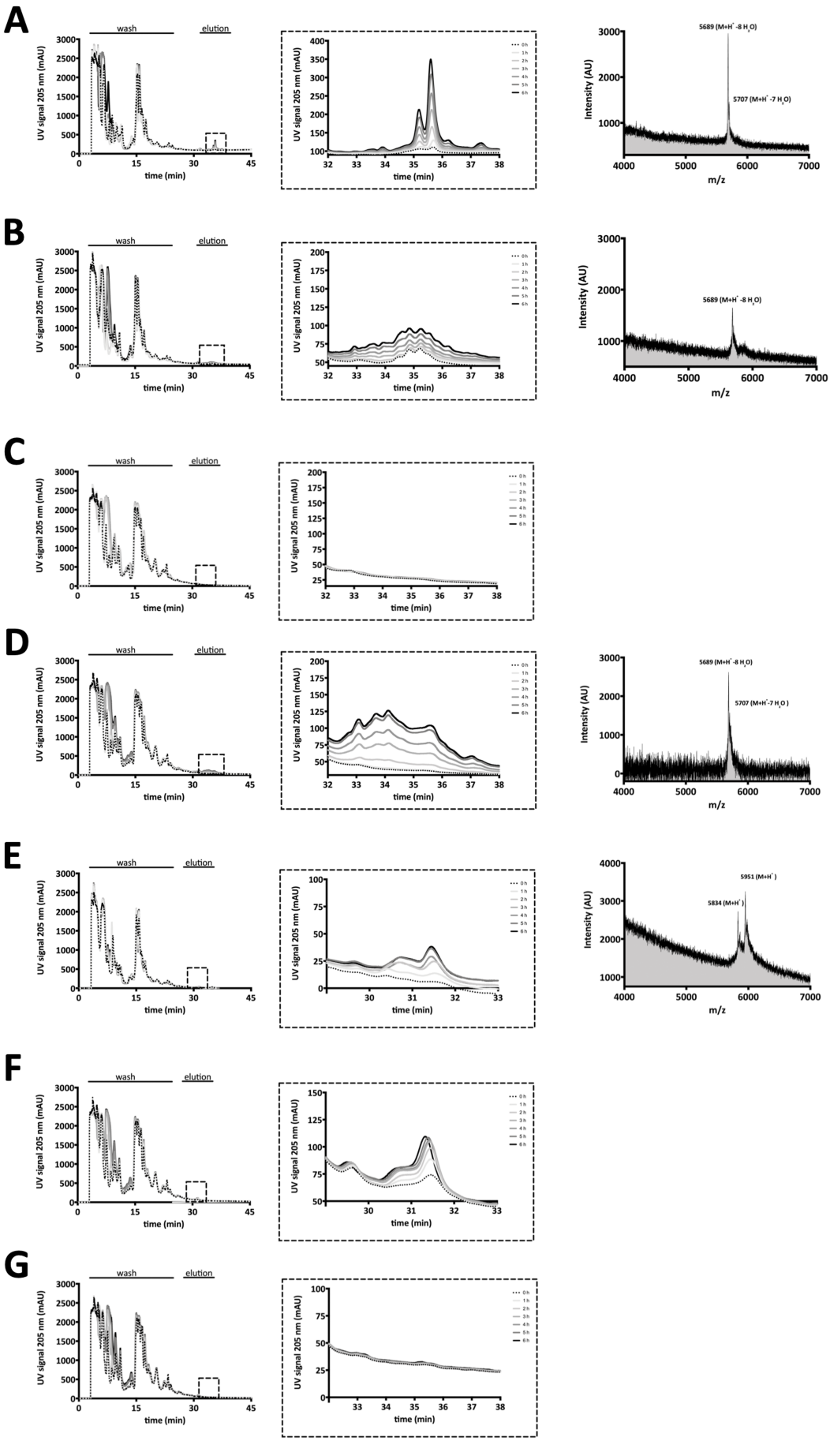

Figure S1: RP-HPLC chromatograms and MALDI-ToF-MS spectra of *in vivo* secretion assay.
The supernatants of pre-NisA secreting *L. lactis* NZ9000 strains were employed to an RP-HPLC analysis. The pre-NisA variants (mNisA, dNisA and uNisA) were separated from other peptides in the supernatant by an acetonitrile/water gradient on a C-18 RP-HPLC column (left panel). The elution fractions (dashed square; middle panel) were further analyzed by MALDI-ToF-MS (right panel) to verify the correct mass. Integration of the corresponding peaks enables the determination of peptide amounts (nmol). Supernatant analysis of *L. lactis* strains (**A**) NZ9000BTC, (**B**) NZ9000BTC$_{H331A}$, (**C**) NZ9000BT$_{H551A}$C, (**D**) NZ9000BT, (**E**) NZ9000T, (**F**) NZ9000TC and (**G**) NZ9000BC.

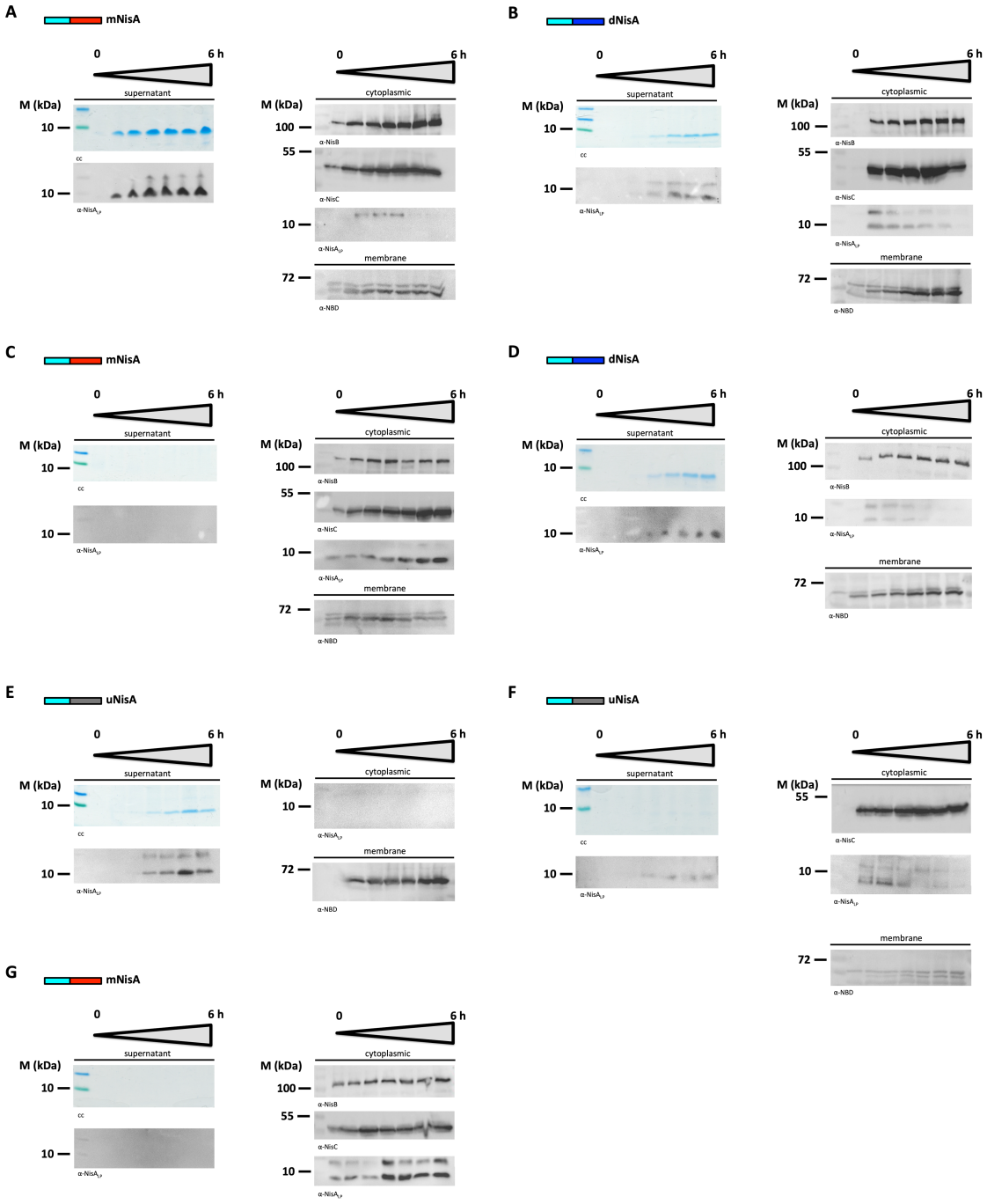

Figure S2: Tricine-SDS-PAGE and Western blot analysis of *in vivo* secretion assay.
The supernatants of pre-NisA secreting *L. lactis* NZ9000 strains from different time points were TCA-precipitated and the pellets were analyzed by Tricine-SDS-PAGE and Western blot with a α-NisA$_{LP}$ antibody (left panel). The Tricine-SDS-PAGE gels were stained by colloidal coomassie (cc). The cytoplasmic and membrane fractions of the cell from different time points were analyzed by Western blot (right panel) with the specific

antibodies (α-NisB, α-$NisA_{LP}$, α-NisC and α-NBD). Sample analysis of *L. lactis* strains **A**) NZ9000BTC, (**B**) $NZ9000BTC_{H331A}$, (**C**) $NZ9000BT_{H551A}C$, (**D**) NZ9000BT, (**E**) NZ9000T, (**F**) NZ9000TC and (**G**) NZ9000BC.
M: marker protein bands

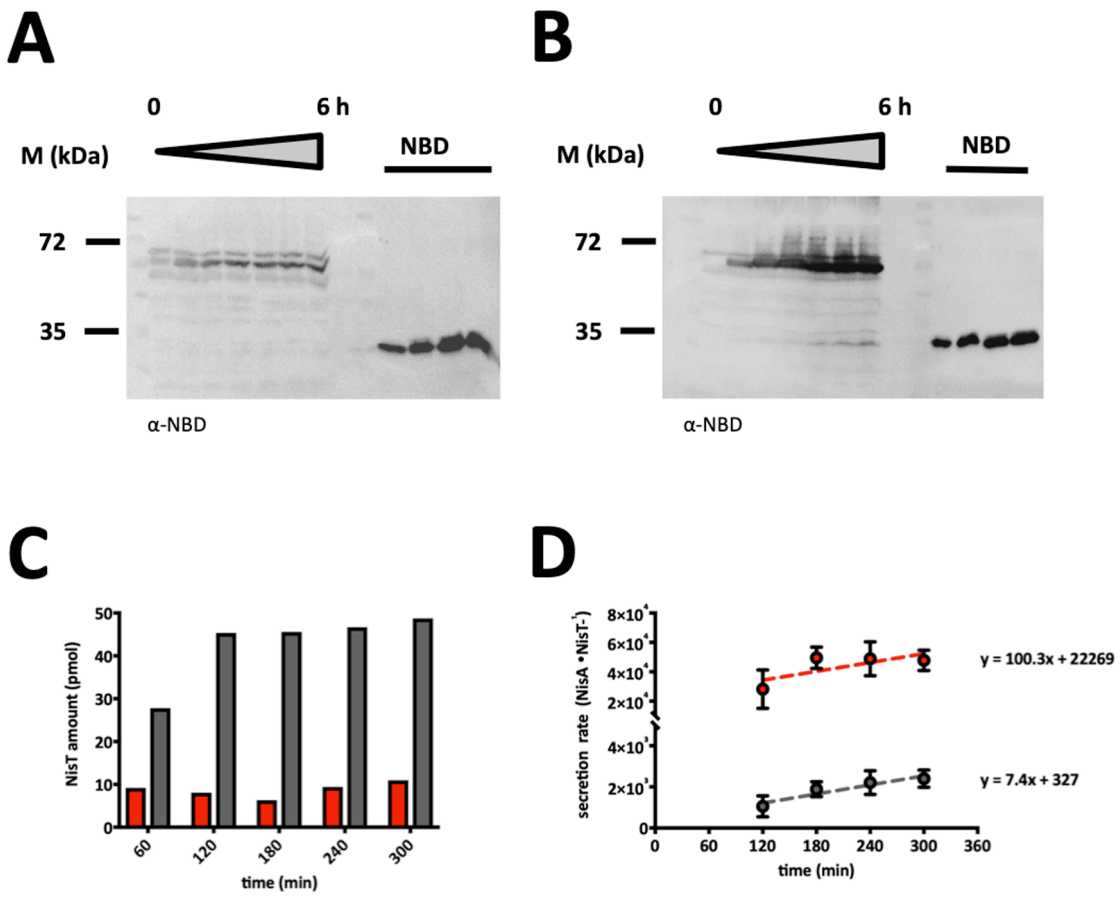

Figure S3: Determination of NisT amount of *in vivo* secretion assay samples.
Western blot analysis of membrane fractions from strains (**A**) NZ9000BTC, (**B**) NZ9000BT and (**C**) NZ9000T with specific antibody α-NBD. Standard of known amount of T_{NBD} is used to determine (**D**) NisT amount in membrane (pmol) of time points 2-5 h. (**E**) A linear regression of plotted values of nmol NisA per nmol NisT against the time (min) results in a apparent secretion rate ($V_{s\ app}$).
M: marker protein bands

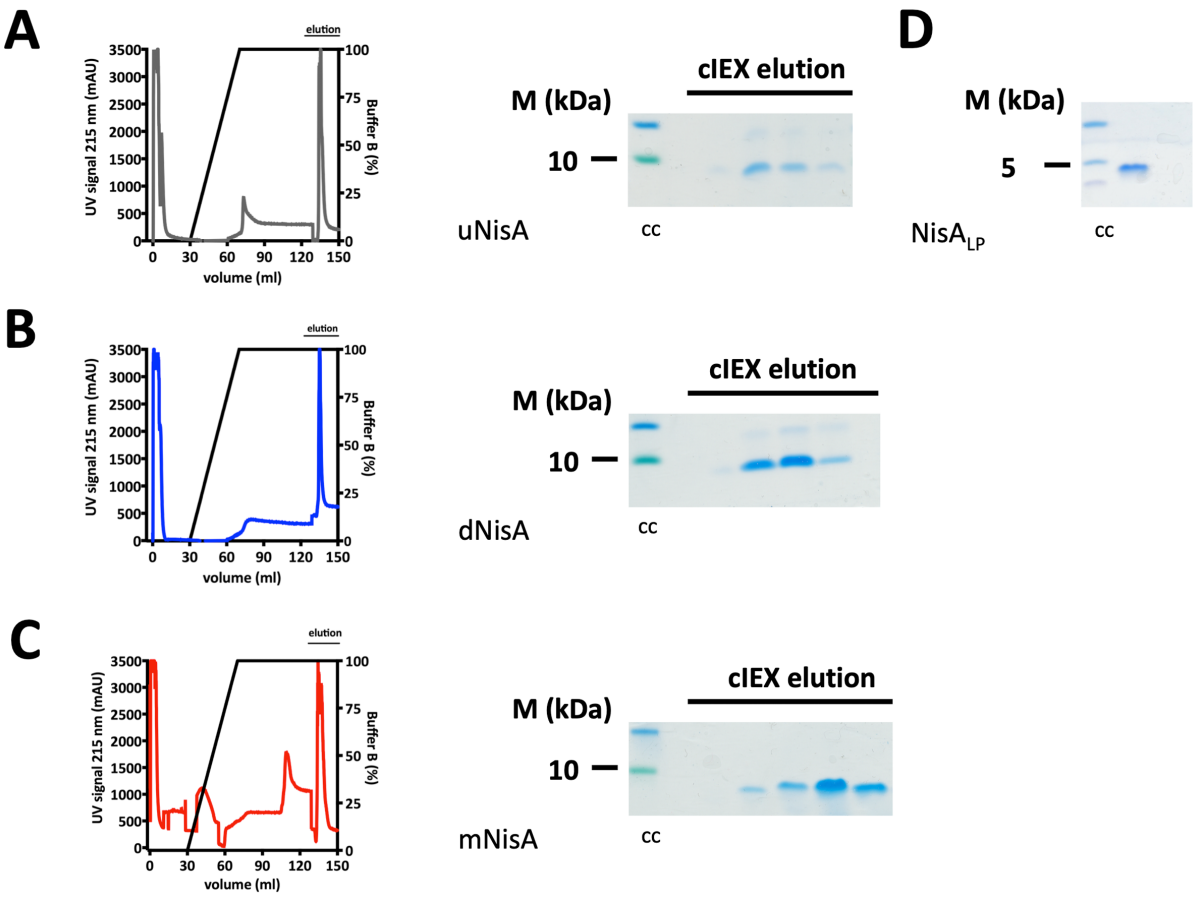

Figure S4: Chromatograms of cIEX from uNisA, dNIsA and mNisA.
The cIEX chromatograms and Tricine-SDS-PAGE of pre-NisA variants (**A**) uNisA (grey), (B) dNisA (blue) and (C) mNisA (red). (**D**) The purity of synthesized NisA leader peptide (NisA$_{LP}$) was controlled by Tricine-SDS-PAGE. The Tricine-SDS-PAGE gels were stained by colloidal coomassie (cc).
M: marker protein bands

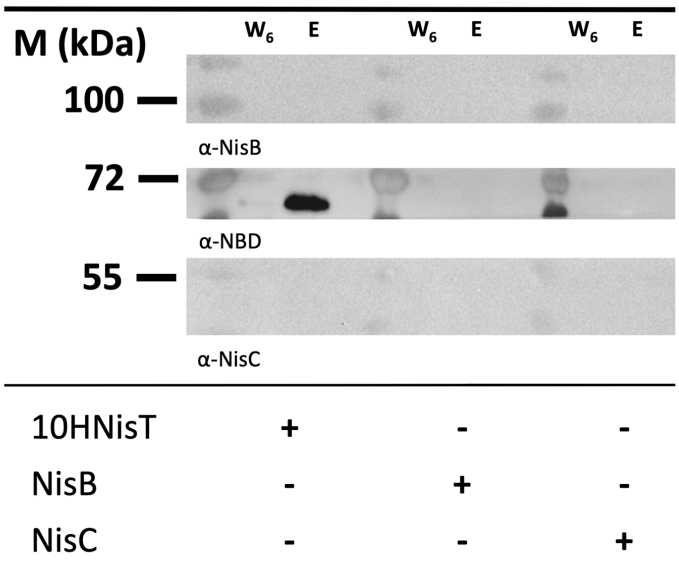

Figure S5: Western blot of pull-down assay with 10HNisT, NisB and NisC.
Controls of the pull-down assay were blotted and analyzed with the specific antibodies of α-NisB, α-NBD and α-NisC. Samples of the last washing step (W$_6$) and the elution fractions (E) were used to detect 10HNisT, NisB or NisC in the samples.
M: marker protein bands; +: addition of protein; -: no protein added

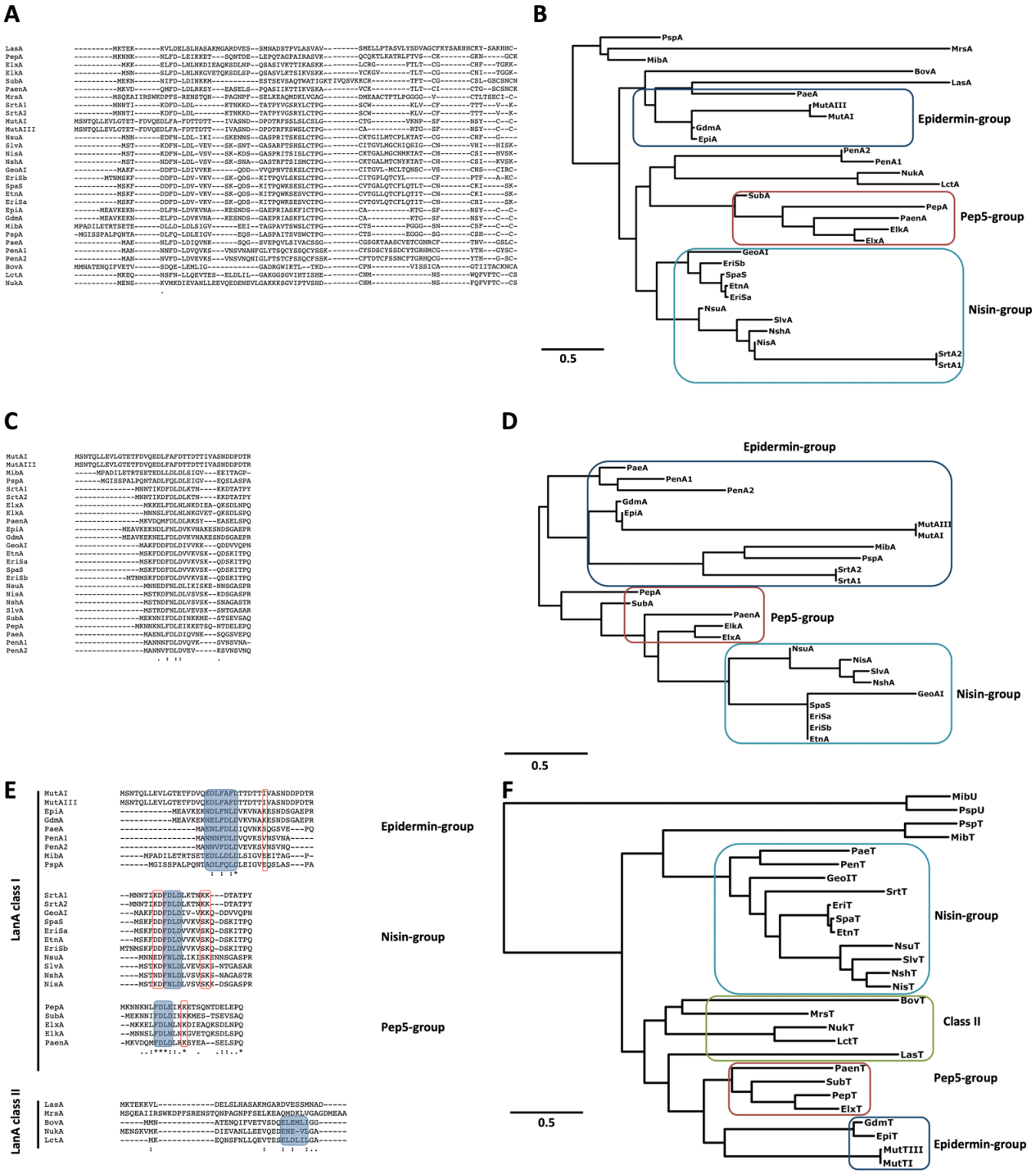

Figure S6: Sequence alignment and phylogenetic analysis of LanA and corresponding LanT.
For the sequence alignment and phylogeny analysis of selected class I and class II lanthipeptides (LanA, see Tab. S4) the web services phylogeny.fr was used (Dereeper, Guignon et al. 2008). There, the multiple sequence alignment is performed with MUSCULE. Following, PhyML sets up the phylogeny, whereas TreeDyn does the tree rendering. Finally, the phylogenetic tree is visualized with iTOL (Letunic and Bork 2016). (**A**) Sequence alignment and (**B**) phylogenetic tree of LanA. (**C**) Sequence alignment and (**D**) phylogenetic tree of $LanA_{LP}$. (**E**) Sequence alignment of $LanA_{LP}$ groups, where the conserved enzyme recognition site is marked by a blue box and a red box marks other conserved positions. (**F**) Phylogenetic tree of the corresponding LanT or $Lan_{C39P}T$. In the phylogenetic tree the relative branch length is indicated by a black line, which displays the value 0.5. Further, are the different groups of lanthipeptides shown by colored boxes (nisin-group: light blue; Pep5-group: red; epidermin-group: dark blue; class II lanthipeptides: green).

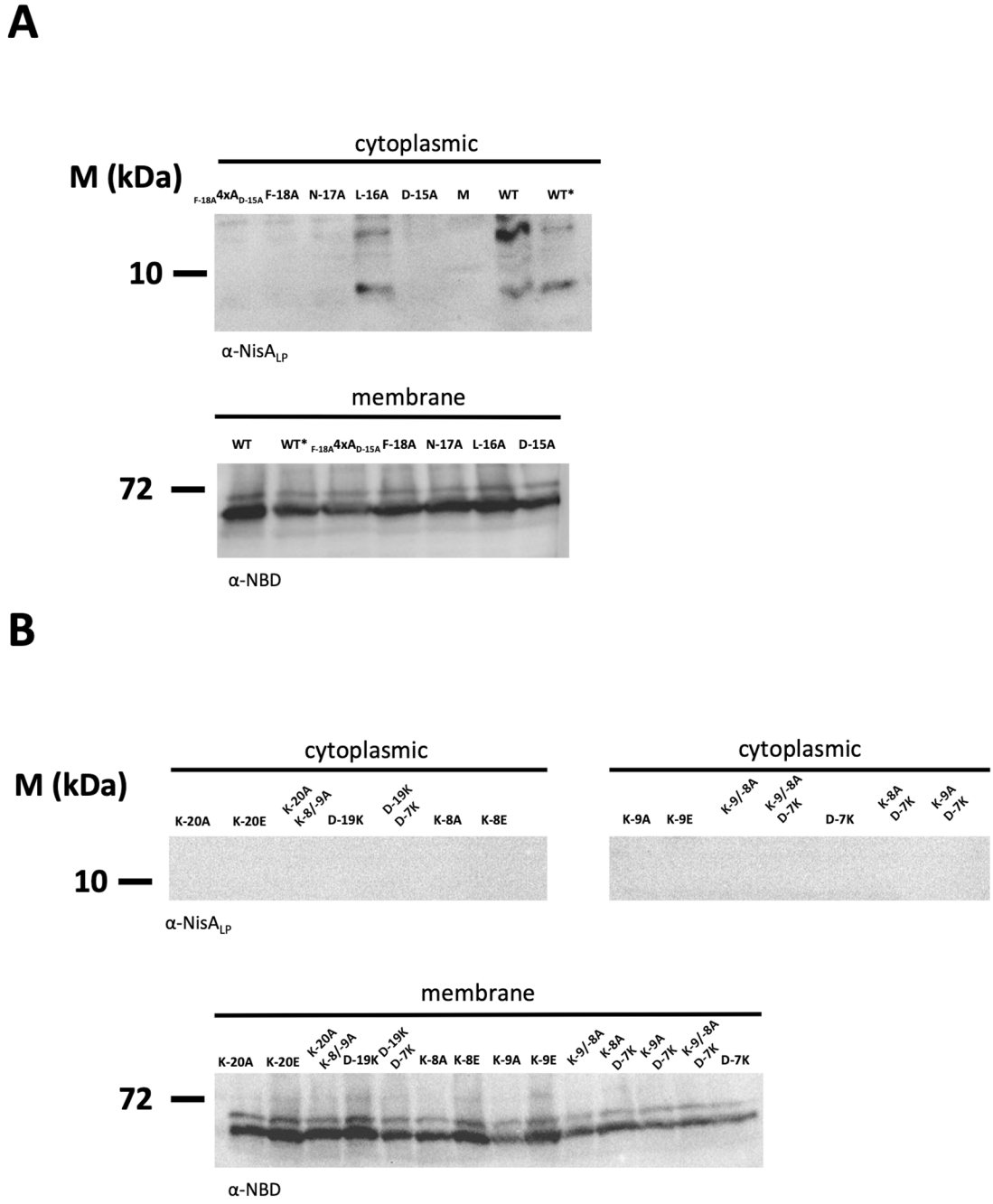

Figure S7: Western blot of *in vivo* secretion assay of $NisA_{LP}$ mutants.
The cytoplasmic and membrane fraction were blotted and analyzed against the specific antibodies of α-$NisA_{LP}$ and α-NBD. (**A**) FNLD-box mutants secreted by NZ9000T. (**B**) Lys and Asp residue mutants secreted by NZ9000T.
M: marker protein bands; WT: NZ9000T ; WT*: $NZ9000T_{H551A}$

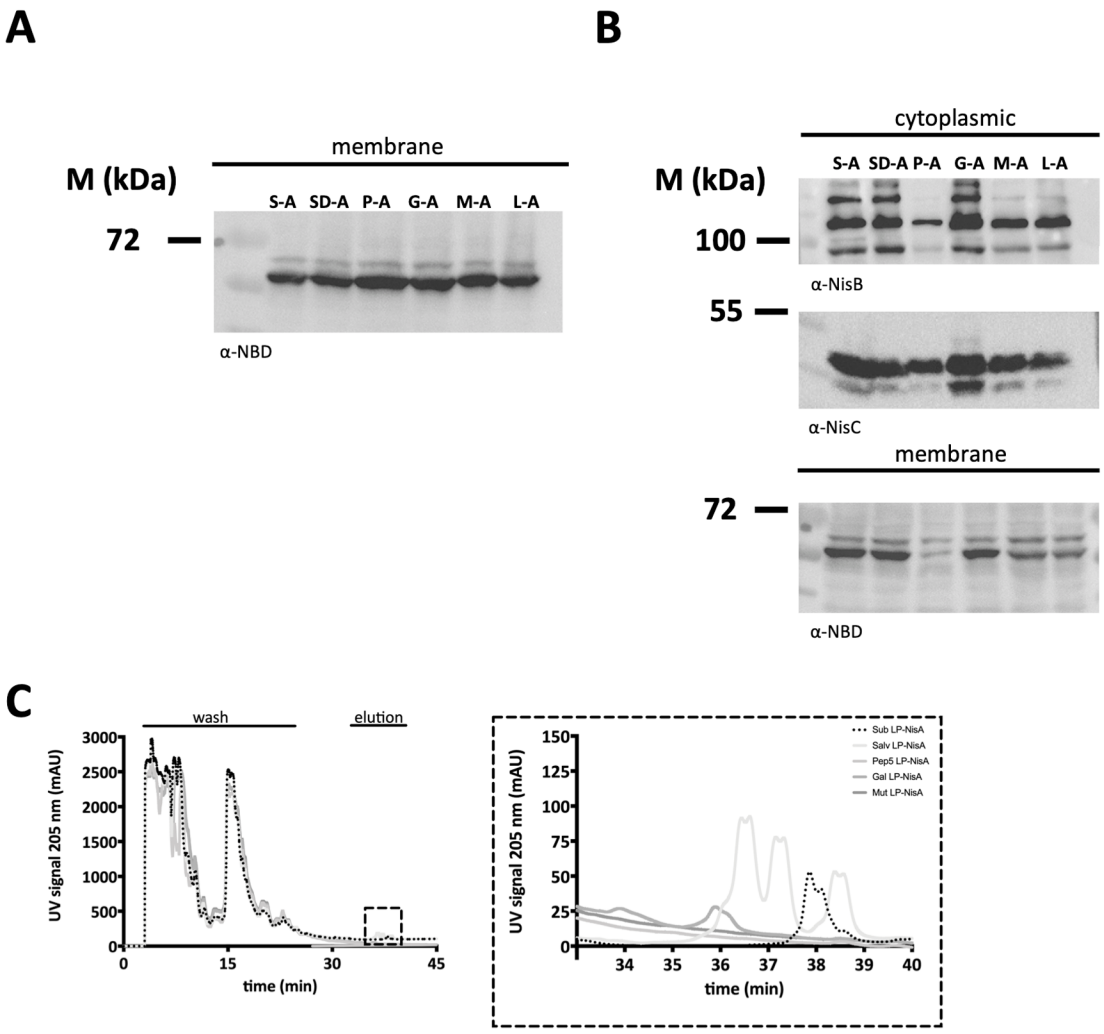

Figure S8: Western blot and RP-HPLC chromatogram of *in vivo* secretion assay of LanA$_{LP}$-NisA hybrids.
The cytoplasmic and membrane fraction were blotted and analyzed against the specific antibodies of α-NisA$_{LP}$, α-NisB, α-NisC and α-NBD. (**A**) LanA$_{LP}$-NisA hybrids secreted by NZ9000T. (**B**) LanA$_{LP}$-NisA hybrids secreted by NZ9000BTC. (**C**) RP-HPLC chromatograms of five hour samples of LanA$_{LP}$-NisA hybrids secreting strain NZ9000BTC. Dashed square exhibit section of 33 to 40 min.
M: marker protein bands; S-A: SpaS$_{LP}$-NisA; SD-A: SlvA$_{LP}$-NisA; P-A: PepA$_{LP}$-NisA; G-A: GdmA$_{LP}$-NisA; M-A: MutAIII$_{LP}$-NisA; L-A: LasA$_{LP}$-NisA

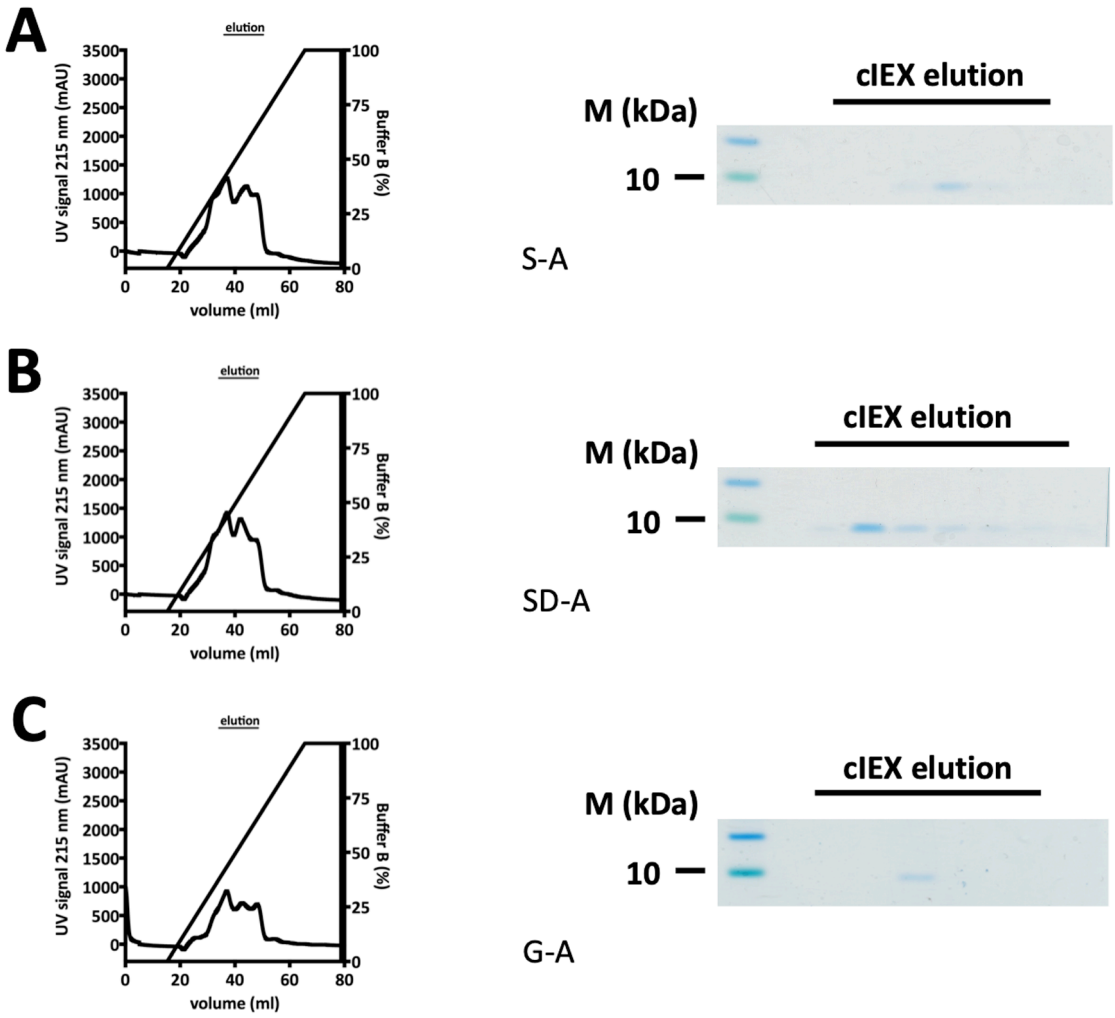

Figure S9: Chromatograms of cIEX from $SpaS_{LP}$-NisA, $SlvA_{LP}$-NisA and $GdmA_{LP}$-NisA.
The cIEX chromatograms and Tricine-SDS-PAGE of the secreted $LanA_{LP}$-NisA hybrids (**A**) $SpaS_{LP}$-NisA (S-A), (**B**) $SlvA_{LP}$-NisA (SD-A) and (**C**) $GdmA_{LP}$-NisA (G-A). The Tricine-SDS-PAGE gels were stained by colloidal coomassie.
M: marker protein bands

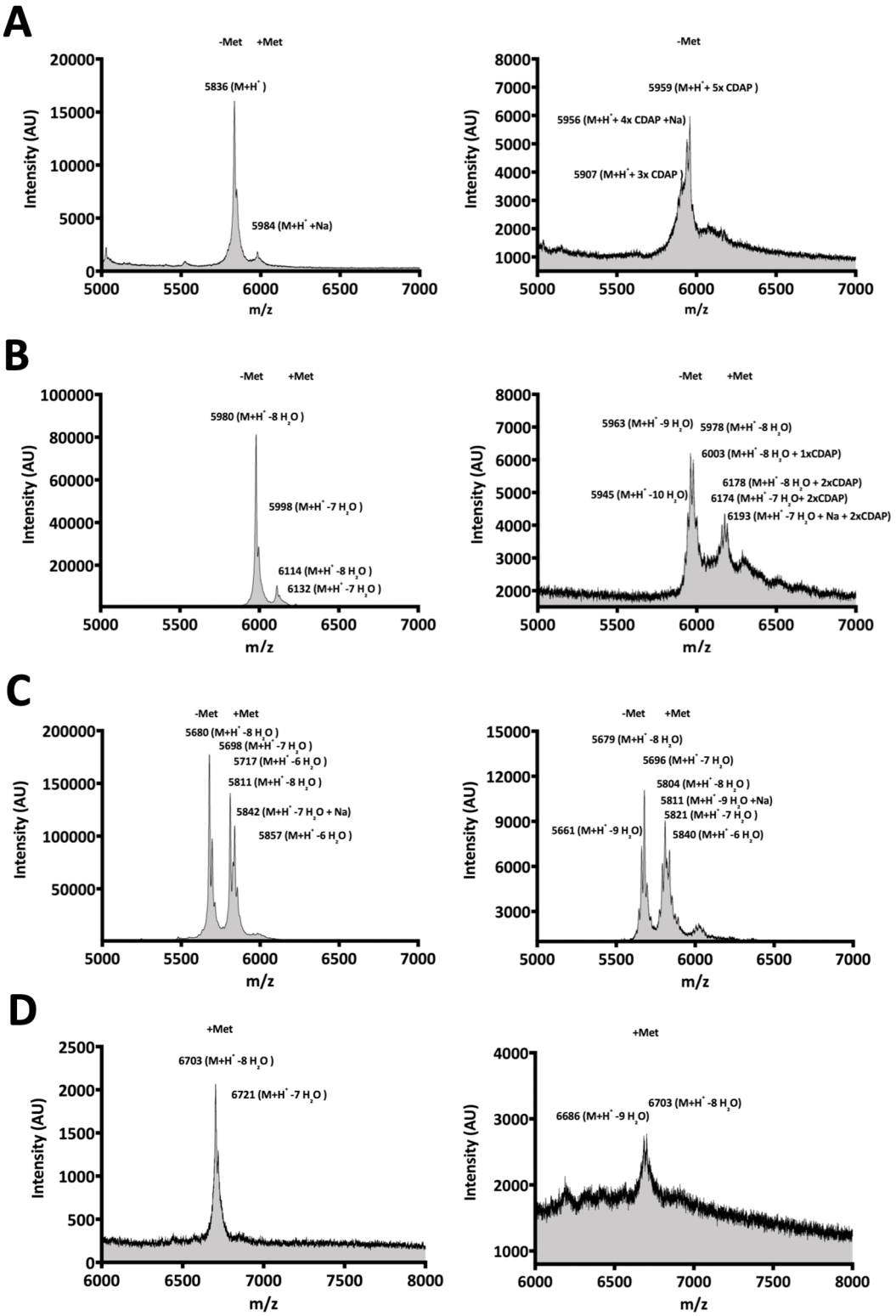

Figure S10: MALDI-ToF-MS spectra of $LanA_{LP}$-NisA hybrids after CDAP coupling assay.
The modification of MeLan or Lan was detected by CDAP coupling assay, resulting in a mass shift of 25 Da per free Cys residue. (**A**) Control of the coupling by using uNisA with five maximal additions (125 Da). Purified and zip-tipped $LanA_{LP}$-NisA hybrids (**B**) $SpaS_{LP}$-NisA, (**C**) $SlvA_{LP}$-NisA and (**D**) $GdmA_{LP}$-NisA were employed to the same assay conditions. The left panel shows the control without CDAP and the right panel shows the detected mass after CDAP coupling.

Table supplemental information

Table S1: Strains and plasmids used in this study.

strain	plasmid	name	properties	reference
Escherichia coli DH5α		DH5α	F– Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK–, mK+) *pho*A *sup*E44 λ– *thi*-1 *gyr*A96 *rel*A1	(Grant, Jessee et al. 1990)
Lactococcus lactis NZ9000	-	NZ9000	expression strain; *nisR* and *nisK* on the chromosome (*pepN*::*nisRnisK*)	(Kuipers, de Ruyter et al. 1997)
L. lactis NZ9000	pIL-SV nisBTC pNZ-SV nisA	NZ9000BTC	mNisA secreting strain	This study
L. lactis NZ9000	pIL-SV nisBTC$_{H331A}$ pNZ-SV nisA	NZ9000BTC$_{H331A}$	dNisA secreting strain	This study
L. lactis NZ9000	pIL-SV nisBT$_{H551A}$C pNZ-SV nisA	NZ9000BT$_{H551A}$C	non NisA secreting strain	This study
L. lactis NZ9000	pIL-SV nisBT pNZ-SV nisA	NZ9000BT	dNisA secreting strain	This study
L. lactis NZ9000	pIL-SV nisT pNZ-SV nisA	NZ9000T	uNisA secreting strain	This study
L. lactis NZ9000	pIL-SV nisT$_{H551A}$ pNZ-SV nisA	NZ9000T$_{H551A}$	non NisA secreting strain	This study
L. lactis NZ9000	pIL-SV nisTC pNZ-SV nisA	NZ9000TC	uNisA secreting strain	This study
L. lactis NZ9000	pIL-SV nisBC pNZ-SV nisA	NZ9000BC	non NisA secreting strain	This study

Table S2: MALDI-ToF-MS analysis of RP-HPLC fractions from *in vivo* secretion assay.
The fractions of RP-HPLC were analyzed by MALDI-ToF-MS and are displayed as masses ($M+H^+$) without or with start methionine. The modified peptides display eight or seven dehydrations and are expected for normally modified pre-NisA by the nisin modification system.

peptide	sample	calc. mass (Da)	observ. mass (+Met*) (Da)
mNisA	RP-HPLC run Fr. 33 from BTC+A supernatant	5688 5706#	5689 5707#
dNisA	RP-HPLC run Fr. 33 from BTC_{H331A}+A supernatant	5688 5706#	5689#
dNisA	RP-HPLC run Fr. 33 from BT+A supernatant	5688 5706#	5689 5707#
uNisA	RP-HPLC run Fr. 31 From T+A supernatant	5832	5834 (5951)
uNisA	RP-HPLC run Fr. 31 From TC+A supernatant	5832	n.d.

8x /7x dehydration
* plus start-methionine
n.d.: not determined

Table S3: The determined kinetic parameter of the *in vivo* secretion assay.
The supernatant of pre-NisA secreting *L. lactis* strains was analyzed by RP-HPLC. The amount of pre-NisA (nmol) plotted against the time (min) was fitted with an allosteric sigmoidal fit and kinetic parameter were determined. There, V_{max} is the maximal amount of secreted pre-NisA, h is the Hill-coefficient and $K_{0.5}$ is the time point, where half of the pre-NisA is secreted. By determine the amount of NisT an apparent secretion rate ($V_{s\ app}$) NisA•NisT^{-1}•min^{-1} was calculated.

strain	V_{max} (nmol)	$K_{0.5}$ (min)	h	$V_{s\ app}$ (NisA•NisT^{-1}•min^{-1})
NZ9000BTC	534 ± 44	134 ± 12	2.9 ± 0.7	100.3 ± 35.2
NZ9000BT$_{H551A}$C	n.d.	n.d.	n.d.	n.d.
NZ9000BTC$_{H331A}$	168 ± 16	200 ± 16	4.1 ± 0.9	n.d.
NZ9000BT	247 ± 15	152 ± 9	4.8 ± 1.2	n.d.
NZ9000T	137 ± 30	144 ± 41	1.9 ± 0.8	7.4 ± 1.6
NZ9000TC	38 ± 4	n.d.	n.d.	n.d.
NZ9000BC	n.d.	n.d.	n.d.	n.d.

n.d.: not determined

Table S4: Overview of selected class I and II lanthipeptides (LanA).

The LanA peptides are assigned to their modification enzymes (PTME) and leader peptidase (Pep). Furthermore, the leader peptides of LanA ($LanA_{LP}$) are displayed as amino acid (aa) sequence and their corresponding length. # unknown/ no data available

name	class	abbrev.	PTME	Pep	$LanA_{LP}$ sequence	length (aa)	reference
Bovicin HJ50	II	BovA	BovM	no	MMNATENQIFVETVSDQELEMLIGG	25	(Xiao, Chen et al. 2004)
Entianin	I	EtnA	EtnBC	no	MSKFDDFDLDVVKVSKQDSKITPQ	24	(Fuchs, Jaskolla et al. 2011)
Epidermin	I	EpiA	EpiBC	EpiP	MEAVKEKNDLFNLDVKVNAKESNDSGAEPR	30	(Allgaier, Jung et al. 1986)
Epilancin 15X	I	ElxA	ElxBC	ElxP	MKKELFDLNLNKDIEAQKSDLNPQ	24	(Ekkelenkamp, Hanssen et al. 2005)
Epilancin K7	I	ElkA	ElkBC	ElkP	MNNSLFDLNLNKGVETQKSDLSPQ	24	(van de Kamp, van den Hooven et al. 1995)
Ericin A	I	EriS2	EriBC	EciP	MTNMSKFDDFDLDVVKVSKQDSKITPQ	27	(Stein, Borchert et al. 2002)
Ericin S	I	EriS1	EriBC	EciP	MSKFDDFDLDVVKVSKQDSKITPQ	24	(Stein, Borchert et al. 2002)
Gallidermin	I	GdmA	GdmBC	Gdm P	MEAVKEKNELFDLDVKVNAKESNDSGAEPR	30	(Kellner, Jung et al. 1988)
Geobacillin I	I	GeoA I	GeoBC	no	MAKFDDFDLDIVVKKQDDVVQPN	23	(Garg, Tang et al. 2012)
Lactocin S	II	LasA	LasM	LasP	MKTEKKVLDELSLHASAKMGARDVESSMNAD	31	(Mortvedt, Nissen-Meyer et al. 1991)
Lacticin 481	II	LctA	LctM	no	MKEQNSFNLLQEVTESELDLILGA	24	(Rince, Dufour et al. 1994)
NAI-107	I	MibA	MibBC	#	MPADILETRTSETEDLLDLDLSIGVEEITAGP	32	(Castiglione, Lazzarini et al. 2008)
Mersacidin	II	MrsA	MrsM	no	MSQEAIIRSWKDPFSRENSTQNPAGNPFSELKEAQMD KLVGAGDMEAA	48	(Bierbaum, Brotz et al. 1995)
Mutacin I	I	MutAI	MutBC I	MutP	MSNTQLLEVLGTETFDVQEDLFAFDTTDTTIVASNDDP DTR	41	(Qi, Chen et al. 2000)
Mutacin III	I	MutAIII	MutBC III	MutP	MSNTQLLEVLGTETFDVQEDLFAFDTTDTTIVASNDDP DTR	41	(Qi, Chen et al. 1999)

name	class	abbrev.	PTME	Pep	$LanA_{LP}$ sequence	length (aa)	reference
Nisin A	I	NisA	NisBC	NisP	MSTKDFNLDLVSVSKKDSGASPR	23	(Kaletta and Entian 1989)
Nisin H	I	NshA	NshBC	NshP	MSTNDFNLDLVSVSKSNAGASTR	23	(O'Connor, O'Shea et al. 2015)
Nisin U	I	NsuA	NsuBC	NsuP	MNNEDFNLDLIKISKENNSGASPR	24	(Wirawan, Klesse et al. 2006)
Nukacin ISK-1	I	NukA	NukM	no	MENSKVMKDIEVANLLEEVQEDENEVLGA	29	(Sashihara, Kimura et al. 2000)
Paenicidin A	I	PaeA	PaeBC	#	MAENLFDLDIQVNKSQGSVEPQ	23	(Lohans, Huang et al. 2012)
Paenibacillin	I	PaenA	PaenBC	PaenP	MKVDQMFDLDLRKSYEASELSPQ	24	(He, Kisla et al. 2007)
Penisin I	I	PenA1	PenBC	#	MANNNFDLDVQVKSVNSVNA	20	(Baindara, Chaudhry et al. 2016)
Penisin II	I	PenA2	PenBC	#	MANNVFDLDVEVKSVNSVNQ	20	(Baindara, Chaudhry et al. 2016)
Pep 5	I	PepA	PepBC	PepP	MKNNKNLFDLEIKKETSQNTDELEPQ	26	(Kaletta, Entian et al. 1989)
Planosporicin	I	PspA	PspBC	#	MGISSPALPQNTADLFQLDLEIGVEQSLASPA	32	(Sherwood, Hesketh et al. 2013)
Salivaricin D	I	SlvA	SlvBC	SlvP	MSTKDFNLDLVEVSKSNTGASAR	23	(Birri, Brede et al. 2012)
Streptin 1	I	StrA1	StrBC	#	MNNTIKDFDLDLKTNKKDTATPY	23	(Karaya, Shimizu et al. 2001)
Streptin 2	I	StrA2	StrBC	#	MNNTIKDFDLDLKTNKKDTATPY	23	(Wescombe and Tagg 2003)
Subtilin	I	SpaS	SpaBC	no	MSKFDDFDLDVVKVSKQDSKITPQ	24	(Klein, Kaletta et al. 1992)
Subtilomycin	I	SubA	SubBC	SubP	MEKNNIFDLDINKKMESTSEVSAQ	24	(Phelan, Barret et al. 2013)

Table S5: Overview about ABC transporters of selected class I and II lanthipeptides.

LanT/ $Lan_{C39P}T$	class	substrate	length (aa)	domain organization (N-term→C-term.)	accession number	reference
BovT	II	BovA	680	C39Pep-TMD-NBD	B1PW41	(Liu, Zhong et al. 2009)
EtnT	I	EtnA	614	TMD-NBD	G0Z2H2	(Fuchs, Jaskolla et al. 2011)
EpiT	I	EpiA	335’	TMD-NBD	O07860	(Allgaier, Jung et al. 1986)
ElxT	I	ElxA	573	TMD-NBD	I6ZHK9	(Ekkelenkamp, Hanssen et al. 2005)
ElkT	I	ElkA	95’	TMD-NBD	Q57072	(Kamp, Hooven et al. 1995)
EriT[#]	I	EriA	614	TMD-NBD	Q93GH7	(Stein, Borchert et al. 2002)
GdmT	I	GdmA	547	TMD-NBD	O07475	(Kellner, Jung et al. 1988)
GeoT I	I	GeoA I	609	TMD-NBD	WP_008881438	(Garg, Tang et al. 2012)
LasT	II	LasA	535	TMD-NBD	Q48851	(Skaugen and Nes 1994)
LctT	II	LctA	691	C39P-TMD-NBD	AAC72259	(Rince, Dufour et al. 1994)
MibT*	I	MibA	316	TMD	E2IHC0	(Foulston and Bibb 2010)
MibU*	I	MibA	290	NBD	E2IHC1	(Foulston and Bibb 2010)
MrsT	II	MrsA	730	C39P-TMD-NBD	Q9RC21	(Altena, Guder et al. 2000)
MutT I	I	MutA I	541	MD-NBD	AAF99583	(Qi, Chen et al. 2000)
Mut III	I	MutA III	541	TMD-NBD	AAD56148	(Qi, Chen et al. 1999)
NisT	I	NisA	600	TMD-NBD	Q03203	(Kuipers, Beerthuyzen et al. 1993)
NshT	I	NshA	600	TMD-NBD	AKB95121	(O'Connor, O'Shea et al. 2015)
NsuT	I	NsuA	598	TMD-NBD	ABA00880	(Wirawan, Klesse et al. 2006)
NukT	II	NukA	694	C39P-TMD-NBD	Q75V16	(Aso, Sashihara

						et al. 2004)
PaeT	I	PaeA	625	TMD-NBD	K7U6S3	(Lohans, Huang et al. 2012)
PaenT	I	PaenA	597	TMD-NBD	M1FLR0	(Huang and Yousef 2015)
PenT#	I	PenA	625	TMD-NBD	ALR96371	(Baindara, Chaudhry et al. 2016)
PepT	I	Pep5	571	TMD-NBD	Q54121	(Kaletta, Entian et al. 1989)
PspT*	I	PspA	322	TMD	CCQ18698	(Sherwood, Hesketh et al. 2013)
PspU*	I	PspA	265	NBD	CCQ18699	(Sherwood, Hesketh et al. 2013)
SlvT	I	SlvA	612	TMD-NBD	H2D758	(Birri, Brede et al. 2012)
SrtT#	I	SrtA	596	TMD-NBD	BAB08163	(Karaya, Shimizu et al. 2001)
SpaT	I	SpaS	614	TMD-NBD	P33116	(Klein, Kaletta et al. 1992)
SubT	I	SubA	585	TMD-NBD	AGL93176	(Phelan, Barret et al. 2013)

‘ gene is disrupted, sequence is only partial ; * ABC transporter is a dimer of homodimers; # ABC transporter has two substrates

Table S6: Leader peptides for $LanA_{LP}$-NisA hybrids.
The selected $LanA_{LP}$ were analyzed towards their length (aa) and sequence identity (%) to the $NisA_{LP}$. The sequence identity was determined with the pairwise sequence alignment tool SIM.

$LanA_{LP}$	**class**	**classification**	**abbrev.**	**length (aa)**	**seq. identity with $NisA_{LP}$ (%)**
Nisin A	I	Nisin-group	NisA	23	100.0
Subtilin	I	Nisin-group	SpaS	24	50.0
Salivaricin D	I	Nisin-group	SlvA	23	70.4
Pep5	I	Pep5-group	PepA	26	29.4
Gallidermin	I	Epidermin-group	GdmA	30	44.1
Mutacin III	I	Epidermin-group	MutAIII	41	22.9
Lactocin S	II	Lacticin-group	LasA	31	33.3

Table S7: MALDI-ToF-MS analysis of peptides from CDAP coupling assay.

The samples of the CDAP coupling assay were analyzed by MALDI-ToF-MS and are displayed as masses ($M+H^+$) without or with start methionine. The unmodified NisA (uNisA) represents the positive control of the assay and indicates a free Cys residue by 25 Da addition (with five maximal couplings in the NisA core peptide). The $LanA_{LP}$-NisA hybrids show coupling, if modification by the nisin modification system was incomplete The eight or seven dehydrations are expected for normally modified NisA core peptide by the nisin modification system.

peptide	sample	calc. mass (Da)	observ. mass (+Met*) (Da)	CDAP coupling
uNisA	- CDAP	5832 (5963)	5836 (5984')	-
uNisA	+ CDAP	5957 [5x CDAP]	5959 [5x CDAP] 5956 [4x CDAP'] 5907 [3x CDAP]	yes
$SpaS_{LP}$-NisA	- CDAP	6120	5980# (6114)# 5998# (6132)#	-
$SpaS_{LP}$-NisA	+ CDAP	6245 [5x CDAP]	5945## 5963## 5978# 6003#[1x CDAP] (6174)# [2x CDAP] (6178)# [2x CDAP] (6193)#, [1x CDAP]	partial
$SalvA_{LP}$-NisA	- CDAP	5820 (5951)	5680# 5698# 5717# (5811)# (5842)#, (5857)#	-
$SalvA_{LP}$-NisA	+ CDAP	5945 [5x CDAP]	5661## 5679# 5696# (5804)# (5811)##, (5821)# (5840)#	no
$GdmA_{LP}$-NisA	- CDAP	6713 (6844)	(6703)# (6721)#	-
$GdmA_{LP}$-NisA	+ CDAP	6838 [5x CDAP]	(6686)## (6703)#	no

10x and 9x dehydration
8x,7x or 6x dehydration
' plus natrium (23 Da)
* plus start methionine (131 Da)

Table S8: Oligonucleotides used in this study.
The forward (for) and reverse (rev) oligonucleotides/primers used for the cloning are displayed in 5'-3' direction. Mutated codons are underlined.

Name	Sequence (5'-3')
K-20Afor	CACCATGAGCACAGCAGATTTTAACTTGGATTTGG
K-20Arev	CCAAATCCAAGTTAAAATCTGCTGTGCTCATGGTG
K-20Efor	CACCATGAGCACAGAAGATTTTAACTTGGATTTGG
K-20Erev	CCAAATCCAAGTTAAAATCTTCTGTGCTCATGGTG
D-19Kfor	CACCATGAGCACAAAAAAATTTAACTTGGATTTGG
D-19Krev	CCAAATCCAAGTTAAATTTTTTTGTGCTCATGGTG
F-18Afor	CACAAAAGATGCAAACTTGGATTTGG
F-18Arev	CCAAATCCAAGTTTGCATCTTTTGTG
N-17Afor	CACAAAAGATTTTGCTTTGGATTTGG
N-17Arev	CCAAATCCAAAGCAAAATCTTTTGTG
L-16Afor	GATTTTAACGCAGATTTGGTATCTGTTTCG
L-16Arev	CGAAACAGATACCAAATCTGCGTTAAAATC
D-15Afor	GATTTTAACTTGGCTTTGGTATCTGTTTCG
D-15Arev	CGAAACAGATACCAAAGCCAAGTTAAAATC
K-9Afor	GTATCTGTTTCGGCAAAAGATTCAG
K-9rev	CTGAATCTTTTGCCGAAACAGATAC
K-9Efor	GTATCTGTTTCGGAAAAAGATTCAG
K-9Erev	CTGAATCTTTTTCCGAAACAGATAC
K-8Afor	GTATCTGTTTCGAAAGCAGATTCA
K-8Arev	CTGAATCTGCTTTCGAAACAGATAC
K-8Efor	GTATCTGTTTCGAAAGAAGATTCAG
K-8Erev	CTGAATCTTCTTTCGAAACAGATAC
D-7Kfor	GTTTCGAAGAAAAAATCAGGTGCATCACCACG
D-7Krev	CGTGGTGATGCACCTGATTTTTTCTTCGAAAC
$_{F\text{-}18}AAAA_{D\text{-}15}$for	GAGCACAAAAGATGCAGCTGCAGCTTTGGTATC
$_{F\text{-}18}AAAA_{D\text{-}15}$rev	GATACCAAAGCTGCAGCTGCATCTTTTGTGCTC
nisAK-9AK-8Afor	GTATCTGTTTCGGCAGCAGATTCAG
nisAK-9AK-8Arev	CTGAATCTGCTGCCGAAACAGATAC
nisAK-9AD-7Kfor	GTATCTGTTTCGGCAAAAAAATCAGGTGCATCAC
nisAK-9AD-7Krev	GTGATGCACCTGATTTTTTTGCCGAAACAGATAC
nisAK-8AD-7Kfor	GTATCTGTTTCGAAGGCAAAATCAGGTGCATCAC
nisAK-8AD-7Krev	GTGATGCACCTGATTTTGCCTTCGAAACAGATAC
nisAK-9AK-8AD-7Kfor	GTATCTGTTTCGGCAGCAAAATCAGGTGCATCAC
nisAK-9AK-8AD-7Krev	GTGATGCACCTGATTTTGCTGCCGAAACAGATAC
EcoRIfor	GTTCGAAGGAATTCCAAAATAAATTATAAG
termlanArev	CTAATTTTGGTTCAAAGAAAGC
10Hfor	CAAAATAAATTATAAGGAGGCAC
10Hrev	CGGATCTCAGTGGTA

infupNZ-SVfor	CACCACCACCACCACTGAGATC
infupNZ-SVrev	TGAGTGCCTCCTTATAATTTATTTTGTAG
infunisTfor	TTTCAGGGCCCATGGATGGATGAAGTGAAAGAATTCA
infunisTrev	GTGGTGGTGTCTAGATTATTCATCATTATCCTCATATTGC
linpNZ-SVfor	TCTAGACACCACCACCACCACCACTG
linpNZ-SVrev	CCATGGGCCCTGAAAATACAGGTTTTCGG
termpNZfor	GAAAACCAAGGCTTGAAACG
pnisArev	CAGAGCCGTTTATTATGCTCGCG
infupIL-SVfor	CGCGAGCATAATAAACGGCTCTGATTAAATTCTGAAGTTTG
infupIL-SVrev	CGTTTCAAGCCTTGGTTTTCTAATTTTGGTTCAAAGAAAG
Δ10Hfor	ATGGATGAAGTGAAAGAATTCACATCAAAAC
Δ$nisT_{TMD}$for	CATATAGGAACTGTTAAAGTAATTAATTTATCATATG
Δ$nisT_{TMD}$rev	CCATGGGCCCTGAAAATACAGGTTTTCGG
ΔnisTfor	CTTTATTATTCAGAGCAATATGAGGATAATGATG
ΔnisTrev	GTTTTGATGTGAATTCTTTCACTTCATCCATC
ΔnisBfor	GACTAATAGATGGATGAAGTGAAAGAATTCAC
ΔnisBrev	GTTTTTTCCTCTCTTTATTTTTATAAGCTATTTAGCAAC
ΔnisCfor	GACCCAGCTTTCTTGTACAAAG
ΔnisCrev	CCCATTGAGCAATAATTTTTTC
$nisT_{H551A}$_for	CAATTTTCATTTCTGCAAGTTTGAATGCTG
$nisT_{H551A}$_rev	CAGCATTCAAACTTGCAGAAATGAAAATTG
$nisC_{H331A}$for	CATATATGATTTGCGCAGGCTATTCTGGTTTAATAG
$nisC_{H331A}$rev	CTATTAAACCAGAATAGCCTGCGCAAATCATATATG

Table S9: Additional plasmids used in this study.

name	properties	reference
pNZ-SV	*E. coli* /*L. lactis* shuttle vector; ErmR; pnisA promoter; empty vector for cloning	(AlKhatib, Lagedroste et al. 2014)
pNZ-SVnisA	NisA expression	(AlKhatib, Lagedroste et al. 2014)
pNZ-SVnisAK-20A	NisA expression; single alanine substitution at pos. K-20 in $NisA_{LP}$	This study
pNZ-SVnisAK-20E	NisA expression; single glutamate substitution at pos. K-20 in $NisA_{LP}$	This study
pNZ-SVnisAK-20A K-9/-8A	NisA expression; single alanine substitution at pos. K-20, K-9 and K-8 in $NisA_{LP}$	This study
pNZ-SVnisAD-19K	NisA expression; single lysine substitution at pos. D-19 in $NisA_{LP}$	This study
pNZ-SVnisAD-19K D-7K	NisA expression; single lysine substitution at pos. D-19 and D-7 in $NisA_{LP}$	This study
pNZ-SVnisAF-18A	NisA expression; single alanine substitution at pos. F-18 in $NisA_{LP}$	This study, (Plat, Kluskens et al. 2011)
pNZ-SVnisAN-17A	NisA expression; single alanine substitution at pos. N-17 in $NisA_{LP}$	This study, (Plat, Kluskens et al. 2011)
pNZ-SVnisAL-16A	NisA expression; single alanine substitution at pos. L-16 in $NisA_{LP}$	This study, (Plat, Kluskens et al. 2011)
pNZ-SVnisAD-15A	NisA expression; single alanine substitution at pos. D-15 in $NisA_{LP}$	This study, (Plat, Kluskens et al. 2011)
pNZ-SV$nisA_{F\text{-}18}AAAA_{D\text{-}15}$	NisA expression; single alanine substitution at pos. F-18 till D-15 in $NisA_{LP}$	This study, (Plat, Kluskens et al. 2011)
pNZ-SVnisAK-9A	NisA expression; single alanine substitution at pos. K-9 in $NisA_{LP}$	This study
pNZ-SVnisAK-9E	NisA expression; single glutamate substitution at pos. K-9 in $NisA_{LP}$	This study
pNZ-SVnisAK-8A	NisA expression; single alanine substitution at pos. K-8 in $NisA_{LP}$	This study
pNZ-SVnisAK-8E	NisA expression; single glutamate substitution at pos. K-8 in $NisA_{LP}$	This study
pNZ-SVnisAK-9A K-8A	NisA expression; single alanine substitution at pos. K-9 and K-8 in $NisA_{LP}$	This study
pNZ-SVnisAD-7K	NisA expression; single alanine substitution at pos. D-7 in $NisA_{LP}$	This study, (Plat, Kuipers et al. 2017)
pNZ-SVnisAK-9A D-7K	NisA expression; single alanine substitution at pos. K-9 and single lysine substitution at pos- D-7 in $NisA_{LP}$	This study
pNZ-SVnisAK-8A D-7K	NisA expression; single alanine substitution at pos. K-8 and single lysine substitution at pos- D-7 in $NisA_{LP}$	This study

pNZ-SVnisAK-9A K-8A D-7K	NisA expression; single alanine substitution at pos. K-9, K-8 and single lysine substitution at pos- D-7 in $NisA_{LP}$	This study
pNZ-SV$spaS_{LP}$-nisA	$SpaS_{LP}$-NisA expression	This study
pNZ-SV$slvA_{LP}$-nisA	$SlvA_{LP}$-NisA expression	This study
pNZ-SV$pepA_{LP}$-nisA	$PepA_{LP}$-NisA expression	This study
pNZ-SV$gdmA_{LP}$-nisA	$GdmA_{LP}$-NisA expression	This study
pNZ-SV$mutAIII_{LP}$-nisA	$MutAIII_{LP}$-NisA expression	This study
pNZ-SV$lasA_{LP}$-nisA	$LasA_{LP}$-NisA expression	This study
pNZ-SV10HnisT	NisT expression, deca-histidine tag at N-terminus; single alanine substitution at pos. H551 in the T_{NBD}: ATP hydrolysis deficient mutant	This study
pNZ-SV10H$nisT_{H551A}$	NisT expression, deca-histidine tag at N-terminus	This study
pIL-SV	*E. coli* /*L. lactis* shuttle vector; CmR; pnisA promoter; empty vector for cloning	(AlKhatib, Lagedroste et al. 2014)
pIL-SVnisT	NisT expression	This study, (van den Berg van Saparoea, Bakkes et al. 2008)
pIL-SV$nisT_{H551A}$	NisT expression; single alanine substitution at pos. H551 in the T_{NBD}: ATP hydrolysis deficient mutant	This study
pIL-SVnisBTC	NisBTC expression	This study, (Rink, Kuipers et al. 2005)
pIL-SV$nisBTC_{H331A}$	NisBTC expression; single alanine substitution at pos. H331 in the NisC; catalytically inactive NisC	This study, (Lubelski, Khusainov et al. 2009)
pIL-SV$nisBT_{H551A}$C	NisBTC expression; single alanine substitution at pos. H551 in the T_{NBD}: ATP hydrolysis deficient mutant	This study
pIL-SVnisBT	NisBT expression	This study, (van den Berg van Saparoea, Bakkes et al. 2008)
pIL-SVnisTC	NisTC expression	This study, (van den Berg van Saparoea, Bakkes et al. 2008)
pIL-SVnisBC	NisBC expression	This study, (van den Berg van Saparoea, Bakkes et al. 2008)

pNGnisB6His	*L. lactis* expression vector; CmR; NisB expression; hexa-histidine tag and TEV-protease cleavage site at C-terminus	(Mavaro, Abts et al. 2011, Reiners, Abts et al. 2017)
pET28b-AA-nisC	*E. coli* expression vector; KanR; NisC expression; hexa-histidine tag and thrombin cleavage site at N-terminus	(Abts, Montalban-Lopez et al. 2013)

ErmR: erythromycin resistance
CmR: chloramphenicol resistance
KanR: kanamycin resistance
T_{NBD}: nucleotide binding domain of NisT

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3.4 Chapter IV – *In vitro* activity of the leader peptidase NisP

Title Substrate Specificity of the Secreted Nisin Leader Peptidase NisP

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Substrate Specificity of the Secreted Nisin Leader Peptidase NisP

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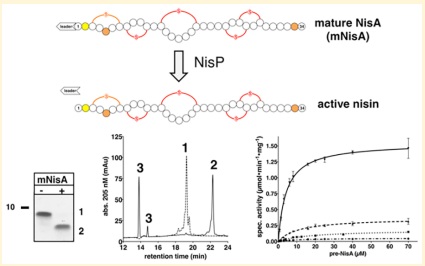
Supporting Information

ABSTRACT: Nisin (NisA) is an antimicrobial peptide produced by *Lactococcus lactis* and belongs to the class of lanthipeptides, more specifically to the class of lantibiotics. They are ribosomally synthesized as a precursor peptide and are comprised of an N-terminal leader peptide and a C-terminal core peptide. The core peptide is post-translationally modified and contains dehydrated amino acids in addition to five (methyl)-lanthionine rings, which are crucial for its activity. The leader peptide serves as a signal sequence and ensures that NisA remains inactive but secretion-competent within the cell. After translocation into the extracellular space, the leader peptide is cleaved by the leader peptidase NisP, resulting in active nisin. NisP is an extracellular subtilisin-like serine protease, which recognizes the cleavage site GASPRIT located at the C-terminal end of the leader peptide. Here, we present the biochemical characterization of secreted and purified NisP ($NisP_s$) with its natural substrate, the fully modified NisA (mNisA). Furthermore, we determined the kinetic parameters of $NisP_s$ in the presence of NisA containing different modification states. Additionally, *in vitro* data revealed that $NisP_s$ can efficiently cleave the leader peptide of mNisA. However, it is strictly dependent on the modification state of the core peptide. Thus, $NisP_s$ has a sequence-based cleavage activity, and the presence of at least one lanthionine ring is crucial for optimal substrate recognition and subsequent cleavage.



Lanthipeptides (lanthionine-containing peptides) are ribosomally synthesized as precursor peptides, which undergo post-translational modifications (PTM). They belong to the family of ribosomally synthesized and post-translationally modified peptides (RiPPs).[1] Lanthipeptides can be classified on the basis of their maturation pathway into classes I–IV, e.g., nisin or epilancin 15X belongs to class I.[2] The dehydration of serine and threonine residues resulting in 2,3-didehydroalanine (Dha) and 2,3-didehydrobutyrine (Dhb), respectively, represents the most common post-translational modification among lanthipeptides. Their subsequent Michael-like condensation with the side chain of a cysteine residue results in the formation of either a lanthionine (Lan) or methyl-lanthionine (MeLan) ring. Lanthipeptides that display antimicrobial activity are called lantibiotics (lanthionine-containing antibiotics).[3] One of the most extensively studied members of these lantibiotics is nisin.[4,5] Nisin (NisA) is produced by the Gram-positive bacterium *Lactococcus lactis* and is ribosomally synthesized as a precursor peptide (pre-NisA) consisting of 57 amino acids. It is encoded on a gene cluster together with its biosynthetic enzymes.[6−8] Like most known lanthipeptides, nisin is comprised of an N-terminal leader peptide and a C-terminal core peptide.[9] The core peptide is post-translationally modified by the specific dehydratase NisB and cyclase NisC, resulting in the formation of five (methyl)-lanthionine rings.[10−12] For both of these enzymes, the leader peptide, especially the FNLD box within the leader, was shown to be essential.[13−15] Recently, the molecular mechanisms of the dehydration reaction of NisB and the formation of the NisA, NisB, and NisC complex were determined.[16,17]

The fully modified precursor, called mature NisA (mNisA), is subsequently secreted in a leader peptide-dependent manner into the extracellular space catalyzed by the ATP binding cassette (ABC) transporter NisT.[18−20] After secretion, mNisA is processed proteolytically by removal of the leader peptide, resulting in nisin activation. This last step of the nisin maturation process is performed by an extracellularly located, subtilisin-like serine peptidase (GenBank accession number Q48674; Pfam entry Peptidase S8) NisP[7] (Figure 1A).

NisP is ribosomally synthesized as a prepro-polypeptide of 682 amino acid residues.[7,21] After secretion via the Sec translocon, autocatalytic and signal peptidase I-mediated processing takes place[22−24] and NisP is anchored to the peptidoglycan by sortase A. Alternatively, an autocleavage reaction at position 647 prohibits this anchoring.[25,26] After these maturation steps, amino acid residues 196–657 (or amino acids 196–647) encode the active, extracellularly located protease NisP, resulting in a molecular mass of approximately 54 kDa.[7,23]

Peptidases of class I–IV lanthipeptides are located extracellularly (e.g., NisP and EpiP[7,27]), cytoplasmically (e.g.,

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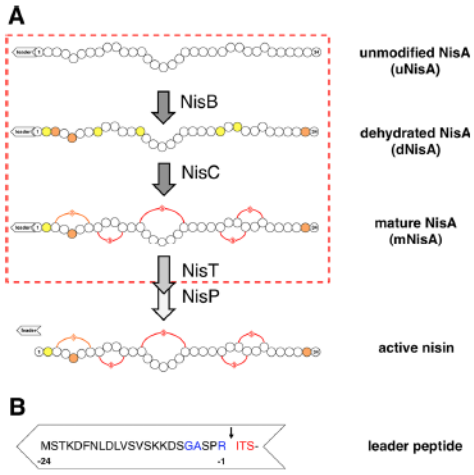

Figure 1. Schematic representation of the nisin biosynthesis pathway. (A) Nisin is ribosomally synthesized as a precursor peptide (pre-NisA), which is first dehydrated by NisB. The resulting dehydrated serines and threonines are highlighted as yellow (Dhb) and orange circles (Dha), respectively. Formation of the (methyl-) lanthionine ring (red) and lanthionine ring (orange) is catalyzed by NisC, and subsequently, mNisA is secreted by NisT. (B) The final step is the removal of the leader peptide by subtilisin-like serine protease NisP, which recognizes the cleavage site GASPRITT and releases active, mature nisin.

PepP and ElxP[28,29]) or as a domain fused to the ABC transporter involved in secretion (e.g., NukT and LctT[30,31]).

In general, subtilisin-like serine proteases show a relatively low degree of sequence homology (<50%) because of deletions and insertions throughout their family, but they do display a high level of sequence identity with respect to the active site and the amino acid residues located in its proximity.[32,33] For NisP, a high-resolution crystal structure is available, which shows the expected fold of a subtilisin-like serine protease.[23,25,34] The important residues of the active site of NisP are the conserved catalytic triad (Asp259, His306, and Ser512) and residue Asn407, which form the oxyanion hole.[7,23] Additional important residues are found in the substrate binding regions (substrate pockets S1−S4; notation from ref 35), where S1 is mainly interacting via electrostatic interactions with residue Arg-1 of the mNisA leader peptide (P1). Furthermore, substrate binding is proposed to occur via hydrophobic binding pocket S4 and binding pockets S2 and S3, which possess a mixed hydrophobic/hydrophilic character.[23]

Within the leader peptide of pre-NisA, NisP recognizes the cleavage site GASPRITT located at the C-terminus (Figure 1B). The influence of these conserved residues of the leader peptide on the activity of NisP was investigated in various studies.[36,37] For example, changing residues at certain positions of the leader peptide (R-1Q and A-4D[37] or R-1N and A-4D[36]) prevented cleavage by NisP.

Because the secretion of nisin is independent of the peptidase reaction and the modification complex, the precursor of nisin can be produced in three different maturation states [unmodified (uNisA), dehydrated (dNisA), and fully modified (mNisA)],[18,36] but the secretion rate of the nisin precursor is dependent on the modification complex.[38,39] The leader peptide of these precursors can be cleaved *"in vitro"* by the addition of the supernatant of cells secreting NisP. This study[18] revealed that NisP is capable of cleaving only the fully modified precursor peptide by recognition of at least one MeLan/Lan ring.

Studies of other leader peptidases revealed that some peptidase can cleave precursor peptides in the modified or unmodified maturation state (e.g., ElxP, EpiP, and LicP).[27,29,31,40] However, the class II lanthipeptide nukacin ISK-1 and class III lanthipeptide flavipeptin are cleaved by their leader peptidases only in their modified maturation state.[30,41]

In this study, the *in vitro* activity of the nisin leader peptidase $NisP_s$ (secreted variant, amino acids 196−572) and its natural substrate, mNisA, was analyzed. Here, kinetic parameters of the cleavage reaction were determined, representing the first kinetic characterization of a lanthipeptide class I (NisA group) peptidase.

Furthermore, the cleavage reaction was investigated with respect to substrate specificity. Therefore, the unmodified and dehydrated pre-NisA and ring mutants of mNisA were employed as substrates. Additionally, we also demonstrate that $NisP_s$ can cleave different kinds of substrates. However, the efficiency of the cleavage reaction is strictly dependent on the modification state of the peptide.

■ MATERIALS AND METHODS

Microorganisms, Media, and Culture Conditions. Strains of *Escherichia coli* and *L. lactis* and the corresponding plasmids used in this study are listed in Table S1. *L. lactis* was grown in M17[42] or minimal medium[43] at 30 °C under semiaerobic conditions supplemented with 0.5% glucose and appropriate antibiotics (erythromycin or/and chloramphenicol at a final concentration of 5 μg/mL).

E. coli was grown in LB medium at 37 °C under aerobic conditions with appropriate antibiotics (30 μg/mL kanamycin or 100 μg/mL ampicillin).

Cloning of mNisA Ring Mutants. The cloning of constructs for expressing mNisA ring mutants was performed as described in ref 44. To generate mNisA ring mutants, each cysteine residue (at positions C7, C11, C19, C26, and C28) was substituted with alanine by site-directed mutagenesis, which resulted in precursor variants of nisin that lacked the MeLan/Lan ring at the corresponding positions. To obtain the corresponding plasmids, a polymerase chain reaction using Pfu DNA polymerase (Fischer Thermo Scientific), template pNZ-SVnisA, and the appropriate pair of oligonucleotides (Table S2) was performed according to standard procedures.

For transformation of *L. lactis* NZ9000/NZ9000 pIL3nisT, pIL3nisBT, and pIL3nisBTC, the standard procedure for preparation of competent cells and electroporation was used as described elsewhere.[45]

Expression and Purification of Pre-NisA. All pre-NisA variants used in this study were expressed and purified as described previously.[14,38] The precursor peptide was purified after expression from 2 L of a minimal medium supernatant via cation-exchange chromatography (cIEX). The cell-free supernatant was diluted 1:1 with 50 mM lactic acid (pH 3) and applied to a HiTrap SP Sepharose column (5 mL, GE Healthcare). After a washing step with 50 mM HEPES-NaOH (pH 7), the peptide was eluted with 50 mM HEPES-NaOH

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(pH 7) and 1 M NaCl. Fractions containing pre-NisA were pooled and filtered through an Amicon ultracentrifugal filter. Initially, a 30 kDa molecular weight cutoff (MWCO) and then a 3 kDa MWCO filter were used in the second step to concentrate the peptides. Aliquots of the peptide were stored at −80 °C until further use. The purity of all pre-NisA variants was analyzed by Tricine−sodium dodecyl sulfate−polyacrylamide gel electrophoresis (Tricine−SDS−PAGE),[46] and its concentration was determined by RP-HPLC.[13]

Expression and Purification of NisP$_s$. The serine protease NisP (GenBank accession number Q48674) was homologously expressed in *L. lactis* NZ9000 harboring plasmid pNGnisP8His, and purification was performed as previously described.[13] NisP is secreted as a shorter variant (amino acids 196−572) with a C-terminal octahistidine tag (named NisP$_s$).

In brief, *L. lactis* strain NZ9000 pNGnisP8His was grown in GM17 medium supplemented with 5 μg/mL chloramphenicol overnight at 30 °C. The cells were harvested the next day and transferred into minimal medium with a starting OD_{600} of 0.1 and supplemented with 0.1 ng/mL nisin for induction. After 16 h, cells were removed by centrifugation (5000*g* for 30 min at 4 °C) and the supernatant was adjusted to pH 8.0 with 3 M Tris-HCl (pH 10). The supernatant was further filtered (pore size of 0.45 μm) before it was applied to an IMAC HP column (GE Healthcare) loaded with Co^{2+} and pre-equilibrated with low-IMAC buffer [50 mM HEPES-NaOH (pH 8.0) and 150 mM NaCl]. After being washed with low-IMAC buffer, NisP$_s$ was eluted with 50 mM HEPES-NaOH (pH 8.0), 150 mM NaCl, and 300 mM imidazole. Elution fractions containing the target protein were pooled and concentrated by ultracentrifugation (10 kDa MWCO). To remove imidazole, the protein was applied to a Superdex 200 10/300 GL column (GE Healthcare) and the buffer was changed to 50 mM HEPES-NaOH (pH 7.0), 150 mM NaCl, and 10% (v/v) glycerol or to *in vitro* assay buffer [50 mM citrate (pH 5.0) and 150 mM NaCl]. Desalted and homogeneous protein was further concentrated by ultracentrifugation (10 kDa MWCO), aliquoted, and stored at −80 °C until further use. The progress of purification and purity of NisP$_s$ was analyzed by SDS−PAGE (preformed according to standard procedures). The concentration of the purified protein was determined by a Nanodrop 1000 (peqlab), using a molar extinction coefficient of 60280 M^{-1} cm^{-1} and a molecular weight of 42.5 kDa.

HPLC Analysis of Pre-NisA Variants. Mature nisin and its precursor variants were analyzed by RP-HPLC (Agilent Technologies 1260 Infinity II). Therefore, a LiChrospher WP 300 RP-18 end-capped column and an acetonitrile/water solvent system were used as described previously.[13]

The RP-HPLC run was monitored at 205 nm, and the concentration of analyzed peptides was determined using the peak area. Therefore, a calibration with known amounts of nisin (lyophilized powder obtained from Sigma-Aldrich) or a synthetic leader peptide (obtained from JPT peptide technologies) was performed. Absorption integrals of different nisin or leader peptide concentrations were determined using the Agilent Lab Advisor software and plotted against the known amounts. Guided by the resulting calibration line, we calculated the specific concentration of analyzed peptides (Figure S4).

Activity Assay of NisP$_s$: Determination of Kinetic Parameters and Substrate Specificity. For the cleavage reaction of different pre-NisA variants, optimized assay conditions were used to determine the kinetic parameters and substrate specificity. Depending on the experimental setup, either the concentration of substrate, the time, or the concentration of the leader peptidase was changed (see the Supporting Information for details). For all assays, NisP$_s$ containing an octahistidine tag was used unless stated otherwise.

With a change in the substrate concentration (from 0.5 to 70 μM) of pre-NisA, the apparent K_m value of NisP$_s$ was determined. The reaction was performed in a 100 μL sample volume with 10 nM NisP$_s$ at 30 °C for 5 min. After the reactions had been quenched with 1 mg/mL AEBSF and 1% HCl, the samples were analyzed using an RP-HPLC system. The specific activity of the leader peptidase was plotted against various substrate concentrations and fitted using the Michaelis−Menten equation (eq 1[47]). Note that y is the velocity of the cleavage reaction whereas x is the substrate concentration. The analysis was performed using Prism 5.0a (GraphPad).

$$Y = V_{max}\frac{X}{K_m + X} \quad (1)$$

To analyze the substrate specificity of NisP$_s$, different maturation states (uNisA or dNisA) and ring mutants of the precursor peptide (dNis$_{AAAAA}$, mNisA$_{CAAAA}$, mNis$_{CCAAA}$, mNisA$_{CCCAA}$, and mNisA$_{CCCCA}$) were incubated with a fixed amount of leader peptidase. The purified peptides were buffer exchanged with an *in vitro* activity assay buffer (citrate buffer) using PD10 columns (GE Healthcare) and concentrated to 40 μM stocks. For the activity assay, final concentrations of 20 μM peptide and 10 nM NisP$_s$ were used in a reaction volume of 100 μL. The reaction was directly quenched with 1 mg/mL AEBSF and 1% HCl, after incubation at 30 °C for 0 min, 5 min, 30 min, 1 h, 3 h, and 23 h. The progress of cleavage was analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS), RP-HPLC, and Tricine−SDS−PAGE. For each reaction, the specific activity (micromoles per minute per milligram of protein) of NisP$_s$ or the velocity of the reaction (micromoles per minute) was calculated on the basis of the amount of released peptides (leader peptide or core peptide of the nisin variant) as determined by HPLC analysis.

MS Analysis of Pre-NisA Variants. The samples of the activity assay (reaction time of 1 or 23 h) were desalted using zip-tip concentrators (Millipore). The eluted peptides were vacuum-dried, and the corresponding pellets were dissolved in acetonitrile and 0.1% TFA. The resulting suspension was mixed in a 1:10 ratio with α-cyano-4-hydroxycinnamic acid, spotted on a plate, and dried. Mass spectra were recorded with an Ultraflex I (Bruker Daltonics) MALDI-TOF mass spectrometer in linear positive mode.

■ RESULTS

Purification and *in Vitro* Activity of Secreted NisP$_s$. Subtilisin-like serine protease NisP was expressed as a preproprotein and secreted via the Sec protein translocation pathway. Like other subtilisin-like serine proteases, wild-type NisP undergoes an autocleavage reaction, wherein the propeptide sequences are cleaved.[24] In the final step, it is suggested that similar to other proteins in Gram-positive bacteria containing a C-terminal LPXTG motif, NisP is also linked to the peptidoglycan by a sortase A from *L. lactis*.[22] Instead of wild-type NisP, we used a soluble and shorter version of NisP[13] wherein the C-terminal part harboring the LPXTG motif was

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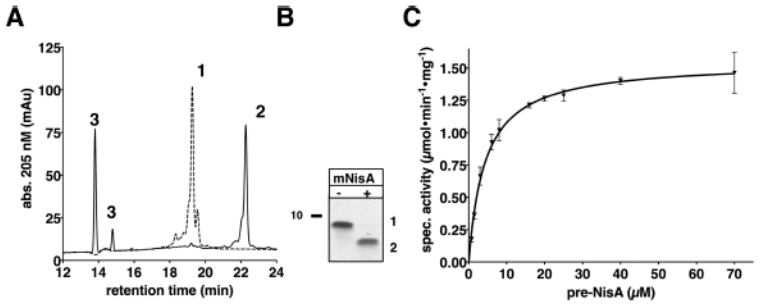

Figure 2. *In vitro* activity of $NisP_s$ with the substrate mNisA. Cleavage of mNisA (**1**) by $NisP_s$ results in the formation of mature nisin (**2**) and the leader peptide (**3**), which was monitored via RP-HPLC (A) and analyzed by Tricine–SDS–PAGE (B). Complete cleavage is achieved after 30 min using 10 nM $NisP_s$ and 50 μM mNisA. For kinetic analysis, the reaction time was decreased to 5 min with various substrate concentrations. (C) The specific activity of the leader peptidase was plotted against the various substrate concentrations and analyzed by the Michaelis–Menten equation (eq 1). Results show the fit of mNisA (triangles, solid line) of at least three independent experiments. Error bars represent the standard error of the mean.

Table 1. Kinetic Parameters of $NisP_s$[a]

pre-NisA variant	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	relative k_{cat}/K_m
mNisA	4.4 ± 0.5	11 ± 0.3	(2.5 ± 0.4) × 10^6	1
uNisA	12.2 ± 3.7	0.39 ± 0.04	(2.8 ± 1.1) × 10^4	0.01
dNisA	17.7 ± 1.4	1.27 ± 0.04	(7.1 ± 0.8) × 10^4	0.03
$dNisA_{AAAAA}$	11.2 ± 2.1	2.6 ± 0.17	(2.3 ± 0.6) × 10^5	0.09

[a]Different variants of pre-NisA (uNisA, dNisA, and $dNisA_{AAAAA}$) and mNisA were used as substrates to determine the kinetic parameters of nisin leader peptidase $NisP_s$. The K_m values of the tested substrates were determined using optimized *in vitro* assay conditions. Furthermore, a turnover number k_{cat}, the catalytic efficiency, and a relative k_{cat}/K_m compared to mNisA were calculated.

deleted, and $NisP_s$ was secreted into the extracellular space after translocation via the Sec translocon.

Additionally, our variant of $NisP_s$ (amino acids 196–572) contains a C-terminal octahistidine tag for the purpose of purification via immobilized metal ion affinity (IMAC) chromatography. $NisP_s$ was purified from the cell-free supernatant with a purity of >95% as determined by Coomassie Brilliant Blue-stained SDS–PAGE. Furthermore, it also shows a high degree of homogeneity during size-exclusion chromatography (Figure S1).

To determine whether $NisP_s$ is capable of cleaving the leader peptide of mNisA, initial activity tests and optimizations of the assay conditions were performed (compare Figures S2 and S3 and supplemental data). The cleavage reaction was analyzed by either RP-HPLC (Figure 2A), MS analysis (Table 2), or Tricine–SDS–PAGE (Figure 2B) and demonstrated that $NisP_s$ displayed cleavage activity. It completely cleaved 50 μM fully modified mNisA within 10 min (at 50 nM enzyme), and the formation of the leader peptide (retention time of 14 min) and mature nisin (retention time of 22 min) was detected (Figure 2A, black graph) in the HPLC chromatogram. Only a single peak corresponding to mNisA (retention time of 19 min) was observed (Figure 2A, dotted graph) in cases in which $NisP_s$ was absent from the reaction mixture or the reaction was stopped immediately by adding 1 mg/mL AEBSF and 1% HCl. After optimization of the activity assay conditions, a citrate buffer (pH 5) supplemented with 150 mM NaCl was used in all further experiments.

To determine the kinetic parameters of $NisP_s$, <20% of the substrate should be cleaved by the protease within the reaction time. Therefore, an enzyme:substrate molar ratio of at least 1:2000 was chosen (Figure S2). In contrast, at higher enzyme concentrations, the linearity of the initial rates of the reaction was not observed.

Kinetic Characterization of $NisP_s$ with mNisA. To determine the kinetic parameters of $NisP_s$ with its natural substrate, mNisA, fully modified mNisA was incubated at a fixed enzyme concentration (10 nM) for 5 min at 30 °C. The apparent K_m (Michaelis constant) value and V_{max} values were subsequently determined according to eq 1 by changing the substrate concentration of pre-NisA (from 0.5 to 70 μM). The k_{cat} (turnover rate) was further calculated by dividing the V_{max} value by the known enzyme concentration. Furthermore, the ratio of k_{cat} to K_m determines the catalytic efficiency.

For the leader peptidase $NisP_s$ and its substrate, mNisA, the V_{max} value is 1.54 ± 0.5 μmol min^{-1} mg^{-1} with a K_m value of 4.4 ± 0.5 μM (Figure 2C and Table 1). Accordingly, the k_{cat} value is 11 ± 0.3 s^{-1}, and the catalytic efficiency is (2.5 ± 0.4) × 10^6 M^{-1} s^{-1} (Table 1). $NisP_s$ is nearly saturated at 20 μM mNisA (~80% of V_{max} and 5 times the K_m value), and this concentration was used as a fixed parameter to analyze the substrate specificity of $NisP_s$ for substrates containing the MeLan/Lan ring (see the next section).

Cleavage of mNisA Ring Mutants. To address the substrate specificity of $NisP_s$, the cleavage efficiency of the leader peptidase was analyzed employing the ring mutants of mNisA. Here, starting at the C-terminus, the formation of the MeLan/Lan rings was subsequently prevented by substituting cysteine to alanine in the core peptide. The notation of mNisA ring mutants is as follows. C indicates a (methyl-) lanthionine ring, whereas A stands for a deleted ring. To investigate the cleavage reaction of the ring mutants by $NisP_s$, the same

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conditions were used for these mutants as described previously for fully modified mNisA.

As in the preliminary experiment, the cleavage reaction of the analyzed ring mutants was quenched after 5 min (for Tricine–SDS–PAGE, the time was prolonged to 60 min). The reaction was quenched by the addition of an AEBSF/HCl mixture and directly analyzed by RP-HPLC. On the basis of the resulting data, the activity (micromoles per minute per milligram of protein) was plotted against the individual ring mutant. Fully modified mNisA served as a positive control, while ring mutant $dNisA_{AAAAA}$ (cysteine-less variant; Cys-less) was employed as a negative control.

Cleavage of all mNisA variants that contain thioether rings (Figure 3 and Table 2) was observed under the assay conditions. The activity for substrates with thioether rings was between 1.6 and 2.5 μmol min^{-1} mg^{-1} (Figure 3). Here, ring mutant $mNisA_{CCCAA}$ shows the highest [2.5 ± 0.18 μmol min^{-1} mg^{-1}] and $mNisA_{CCCCC}$ the lowest activity [1.6 ± 0.18 μmol min^{-1} mg^{-1}]. The formation of the leader peptide and mature nisin was confirmed by RP-HPLC and MALDI-TOF MS analyses (Table 2).

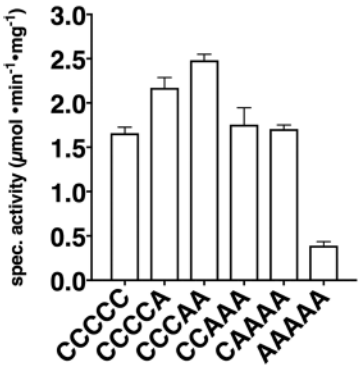

Figure 3. *In vitro* activity of $NisP_s$ with the substrates mNisA and ring mutants. The substrate specificity of $NisP_s$ with different mNisA ring mutants was analyzed via RP-HPLC. The activity of the leader peptidase was plotted against (methyl-) lanthionine ring containing mNisA and Cys-less dNisA (no lanthionine rings). The cleavage reaction (5 min; 20 μM substrate and 10 nM $NisP_s$) was performed in at least three independent experiments, and error bars represent the standard error of the mean.

Surprisingly, $NisP_s$ was capable of cleaving the Cys-less variant (Figure 3 and Table 2). The complete deletion of rings A–E lowers the cleavage activity by a factor of 4 [0.3 ± 0.12 μmol min^{-1} mg^{-1}]. Therefore, we conclude that ring deletion down to a single Lan ring has no influence on $NisP_s$ activity. Furthermore, the Cys-less variant clearly indicates that a substrate without lanthionine rings is recognized and cleaved by $NisP_s$. However, if $NisP_s$ simply recognizes the first lanthionine ring (amino acids 3–7), ring mutant $mNisA_{ACCCC}$ would provide insights. Thus, this pre-NisA was purified and analyzed to determine the modification status, and we determined whether the dehydration pattern and MeLan/Lan ring formation are equivalent to mNisA. Because we observed six or fewer dehydrations (Table 2), which indicated a non-canonical ring formation, we did not further analyze this pre-NisA variant with respect to $NisP_s$ cleavage.

Cleavage of Unmodified and Dehydrated Pre-NisA. We further analyzed whether $NisP_s$ is capable of cleaving other pre-NisA variants. Therefore, we tested pre-NisA in its unmodified and dehydrated state under the established assay conditions. For each reaction, final concentrations of 10 nM $NisP_s$ and 20 μM substrate were used and the reaction was quenched by the addition of an AEBSF/HCl mixture at different time points. The progress of the reaction was analyzed by RP-HPLC.

Tricine–SDS–PAGE and RP-HPLC confirmed cleavage of uNisA and dNisA by $NisP_s$ (Figure 4A,B). During the RP-HPLC analysis, no core peptide (retention time of 22 min) was observed for the tested substrates (even for the $dNisA_{AAAAA}$ ring mutant). Additionally, a peptide with a retention time of 14 min was observed, which corresponds to the leader peptide. In comparison to mNisA, these variants were cleaved less efficiently. For uNisA, 16 ± 0.4% of the substrate was cleaved after 3 h and an incubation time of 23 h resulted in 59 ± 2.0% cleavage (Figure 4B). The dNisA was cleaved faster, with 30 ± 3.8% of the substrate being cleaved after 1 h. A prolonged incubation time of 23 h led to more cleavage of dNisA, which reached 91 ± 4.8% (Figure 3B). For the substrate mNisA, 100% cleavage was observed after 30 min (Figure 3B).

Table 2. Expected and Observed Masses of the Pre-NisA Variants[a]

	before leader cleavage		after leader cleavage			
pre-NisA variant	expected	observed	expected nisin	expected leader	observed	cleavage
uNisA	5832	5856[b]	3498	2354	5856[b]/2976/2352	–[c]
dNisA	5688	5684	3354	2354	5684/3351/2353	–[c]
$dNisA_{AAAAA}$	5528	5523	3194	2354	5529/3191/2353	–[c]
$mNisA_{CAAAA}$	5560	5541	3226	2354	3207[d]/2352	+
$mNisA_{CCAAA}$	5592	5573	3258	2354	3240[d]/2352	+
$mNisA_{CCCAA}$	5624	5604	3290	2354	3272[d]/2352	+
$mNisA_{CCCCA}$	5656	5655	3322	2354	3321/2352	+
$mNisA_{CCCCC}$	5688	5687	3354	2354	3353/2352	+
$mNisA_{ACCCC}$	5656	5699/5719/5738[e]	3322	2354	nd[f]	nd[f]

[a]The average masses ($M + H^+$) of pre-NisA lacking the start methionine (−Met1) in daltons before and after leader peptide cleavage by $NisP_s$ (1 h reaction time) are summarized. Masses of mNisA contain eight dehydrations as the main species, if not indicated otherwise. Incomplete cleavage is indicated by a dash, while complete cleavage is represented by a plus. [b]Natrium ions attached. [c]Partial cleavage after 1 h. [d]Nine-fold dehydrations are the main species. [e]Less than 7-fold dehydration are the main species. [f]Not determined.

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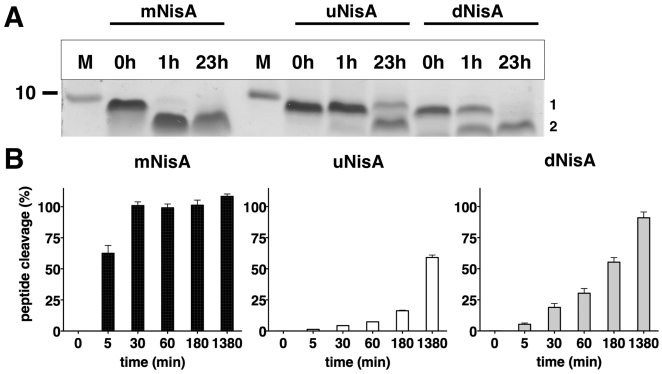

Figure 4. *In vitro* activity of $NisP_s$ with the substrates uNisA and dNisA. (A) Cleavage of pre-NisA by $NisP_s$ was analyzed via Tricine−SDS−PAGE. *In vitro* assay reactions with $NisP_s$ (+) show conversion of pre-NisA over time (0−23 h). Noncleaved peptide NisA is indicated by 1 and mature nisin by 2. The marker protein band (M) corresponds to 10 kDa. (B) The time-dependent cleavage of $NisP_s$ was monitored using three different pre-NisA variants [uNisA (white bars), dNisA (gray bars), and fully modified mNisA (black bars)]. Reactions were performed in three independent experiments, and error bars represent the standard error of the mean.

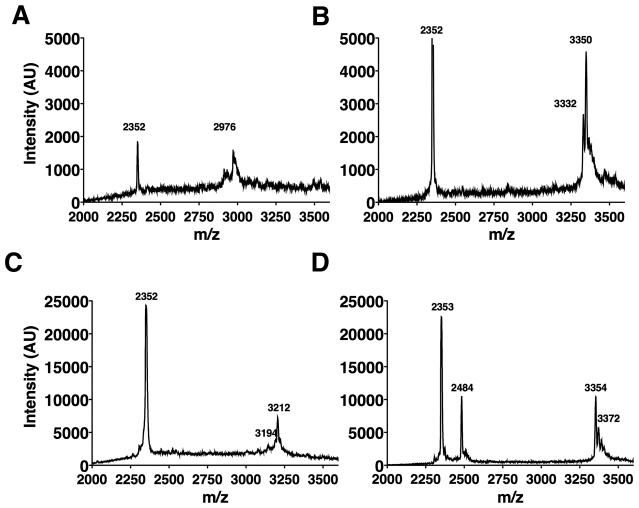

Figure 5. MS analysis of pre-NisA after $NisP_s$ cleavage. MS analysis of the $NisP_s$ substrates [(A) uNisA, (B) dNisA, (C) $dNisA_{AAAAA}$, and (D) mNisA] is shown after cleavage reaction employing *in vitro* assay conditions. After incubation for 23 h, the reaction was quenched and the zip-tip-purified samples were analyzed by MALDI-TOF MS. The most prominent mass peaks were identified as mature nisin or the leader peptide. The corresponding masses are indicated in the spectra.

MALDI-TOF MS analysis also verified the cleavage of uNisA and dNisA by $NisP_s$. In the MS spectra, a peptide with a mass of 2352 Da was identified (Figure 5 and Table 2), which corresponds to the molecular mass of the leader peptides lacking the start methionine (−Met1). Furthermore, molecular masses corresponding to the core peptide of dNisA (3350 Da, peptide with 7-fold dehydrations, and 3332 Da, peptide with 8-fold dehydrations) and of noncleaved dNisA (5688 Da, peptide with 8-fold dehydrations, −Met1) were also identified (Figure 5B and Table 2).

Similar results were obtained for the substrate uNisA (Figure 5A and Table 2). The determined mass was equivalent to the molecular mass of the leader peptide (2352 Da). Furthermore, molecular masses for noncleaved precursor with and without a starting methionine (5963 and 5856 Da, respectively) were also detected. However, no molecular mass corresponding to the core peptide (3480 Da) was identified, but a mass of 2979 Da (Figure 5A) could be observed. This mass corresponds to a part of the core peptide corresponding to positions 4−32

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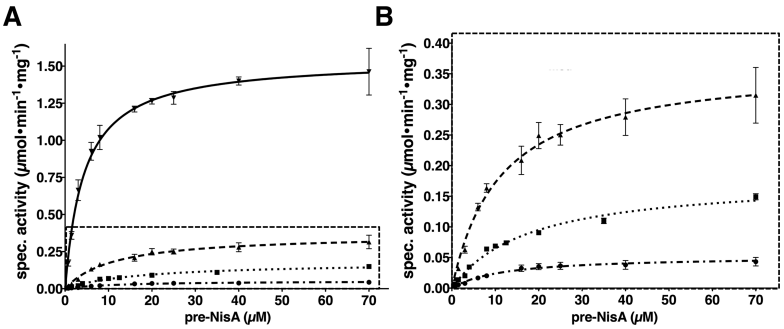

Figure 6. Michaelis–Menten analysis of pre-NisA after $NisP_s$ cleavage. Via analysis of the peak of mature nisin or the leader peptide, the activity of $NisP_s$ was determined. The reaction time for uNisA, dNisA, and $dNisA_{AAAAA}$ was prolonged to 1 h. (A) Afterward, the specific activity of leader peptidase was plotted against various substrate concentrations and analyzed by the Michaelis–Menten equation (eq 1). Results show the fits for mNisA (triangles, solid line), uNisA (circles, dashed–dotted line), dNisA (squares, dotted line), and $dNisA_{AAAAA}$ (triangles, dashed line). (B) Close-up of the plot representing the curves of uNisA, dNisA, and $dNisA_{AAAAA}$. At least three independent experiments were performed, and the error bars represent the standard error of the mean.

[determined by the FindPept tool (expasy.org)], including the serine at either position 3 or position 33.

This suggests that the unmodified peptide is not stable and undergoes an unspecific cleavage during MS analysis. Nevertheless, we demonstrated that $NisP_s$ also cleaved the leader peptide of pre-NisA lacking lanthionine rings. Thus, a determination of the kinetic parameters for pre-NisA in any post-translational maturation state is possible.

Kinetic Characterization of $NisP_s$ with Pre-NisA Variants. To obtain further quantitative insights into the different cleavage efficiencies, we determined the kinetic parameters of $NisP_s$ for three additional pre-NisA variants (besides fully modified, we also used unmodified, dehydrated, and the corresponding Cys-less variant). For this purpose, these variants were incubated at a fixed enzyme concentration (10 nM) for 60 min (for unmodified, dehydrated, and Cys-less variants) at 30 °C.

Upon variation of the substrate concentration of pre-NisA (from 0.5 to 70 μM), the apparent K_m (Michaelis constant) value and the V_{max} value were determined according to eq 1. Although the K_m values for the analyzed variants of pre-NisA ranged only from 11 to 17 μM (Table 1), the V_{max} values differed. For uNisA, the V_{max} is 52.4 ± 4.9 nmol min^{-1} mg^{-1}; for dNisA, it is 179 ± 6.3 nmol min^{-1} mg^{-1}, and for Cys-less, it is 366 ± 23 nmol min^{-1} mg^{-1} (Figure 6). The determined Michaelis constant for uNisA was 12.2 ± 3.7 μM, that for dNisA 17.7 ± 1.4 μM, and that for the Cys-less variant 11.2 ± 2.1 μM. The turnover rates of $NisP_s$ for these pre-NisA variants were 0.39 ± 0.04, 1.27 ± 0.04, and 2.6 ± 0.17 s^{-1} (Table 1), respectively. The resulting K_m and k_{cat} values were used to calculate the catalytic efficiencies.

In comparison to that of fully modified mNisA, the value for the Cys-less variant deviates by 1 order of magnitude [(2.3 ± 0.6) × 10^5 M^{-1} s^{-1} (compare to Table 1)]. For uNisA and dNisA, the difference is 2 orders of magnitude [(2.8 ± 0.9) × 10^4 and (7.1 ± 0.8) × 10^4 M^{-1} s^{-1} (compare to Table 1)]. Although the K_m values are in the same range, their k_{cat} and V_{max} values differ (compare to the section above). uNisA has the lowest k_{cat} value (0.36 s^{-1}), which is one-third of the value for dNisA (1.27 s^{-1}). The value of the Cys-less variant is 7-fold higher (2.6 s^{-1}), and the value for mNisA is even 30-fold higher (11 s^{-1}).

Because these differences might be due to cysteine residues, we investigated the effect of free thiol groups (and a possible disulfide-based dimer formation) in uNisA and dNisA. We added 5 mM DDT to the reaction buffer and incubated the peptides for 30 min prior to adding $NisP_s$. However, this had no effect on the efficiency of the cleavage reaction (not shown).

In summary, the determined kinetic parameters indicate that although pre-NisA variants without MeLan/Lan rings are recognized by $NisP_s$ they are less efficiently cleaved.

■ DISCUSSION

Nisin and other lantibiotics are processed by subtilin-like serine proteases as the final step of their maturation.[7,36] *In vivo*, only the removal of the leader peptide of a fully modified lantibiotic leads to an antimicrobial active peptide.[7] Although the activity of lanthipeptide peptidases (LanP) was studied frequently,[7,27,29−31,41] only little is known how the protease actually interacts with its substrate on a molecular level (besides the modeling from Siezen et al.[23]).

Furthermore, the K_m and k_{cat} values for only one lanthipeptide leader peptidase (ElxP) and its substrate (ElxA) were determined.[29] To the best of our knowledge, no additional detailed kinetic data exist for a class I lanthipeptide leader peptidase such as NisP. Although for NisP, *in vivo* and *in vitro* activity was described,[7] the question of why NisP cleaves only mNisA and not other maturation states of pre-NisA remains.

In 2004, Rink and co-workers demonstrated that the cleavage reaction is independent of secretion and demonstrated the cleavage of MeLan/Lan ring-containing precursor nisin by NisP.[18] They also showed that peptides without MeLan/Lan rings were not cleaved by the leader peptidase. Thus, the recognition site in the leader peptide sequence and the MeLan/Lan rings are both important for the activity of the leader peptidase, because otherwise NisP remains inactive. Similar results were obtained for the prolyl-oligopeptidase FlaP and the peptidase domain of NukT, which cleaves only fully modified precursor peptides.[30,41]

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On the other hand, studies in which a cleavage reaction was observed with a peptide lacking the MeLan/Lan rings (unmodified and also dehydrated peptides) have also been reported.[27,29,31,40] Thus, a leader sequence-based recognition of the substrate by most of the LanPs must be proposed.

In the study presented here, we demonstrate that $NisP_s$ cleaves fully modified NisA (mNisA) as well as different ring mutants, in which rings A−E positioned in the core peptide were subsequently disrupted. Surprisingly, the leader peptide of $dNisA_{AAAAA}$ was still cleaved. Because of the missing cysteines, this variant contains no MeLan/Lan rings. Furthermore, we observed that other peptides such as uNisA and dNisA also act as substrates of $NisP_s$. However, the cleavage reaction of these peptides is slower as indicated by the corresponding k_{cat} values (unmodified, 0.39 s^{-1}; dehydrated, 1.27 s^{-1}; Cys-less, 2.6 s^{-1}; modified, 11 s^{-1}), resulting in a strongly reduced catalytic efficiency of $NisP_s$ in comparison to that of the modified peptide. To obtain better insight into how the catalytic efficiency of $NisP_s$ is changed with respect to magnitude, we expressed it as relative k_{cat}/K_m values (Table 1). Here, the value for mNisA is arbitrarily set to 1, and for peptides without MeLan/Lan rings, the value is <1 [$dNisA_{AAAAA}$ (0.09) > dNisA (0.03) > uNisA (0.01)].

As NisP recognizes the GASPR motif of the leader peptide, a cleavage of peptides without MeLan/Lan rings might also be possible. The reduced turnover rates are likely due to the missing MeLan/Lan rings, which suggest the importance of thioether rings for proper substrate binding. If a simple Michaelis−Menten kinetic is assumed and the k_{cat} values are affected, the stability of the enzyme−substrate complex ([ES]) is altered.[48] Therefore, a decreased k_{cat} value can reflect a larger contribution of binding energy to the [ES], but no additional contribution to the transition state. As a result, the amount of Gibbs free energy is larger and the reaction rate is reduced.[49] From uNisA to dNisA and $dNisA_{AAAAA}$, we observed an increase in k_{cat}, whereas the K_m value remains virtually constant (11−17 μM). Thus, we assume that the binding energy leads to a more stable [ES] complex and is not used for catalysis of these substrates. For cleavage of the substrate, the correct orientation of the cleavage site in the catalytic center represents the rate-limiting step of the reaction. As these substrates lack MeLan/Lan rings, the probability of the cleavage site GASPRlI being oriented correctly within the catalytic center is reduced. Please note that the NisP used in this study is a secreted variant ($NisP_s$, amino acids 196−572) and the kinetic properties and substrate specificity might be influenced in comparison to those of wild-type NisP.

The partial cleavage of the leader sequence of the Cys-less FlaA mutant (C8A, C21A) by FlaP[41] also supports our results. Here, the authors postulated that the polarity of the unmodified hydroxyl and thiol side chains plays a role in preventing hydrolysis of the unmodified and dehydrated peptides. As uNisA, dNisA, and $dNisA_{AAAAA}$ all contain unmodified hydroxyl and thiol side chains, the turnover rate is reduced, resulting in a lower cleavage efficiency. The k_{cat} of peptides with unmodified thiol groups is even further reduced, as $NisP_s$ possesses a lower turnover rate for uNisA and dNisA than for $dNisA_{AAAAA}$ (it contains only unmodified hydroxyl groups). Furthermore, we state that the decrease in the turnover rate is not based on intermolecular or intramolecular dimerization of the substrates uNisA and dNisA, as the addition of a reducing agent did not improve the cleavage efficiency.

In 2014, the crystal structure of NisP was determined and provided insights into how mNisA might interact with the enzyme.[25] On the basis of the interaction of the C-peptide (a part of NisP's C-terminus after autocleavage) within the S2 and S3 binding pockets of NisP, the authors noted that a proline in the P2 position (as in the leader peptide sequence) would influence this interaction. This would be not the case, if another residue such as valine is present at this position as it would further influence the affinity of NisP for its substrate. If proline leads to a lower binding affinity, the change of the recognition site to the autocleavage site would explain why the authors observed cleavage of uNisA. In the absence of Lan rings, NisP can cleave the leader peptide of unmodified nisin if the cleavage site is identical to the putative pro-NisP autocleavage site (VSLR-QP).[37] Thus, the theoretically higher affinity of the leader peptide, with a change in the cleavage site, circumvents the reduced rate of turnover of NisP with substrates lacking the MeLan/Lan rings. Nevertheless, in our *in vitro* assay, we observe cleavage of pre-NisA without MeLan/Lan rings even at the leader peptide cleavage site.

Besides the leader peptidase NisP (class I lanthipeptide peptidase, NisA group), the peptidase ElxP (class I lanthipeptide peptidase, ElxA group) possesses a similar recognition motif, for which a leader peptide sequence-based recognition was suggested,[29] and kinetic data are available only for this leader peptidase. In comparison to $NisP_s$, ElxP shows a catalytic efficiency that is 4 orders of magnitude lower. The value for ElxP is 2.4×10^2 M^{-1} s^{-1}, while we obtained a value of 2.5×10^6 M^{-1} s^{-1} for $NisP_s$. However, one has to keep in mind that ElxA contains a six-histidine tag at the N-terminus, which might influence the interaction of the enzyme with the substrate. This could lead to the 50-fold higher K_m value (ElxP and His_6ElxA, 223 ± 44 μM) and a 200-fold lower turnover rate (ElxP and His_6ElxA, 0.05 s^{-1}). Both the lower K_m value (4.4 μM) and the higher k_{cat} value (11 s^{-1}) result in a higher catalytic efficiency of $NisP_s$ toward its natural substrate, mNisA. In the absence of any other kinetic parameters for other lanthipeptide leader peptidases, further evaluation and comparison are not possible.

The activity of $NisP_s$ with its natural substrate demonstrates that the recognition site is important, but for an efficient cleavage, the peptide has to contain at least one MeLan/Lan ring (in our study, the first lanthionine ring at amino acids 3−7). This highlights the importance of NisP as a tool to cleave therapeutics peptides with a thioether ring.[50] For cleavage of the leader sequence of NisA, trypsin can be used,[7] but it cleaves after Arg or Lys residues in a manner more nonspecific than that of NisP. Moreover, if the peptide has only one Lan ring and Lys residues are accessible for the peptidase, the nisin leader peptidase NisP should be used as it recognizes a defined motif and a substrate with MeLan/Lan rings is cleaved in an efficient manner.

In summary, we demonstrated that the presence of MeLan/Lan rings is essential for NisA recognition by its leader peptidase, which leads to higher turnover rates. The binding of NisP to the leader peptide, mainly via binding pockets S1−S4, contributes to a basal proteolytic activity. Here, the presence of only MeLan/Lan rings increases the turnover rates and decreases the apparent K_m value. Thus, for an efficient cleavage on an appropriate time scale, the substrates for lanthipeptide proteases require MeLan/Lan rings.

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.7b00524.

SDS−PAGE of $NisP_s$ and NisA variants (before and after cleavage), SEC chromatogram of $NisP_s$, RP-HPLC calibration, and time dependency and pH dependency of $NisP_s$ activity (PDF)

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Notes

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■ ABBREVIATIONS

AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; Dha, dehydroalanine; Dhb, dehydrobutyrine; IMAC, immobilized metal ion affinity chromatography; RiPPs, ribosomally synthesized and post-translationally modified peptides; RP-HPLC, reverse phase high-pressure liquid chromatography; SEC, size-exclusion chromatography.

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Supplemental Information

Substrate Specificity of the Secreted Nisin Leader Peptidase NisP

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Running title: *Lantibiotic leader peptidase activity*

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List of Supplemental Information

EXPERIMENTAL PROCEDURES

***In vitro* activity assay of $NisP_s$**

The kinetic parameters of purified $NisP_s$ for its natural substrate mNisA were determined after establishing optimal *in vitro* activity assay conditions. For this purpose, the optimal concentration of enzyme, the time dependent cleavage of substrate (Figure S2) and different buffer conditions (Figure S3) were investigated.

Initially, the molar ratio of enzyme to substrate was varied wherein molar ratios of 1: 1000, 1: 2500 and 1: 5000 were used to determine the possible reactions between $NisP_s$ and the nisin precursor peptide. 50 µM of precursor peptide was incubated with 10, 25 and 50 nM of enzyme in in 50 mM HEPES-NaOH pH 7.0, 150 mM NaCl in a reaction volume of for different time points. Addition of 1 % HCl (or TFA) supplemented with 1 mg/ml AEBSF (VWR) quenched the reaction and the formation of mature nisin was monitored via RP-HPLC. The enzyme activity experiments were conducted in a set of three independent experiments (Figure S2).

Subsequently, a pH screen was performed to investigate the pH dependency of the reaction (Figure S3). The cleavage reaction was analyzed with different buffers ranging from pH 3 to 11 (Table S3) with a fixed $NisP_s$ concentration (10 nM) in a 100 µl reaction volume at 30 °C. Due to the instability of mNisA or mature nisin under acidic pH (1) and also > pH 8 (lead to precipitation of peptide), pH values from 4 to 8 are a good range to test the activity. Furthermore, variations in ionic strength neither lead to a higher nor lower activity of the protease (not shown).

Therefore, a citrate-based buffer at pH 5.0 with physiological salt concentration (150 mM NaCl) was chosen for further activity assays.

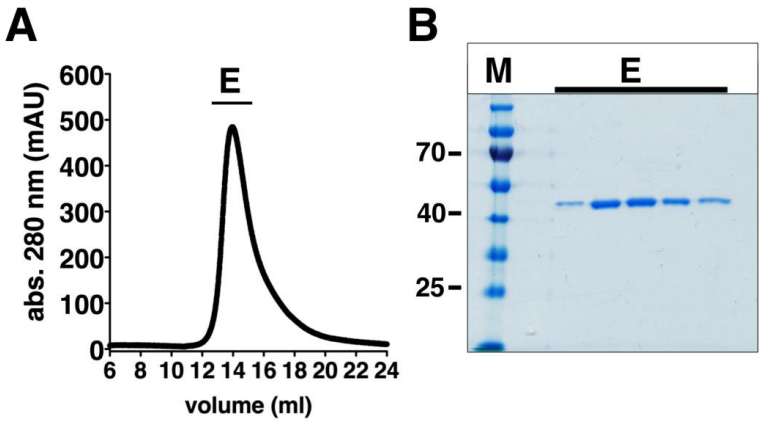

Figure S1. SEC chromatogram and SDS PAGE gel of $NisP_s$ purification.
(**A**) Two step purification of $NisP_s$ via IMAC and SEC. For SEC, $NisP_s$ was applied onto a Superdex 200 10/300 GL column. Purification was monitored via UV absorption at 280 nm (black line). (**B**) SDS-PAGE of the main elution fractions (**E: no. 1-5**) of the SEC stained with colloidal Coomassie. Marker proteins (**M**) confirm the MW (42 kDa) of secreted $NisP_s$.

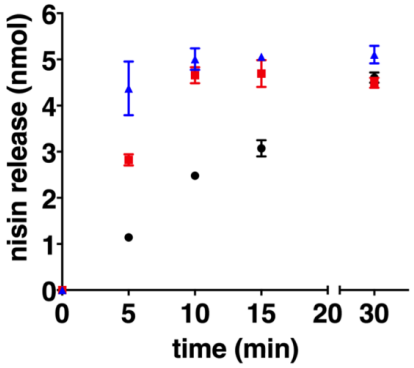

Figure S2. Time dependent nisin formation after incubation with $NisP_s$.
The substrate mNisA (50 µM) was incubated with purified $NisP_s$ in a molar ratio of 1: 1000 (blue), 1: 2500 (red) and 1: 5000 (black).

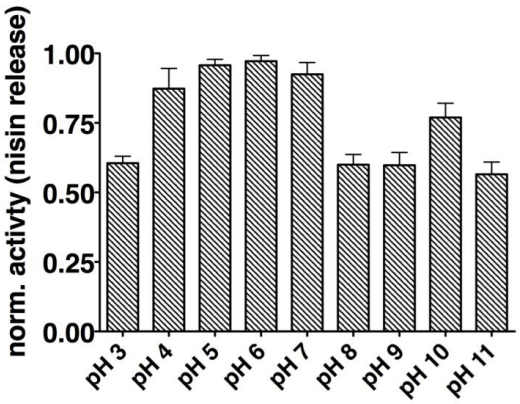

Figure S3. Screening the pH-dependence of the activity of $NisP_s$.
The substrate mNisA (20 μM) was incubated with purified $NisP_s$ (molar ratio of 1: 2000) for 5 minutes under different buffer conditions. The pH ranged from pH 3 to pH 11 using buffers according to their pK_A values. Activity of the protease was normalized and plotted against pH values. Normalized values represent data from three independent experiments with the standard deviation reported as errors bars.

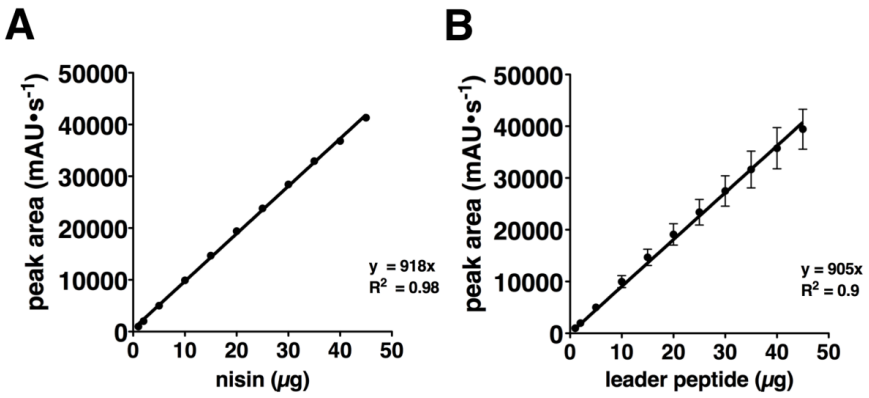

Figure S4. Peptide calibration of unknown peptide concentrations.
The absorption of nisin (**A**) or leader peptide (**B**) concentrations ranging from 0.1 to 45 µg was monitored at 205 nm. A linear correlation between the peak area and the amount of peptide was observed up to 45 µg of peptide. Data plotted represents three independent serial dilutions and measurements on RP-HPLC (Agilent technologies). As a standard, lyophilized nisin powder obtained from a commercial source (Sigma), which contains 2.5 % (w/w) nisin was used. In the case of the leader peptide, a synthetic peptide (JPT peptide technologies) was used.

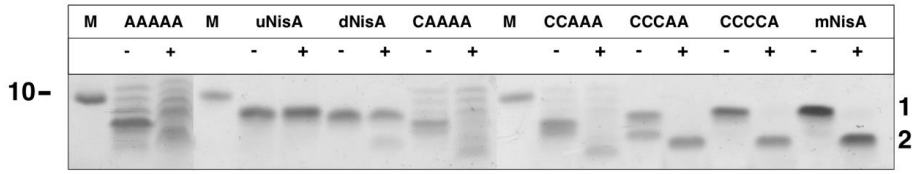

Figure S5. Tricine SDS-PAGE of different pre-NisA variants.
The cleavage of different pre-NisA variants was confirmed by Tricine SDS-PAGE. *In vitro* activity assay reaction without $NisP_s$ (-) and in presence of $NisP_s$ (+) after 1 h of incubation. Pre-NisA is indicated by "1", and mature nisin by "2". The marker protein band (M) represents 10 kDa

Table S1. Microorganisms, strains and plasmids used in this study.

Strains/plasmids	Characteristics	References
Escherichia coli DH5α	F– Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK–, mK+) *pho*A *sup*E44 λ– *thi*-1 *gyr*A96 *rel*A1	(2)
Lactococcus lactis NZ9000	nisRK$^+$	(3)
pIL3nisT	nisT	(4)
pIL3BT	nisBT	(4)
pIL3BTC	nisBTC	(5)
pNGnisP8His	Secreted NisP (NisP$_s$;aa position 196-572) with C-term. 8x His-tag	(6)
pNZ-SVnisA	Shuttle vector E.coli/L.lactis	(7,8)
pNZ-SVnisA ring mutants		(7,8), this study

Table S2. Oligonucleotides for site-directed mutagenesis.

Name	Sequence 5´-3
nisAC7A for	GTATTTCGCTAGCAACACCCGGTTG
nisAC7A rev	CAACCGGGTGTTGCTAGCGAAATAC
nisAC11A for	GTACACCCGGTGCAAAAACAGGAGCTC
nisAC11A rev	GAGCTCCTGTTTTTGCACCGGGTGTAC
nisAC19A for	GCTCTGATGGGTGCAAACATGAAAACAG
nisAC19A rev	CTGTTTTCATGTTTGCACCCATCAGAGC
nisAC26A for	GAAAACAGCAACTGCACATTGTAGTATTC
nisAC26A rev	GAATACTACAATGTGCAGTTGCTGTTTTC
nisAC28A for	CAACTTGTCATGCAAGTATTCACGTAAG
nisAC28A rev	CTTACGTGAATACTTGCATGACAAGTTG

Table S3. Buffers used for pH screening.

Buffer (50mM)	pH
lactic acid-lactate	3
lactic acid-lactate	4
citrate acid-citrate	5
MES-NaOH	6
HEPES-NaOH	7
Tris-HCl	8
Tris-HCl	9
CAPS-NaOH	10
CAPS-NaOH	11

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3.5 Chapter V – ABC transporter of lantibiotic resistance

Title The N–terminal region of nisin is important for the BceAB-type ABC transporter NsrFP from *Streptococcus agalactiae* COH1

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The N-terminal Region of Nisin Is Important for the BceAB-Type ABC Transporter NsrFP from *Streptococcus agalactiae* COH1

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Lantibiotics are (methyl)-lanthionine-containing antimicrobial peptides produced by several Gram-positive bacteria. Some human pathogenic bacteria express specific resistance proteins that counteract this antimicrobial activity of lantibiotics. In *Streptococcus agalactiae* COH1 resistance against the well-known lantibiotic nisin is conferred by, the nisin resistance protein (NSR), a two-component system (NsrRK) and a BceAB-type ATP-binding cassette (ABC) transporter (NsrFP). The present study focuses on elucidating the function of NsrFP via its heterologous expression in *Lactococcus lactis*. NsrFP is able to confer a 16-fold resistance against wild type nisin as determined by growth inhibition experiments and functions as a lantibiotic exporter. Several C-terminal nisin mutants indicated that NsrFP recognizes the N-terminal region of nisin. The N-terminus harbors three (methyl)-lanthionine rings, which are conserved in other lantibiotics.

Keywords: ABC transporter, lanthionine ring, lantibiotic, nisin, resistance

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INTRODUCTION

Lantibiotics are ribosomally synthesized antimicrobial peptides of approximately 19–38 amino acids, which are mainly produced by Gram-positive bacteria (Klaenhammer, 1993). They are characterized by extensive post-translational modifications, which result in the presence of dehydrated amino acids, lanthionine and methyl-lanthionine rings (Chatterjee et al., 2005). Lantibiotics are considered to be promising candidates as antibiotic alternatives due to their capability to inhibit various multidrug-resistant pathogenic bacteria such as Staphylococci, Enterococci, Streptococci and Clostridia species (Dischinger et al., 2014). Several lantibiotics are also effective against Gram-negative bacteria like species of the *Neisseria* and *Helicobacter* genus (Mota-Meira et al., 2000). The pharmaceutical potential of lantibiotics has been extensively studied and some are already in the preclinical and clinical phases of development (Yang et al., 2014). Lantibiotics exhibit different modes of action including binding to the cell wall, which results in growth inhibition, as well as subsequent pore formation leading to immediate cell death (Brötz et al., 1998a; Hasper et al., 2004, 2006; Islam et al., 2012).

Some bacteria, however, are inherently resistant against lantibiotics due to the expression of various protein systems that can detect and subsequently respond to the presence of lantibiotics in the extracellular medium (reviewed in Draper et al., 2015). These broad range resistance systems can either be unspecific such as changes in bacterial cell wall and membrane (Nawrocki et al., 2014;

de Freire Bastos et al., 2015; Draper et al., 2015) or more specific by proteolytic degradation of the lantibiotic itself (Sun et al., 2009).

In the present study, we focused on the lantibiotic nisin, which is produced by some *Lactococcus lactis* and *Streptococcus uberis* strains (Klaenhammer, 1993; Chatterjee et al., 2005). Nisin has a broad antimicrobial spectrum against a wide range of Gram-positive bacteria and exhibits several different modes of action (Ruhr and Sahl, 1985; Brötz et al., 1998b; Hsu et al., 2004; Hasper et al., 2006). One dominant activity is the binding to lipid II, a precursor molecule of peptidoglycan, thereby inhibiting cell wall synthesis (Wiedemann et al., 2001). Secondly, nisin is able to insert into the membrane to form pores (Hasper et al., 2004), which leads to the efflux of ions, nutrients, and subsequently to cell death. This last activity is a very rapid process and occurs almost instantly. Nisin can be structurally dissected in the N-terminus (containing the (methyl)-lanthionine rings A–C), a hinge region with the amino acids NMK and the C-terminus containing rings D and E (Van de Ven et al., 1991) (**Figure 1**). These rings are crucial for the nM activity and deletion of for example only ring E reduces the activity about eightfold (Alkhatib et al., 2014b).

Within the human pathogen *Streptococcus agalactiae* COH1 the expression of a proteogenous resistance system comprising of NSR (nisin resistance protein; a serine protease), an ATP-binding cassette (ABC) transporter (NsrFP) and a two-component system (TCS) (NsrRK) confers resistance against nisin (Khosa et al., 2013). Recently, this NSR operon has been characterized biochemically and structurally. *In vitro* studies showed that NSR expressed in *L. lactis* confers 20-fold resistance against nisin. This is mediated by cleaving off the last six amino acids from nisin, thereby lowering its activity (Sun et al., 2009; Khosa et al., 2013, 2016a). Another component of this nisin resistance operon is the BceAB-type ABC transporter NsrFP. BceAB-type ABC transporters, are putatively involved in antimicrobial peptide (like lantibiotics) removal from the lipid membrane (Gebhard and Mascher, 2011). They have been named after the BceAB transporter system from *Bacillus subtilis* conferring resistance against the antimicrobial peptide bacitracin (Ohki et al., 2003; Rietkötter et al., 2008). Interestingly, within the genomes the lantibiotic BceAB-type ABC transporter are encoded in close proximity to a TCS (Khosa et al., 2013) which senses the presence of the lantibiotic and subsequently up-regulates the expression of the ABC transporter (Dintner et al., 2011). The BceAB from *B. subtilis* has been shown to form a multicomponent complex with its designated TCS BceRS upon binding of bacitracin (Dintner et al., 2014). This highlights that the BceAB transporter from *B. subtilis* is directly involved in bacitracin sensing and consequently triggering the up-regulation of its own gene by the TCS BceRS.

Within NsrFP from *S. agalactiae* COH1, the transmembrane domain NsrP contains 10 predicted transmembrane helices and harbors a 212 amino acid large extracellular domain (ECD) in between helices VII and VIII (Khosa et al., 2013; **Figure 2**). NsrF is the nucleotide-binding domain delivering the energy for the transport by ATP hydrolysis.

In this study, we determined the function of NsrFP from *S. agalactiae* COH1 in conferring nisin resistance. We expressed only the NsrFP transporter without the corresponding NsrR/NsrK TCS and observe that NsrFP can confer resistance up to 80 nM nisin. In comparison to this, a strain lacking this transporter can only survive a nisin concentration of 5 nM. Above this concentration the cells are suffering from pore formation mediated by nisin. Furthermore, we could show that NsrFP works as a lantibiotic exporter by a peptide release assay. Additionally several mutants of nisin were used to investigate the substrate specificity, which highlights that NsrFP recognizes the N-terminal region of nisin. This was confirmed by the observed resistance against nisin H (O'Connor et al., 2015) and gallidermin (Kellner et al., 1988), which both contain a similar N-terminus but differ in the C-terminal part of the peptide.

MATERIALS AND METHODS

Cloning of *nsrfp*

The *nsrfp* gene from *S. agalactiae* COH1 was amplified from the chromosomal DNA using two primers (NsrFP_for 5′-CA TCACCACCACCACTTATTAGAAATCAATCACTTAG-3′ and NsrFP_rev 5′-GTGGTGGTGGTGGTGCATATAATTCTCCTTTA TTTATTATAC-3) and ligated into *pIL-SV* (*E. coli–L. lactis* shuttle vector) (Alkhatib et al., 2014b). The point mutation $NsrF_{H202A}$ was introduced by a standard mutagenesis protocol using the following primers: forward: 5′-GATGGT AACCGCTTCAGCAAATGCTG-3′; reverse: 5′-CAGCATTTGC TGAAGCGGTTACCATC-3′. The resulting plasmid was verified by sequencing and transformed into the *L. lactis* strain NZ9000 for expression (Holo and Nes, 1989) and the corresponding strains were termed NZ9000NsrFP and NZ9000$NsrF_{H202A}$P. An empty vector *pIL-SVCm* was also transformed into the NZ9000 strain and was used as a control (that excludes any possible effect of induction of the plasmid), and this strain was called NZ9000Cm. The expression of the *nsrfp* gene is regulated by the TCS NisR/NisK present in the NZ9000 strain genome.

Expression of NsrFP and $NsrF_{H202A}$P

The NZ9000NsrFP and NZ9000$NsrF_{H202A}$P strains were grown in GM17 media supplemented with 5 μg/ml chloramphenicol. By the addition of nisin (final concentration of 1 ng/ml, which is equivalent to 0.3 nM), the expression was induced and the culture was further grown overnight. To analyze the expression, the cells were harvested at OD_{600} of 2.0 by centrifuging at 5000 × *g* for 30 min. The resulting pellet was resuspended with R-buffer [50 mM HEPES pH 8.0, 150 mM NaCl, 10% (w/v) glycerol] to an OD_{600} of 200. Then 1/3 (w/v) glass beads (0.3 mm) were added and cells were lysed. A cycle of 1 min disruption and 2 min cooling on ice was repeated five to six times. A low centrifugation step at 10,000 × *g* to collect the cytoplasmic part was performed. Followed by a high spin step (100,000 × *g*) to harvest the membranes. To collected cytoplasmic and membrane fractions SDS-loading dye [0.2 M Tris–HCl, pH 6.8, 10% (w/v) SDS, 40% (v/v) glycerol, 0.02% (w/v) bromophenol and β-mercaptoethanol] was added, samples were

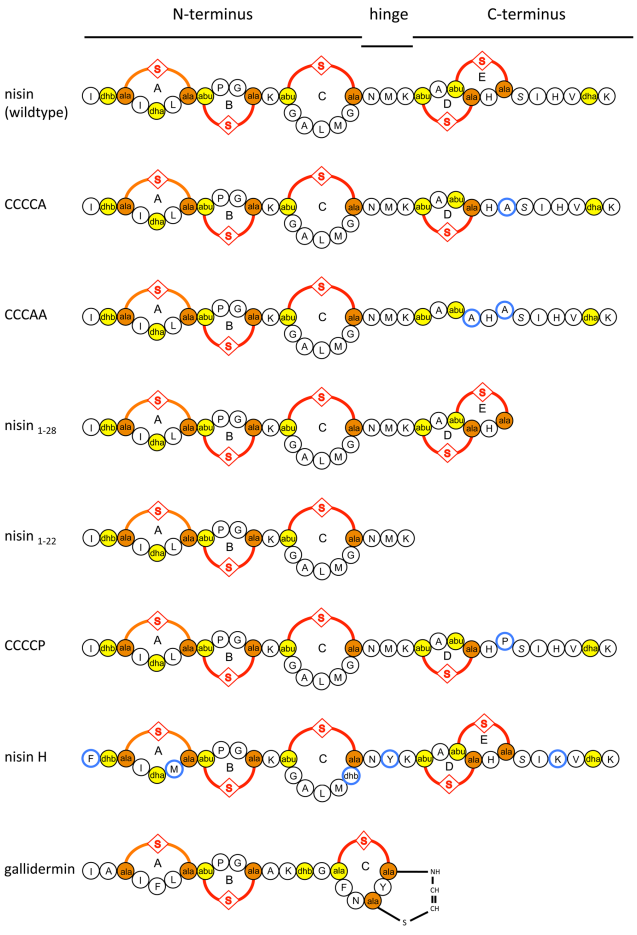

FIGURE 1 | Schematic overview of nisin, the variants of nisin, nisin H, and gallidermin used in this study. Introduced mutations in CCCCA, CCCAA, CCCCP, and the natural variants in nisin H are highlighted in blue. The (methyl)-lanthionine rings (labeled with A, B, C, D, and E) are formed by a dehydrated amino acid residue and a cysteine residue side chain (orange and yellow).

further used for SDS-PAGE and Western blot analysis (20 µl loaded). To detect NsrFP and $NsrF_{H202A}P$ a polyclonal antibody against the large extracellular loop of NsrP was used (Davids Biotechnologie, Regensburg, Germany).

Cloning of the Nisin H and CCCCP Variant

The used variants (CCCCA, CCCAA, $nisin_{1-28}$ and $nisin_{1-22}$) were previously described in Alkhatib et al. (2014b). Nisin H (O'Connor et al., 2015) was created by introducing five point mutations into the *pNZ-SV-nisA* vector (Alkhatib et al., 2014b). For the I_1F-L_6M point mutations we used the following primers (forward: 5′-GTG CATCACCACGCTTTACAAGTATTTCGATGTGTACACCCGG TTG-3′; reverse: 5′-CAACCGGGTGTACACATCGAAATACTT GTAAAGCGTGGTGATGCAC-3′). The $G_{18}T$-$M_{21}Y$ mutations were introduced with the primers (forward: 5′-GTAAAAC AGGAGCTCTGATGACATGTAACTATAAAACAGCAACTTGT CATTG-3′; reverse: 5′-CAATGACAAGTTGCTGTTTTATAGTT ACATGTCATCAGAGCTCCTGTTTTTAC-3′) and the last mutation $H_{31}K$ with the primers (forward: 5′-CTTGTCATTG

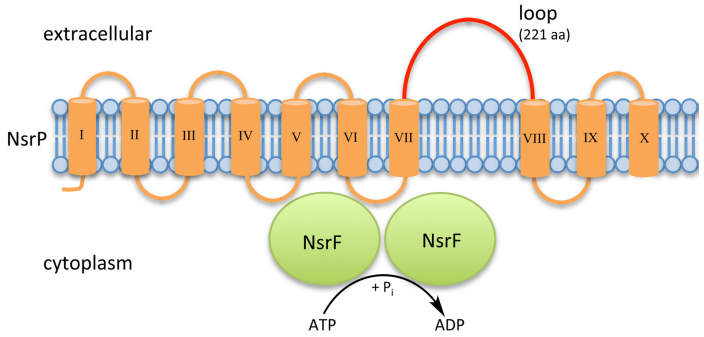

FIGURE 2 | Schematic representation of NsrFP from *S. agalactiae* COH1. NsrFP is an ABC transporter consisting of NsrF (highlighted in green), which hydrolyses ATP and NsrP (highlighted in orange). NsrP is a membrane protein consisting of 10 predicted transmembrane helices with an extracellular domain (depicted in red), which is a characteristic for the BceAB-type of lantibiotic resistance transporters.

TAGTATTAAAGTAAGCAAATAAGCTTTC-3′; reverse: 5′-GAAAGCTTATTTGCTTACTTTAATACTACAATGACAAG-3′).
The CCCCP variant, were the last cysteine was exchanged by a proline was created into the *pNZ-SV-nisA* vector with the primers (forward: 5′-CAGCAACTTGTCATCCAAGTATTCACGTAAG-3′; reverse: 5′-CTTACGTGAATACTTGGATGACAAGTTGCTG-3′).

The resulting plasmids were verified by sequencing and transformed into the *L. lactis* strain NZ9000 (already containing the *pIL3-BTC* vector; Rink et al., 2005) for expression by electroporation as described above.

Expression, Purification of Prenisin Variants

Prenisin was purified as described in Alkhatib et al. (2014b). Activation of purified prenisin was done by overnight cleavage at 8°C with purified NisP (Abts et al., 2013). The efficiency of the reaction was monitored and the concentration of active nisin was determined by RP-HPLC as previously described (Abts et al., 2013). The activated nisin variants were then directly used for IC_{50} assays. Gallidermin is commercially available (Enzo Life Sciences).

Purification of Nisin

Nisin was purified as described in Abts et al. (2011). The concentration of nisin was measured by using RP-HPLC as previously described (Abts et al., 2013).

Determination the Activity of Nisin by Growth Inhibition (IC_{50})

Cells from the different expressing strains were grown overnight in GM17 supplemented with 5 μg/ml chloramphenicol in presence of 1 ng/ml nisin. The diluted cells (final OD_{584} was 0.1) were incubated with a serial dilution of nisin in a 96-well plate. The total volume in each well was 200 μl, consisting of 50 μl nisin and 150 μl GM17 containing the corresponding *L. lactis* strain. The highest concentration of nisin used was adapted to the corresponding maximum resistance displayed by each strain.

The plate was incubated at 30°C. After 5 h, the optical density was measured at 584 nm via 96-well plate reader BMG. The normalized optical density was plotted against the logarithm of the nisin concentration in order to calculate the IC_{50} of nisin and the data was evaluated using the following equation (Eq. 1):

$$y = \mathrm{OD}_{\min} + \frac{\mathrm{OD}_{\max} - \mathrm{OD}_{\min}}{1 + 10^{(\log(\mathrm{IC}_{50}) - x)} \times p}$$

The OD_{max} value describes the normalized optical density value where no nisin was added, while the OD_{min} value corresponds to the normalized optical density of the cells grown in the highest nisin concentrations. The y represents the resulted normalized optical density value and x represents the logarithmic of the nisin concentration added. The IC_{50} value is the concentration of nisin where the growth of the *L. lactis* strain is inhibited by 50% (Abts et al., 2011).

Calculation of the Fold of Resistance

We determined the IC_{50} value of nisin against the NZ9000Cm sensitive strain as well as the strain NZ9000NsrFP and NZ9000$NsrF_{H202A}$P. By dividing these two values the fold of resistance is obtained. For example wild type nisin displayed an IC_{50} of 4.9 nM against NZ9000Cm and 82.2 nM against NZ9000NsrFP. Dividing these two values results in a fold of resistance of 16.7. We used this fold of resistance to obtain a quantitative, comparable value for the nisin variants.

Dependency of Nisin Variants on Induced Expression of NsrFP

We verified the expression level of NsrFP in the corresponding strain NZ9000NsrFP by inducing expression with the different nisin variants. Here, we used half the IC_{50} value, which

was determined for each nisin variant against the sensitive NZ9000Cm strain, to exclude an effect on the expression level of NsrFP. The initial OD_{600} of the NZ9000NsrFP strain was 0.1 and we induced each sample with the half IC_{50} value of the corresponding nisin variants. The strains were further grown for 5 h at 30°C. After harvesting the cells, SDS-PAGE samples were prepared as describe above. The expression of NsrFP was analyzed by Western blot using a polyclonal antibody directed against the extracellular loop.

SYTOX Green Nucleic Acids Binding Assay

SYTOX green nucleic acids binding dye possesses a high binding affinity toward nucleic acids. It enters cells, which contain a pore in the plasma membrane and never crosses the intact membranes of living cells (Roth et al., 1997). The cells of NZ9000NsrFP were grown overnight in GM17 supplemented with 5 μg/ml chloramphenicol in presence of 1 ng/ml nisin. The next day, the overnight culture was diluted to an OD_{600} of 0.1 in fresh media supplemented with 5 μg/ml chloramphenicol. The cultures were grown until the OD_{600} reaches 0.5, the SYTOX green dye was added at a final concentration of 2.5 μM and incubated for 5 min according to the manual of the manufacturer (Invitrogen). The fluorescence signal, which was measured at an excitation and emission wavelength of 504 and 523 nm, respectively, was monitored. After a stable baseline is reached, nisin was added and the fluorescence was monitored over an additional time period.

Nisin Transport Assay

To answer the question whether NsrFP is an importer or exporter we performed a well-known nisin transport assay (Stein et al., 2003, 2005).

We grew the cells of NZ9000NsrFP, NZ9000$NsrF_{H202A}$P, and NZ9000Cm in GM17 supplemented with 5 μg/ml chloramphenicol in presence of 1 ng/ml nisin. We harvested the cells and washed them with 50 mM HEPES, pH 7, 500 mM NaCl, 10 % (v/v) glycerol. The cell density was adjusted to an OD_{600} of 10 in 1 ml of the corresponding strain and incubated with 8 μg nisin at 30°C for 30 min under gently shaking. After centrifugation at 10,000 × *g* for 10 min the supernatant was collected and applied to RP-HPLC for the quantification of nisin as described above.

RESULTS

IC_{50} Determination of NZ9000NsrFP and NZ9000$NsrF_{H202A}$P

We cloned NsrFP and $NsrF_{H202A}$P in a *pIL-SVCm* shuttle vector and induced the expression with a sublethal amount of nisin (0.3 nM). To ensure, that there were no side effects from induction with nisin, we compared all experiments with a control strain. This strain was transformed with an empty plasmid and was treated exactly the same. We observed that the induction using 0.3 nM had no influence on the morphology or growth behavior of the *L. lactis* strains. This expression system has been used in the past for several proteins involved in nisin modification as well as immunity and resistance (Kuipers et al., 2004; Plat et al., 2011; AlKhatib et al., 2014a; Khosa et al., 2016a). Nisin was purified as previously described (**Figure 3**; Abts et al., 2011). To address the activity of nisin against the NZ9000Cm, NZ9000NsrFP, and NZ9000$NsrF_{H202A}$P strains, growth experiments were performed using an increasing concentration of nisin. From these the IC_{50} values were determined, which reflects the growth inhibition of the corresponding strain by 50% using Eq. 1.

Nisin is highly active against the NZ9000Cm strain, as observed by the IC_{50} value of 4.9 ± 0.4 nM (**Figure 4** and **Table 1**). The NZ9000NsrFP strain exhibited a higher IC_{50} value of 82.2 ± 6.7 nM (**Figure 4** and **Table 1**). By dividing the two values a 16.7-fold of resistance was calculated (see Materials and Methods). This highlights that NsrFP expressed in *L. lactis* confers resistance against nisin. We cloned a variant of NsrFP termed $NsrF_{H202A}$P, where the histidine at position 202 of NsrF is mutated to an alanine. By sequence alignments this histidine residue was identified as the catalytically important residue for ATP hydrolysis, generally termed as H-loop (Zaitseva et al., 2005). The corresponding NZ9000$NsrF_{H202A}$P strain displayed a lower IC_{50} value of 5.1 ± 0.8 nM, which within experimental error represents the same value as obtained for the NZ9000Cm strain (**Figure 4** and **Table 1**). This suggests that NsrFP relies on ATP hydrolysis to confer resistance against nisin. Here, we have to note that the expression of $NsrF_{H202A}$P led to a reduced final OD (0.8 compared to 1.1 for the wild type strain) in our growth experiments. The observed difference does not rise from different expression levels of NsrFP and the $NsrF_{H202A}$P variant as shown by Western blot analysis using a polyclonal antibody directed against the large ECD of NsrP (Davids Biotechnology, Regensburg, Germany) (**Figure 3A**).

Pore Formation of Nisin in the NZ9000NsrFP Strain

Nisin is able to form pores in the membrane of Gram-positive bacteria initiated by the initial binding to lipid II and subsequently reorientation of the C-terminal part of nisin into the membrane (Hasper et al., 2004). This leads to membrane leakage and rapid cell death. We monitored this pore formation using a SYTOX green nucleic acid dye (Roth et al., 1997). When pores are formed in the membrane the SYTOX dye enters the cells and binds to the DNA, resulting in an increased fluorescence signal. This is an almost instant effect, which can be monitored in real time. We monitored the pore forming action of nisin against the NZ9000NsrFP, using different nisin concentrations, which were based on the IC_{50} values of the corresponding strains determined above. As a control, we added only buffer without nisin, which resulted in no increase of the fluorescence signal as observed by the black line in **Figure 5**. This control indicates that no cells are spontaneously lysed under this experimental setup.

When 40 nM nisin (corresponding to half the IC_{50} value determined for the NZ9000NsrFP strain) was added to the

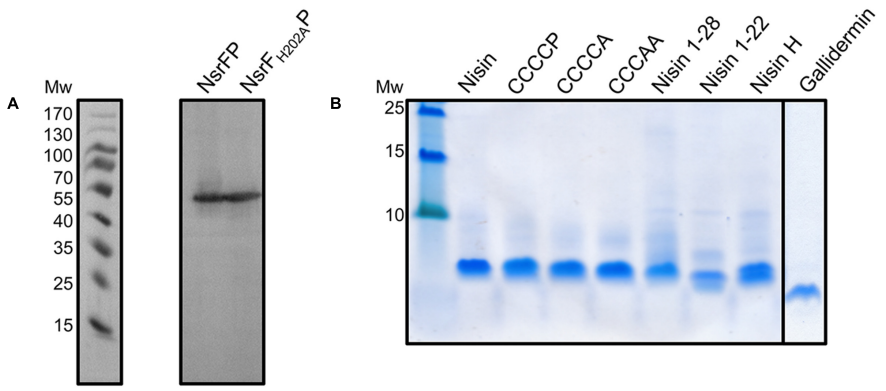

FIGURE 3 | Expression of NsrFP and NsrF$_{H202A}$P in *L. lactis* and purification of nisin variants. **(A)** The expression of NsrFP and NsrF$_{H202A}$P was monitored by Western blot using a polyclonal antibody against the extracellular domain. As observed both proteins are expressed at a similar level in *L. lactis* NZ9000. **(B)** Nisin and its variants were purified and activated by using a standard procedure (see Materials and Methods). All substrates display a similar purity, judged by a 20% Tricine-SDS-PAGE.

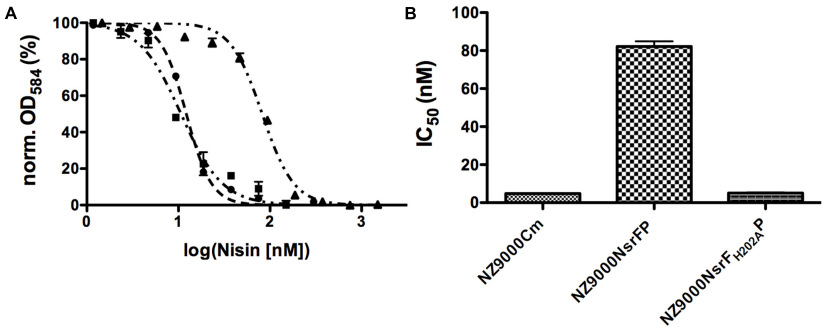

FIGURE 4 | Activity of nisin against the NZ9000Cm, NZ9000NsrFP, and NZ9000NsrF$_{H202A}$P strains. **(A)** The IC$_{50}$ of nisin against the NZ9000Cm (●), NZ9000NsrFP (▲), and strain NZ9000NsrF$_{H202A}$P (■) was determined. As observed the curve shifted to higher nisin concentration indicating that the NZ9000NsrFP strain is more resistant toward nisin. **(B)** The calculated IC$_{50}$ values of nisin against the NZ9000Cm, NZ9000NsrFP, and the NZ9000NsrF$_{H202A}$P strain are highlighted.

NZ9000NsrFP strain, no increase of the fluorescence signal was observed (**Figure 5**, green line). This indicates that the NZ9000NsrFP strain can survive a nisin concentration of 40 nM. Only a small linear increase was visible after 400 s, which reflects to a less extent cell lysis after some time. A nisin concentration equivalent to the IC$_{50}$ value (80 nM) resulted in a slightly stronger increase of the signal after a delay time (**Figure 5**, blue line). Finally, after adding a nisin concentration of two-times the IC$_{50}$ value (e.g., 160 nM to the NZ9000NsrFP strain) a rapid increase of the fluorescence signal was observed and reaches a stable plateau already after a couple of seconds. This shows that NsrFP is not able to confer resistance above the determined IC$_{50}$ concentration (**Figure 5**, red line).

Nisin Transport Assay–Peptide Release Assay

We performed a peptide release assay to verify the transport direction of NsrFP. Previously, the same assay was used to characterize NisFEG and SpaFEG, two exporting systems from lantibiotic producing strains (Stein et al., 2003, 2005). Here, we incubated the NsrFP expressing strain with 8 μg nisin for 30 min. After centrifugation of the cell, the supernatant was analyzed via RP-HPLC to determine the amount of nisin. From 8 μg nisin, 4.3 μg nisin was recovered from the supernatant (**Figure 6**). As a control, we used the NsrF$_{H202A}$P and the sensitive NZ9000Cm strain. There only ∼2 μg nisin was recovered from the supernatant (**Figure 6**). This shows that NsrFP is able to

TABLE 1 | IC_{50} values of nisin and its variants against the NZ9000Cm, NZ9000NsrFP, and NZ9000$NsrF_{H202A}$P strains.

	NZ9000Cm	NZ9000NsrFP		NZ9000$NsrF_{H202A}$P	
Nisin variant	**IC_{50} (nM)**	**IC_{50} (nM)**	**Fold of resistance**	**IC_{50} (nM)**	**Fold of resistance**
Wild type	4.9 ± 0.4	82.2 ± 6.7	16.7	5.1 ± 0.8	1.1
CCCCP	39.7 ± 1.5	238.4 ± 11.7	6.0	37.8 ± 3.9	0.9
CCCCA	64.4 ± 8.4	2023 ± 143	31.4	38.9 ± 5.9	0.6
CCCAA	278.6 ± 18.8	36346 ± 3632	130.5	154.6 ± 30.8	0.5
$Nisin_{1-28}$	157.0 ± 8.7	5243 ± 1225	33.4	65.3 ± 11.4	0.4
$Nisin_{1-22}$	309.9 ± 51.4	12220 ± 804	39.4	209.0 ± 39.9	0.7
Nisin H	7.0 ± 0.4	86.5 ± 3.7	12.3	7.5 ± 0.8	1.1
Gallidermin	67.1 ± 9.1	840 ± 87.0	12.5	59.7 ± 7.3	0.9

Besides the IC_{50} values also the fold of resistance against the nisin variants mediated by NsrFP are shown. The fold of resistance is calculated by the division of the IC_{50} value obtained of the NZ9000NsrFP by the value for the NZ9000Cm strain. The values represent the average and standard deviation of at least four different experiments.

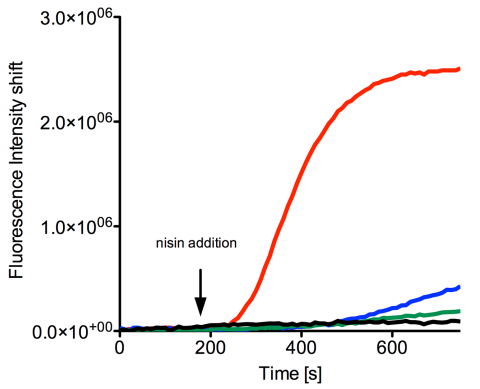

FIGURE 5 | SYTOX green assay to visualize pore formation mediated by nisin. The NZ9000NsrFP strain was grown until OD_{600} of 0.5 and then incubated with the SYTOX dye. After a stable baseline was reached nisin (indicated with an arrow) was added at various concentrations: 40 nM (green line), 80 nM (blue line), and 160 nM (red line). As a control only buffer was added (black line). The fluorescence signal was monitored online using a fluorolog (Horiba III) and the rapid increase indicates pore formation. The curves are representatives of at least four biological replicates.

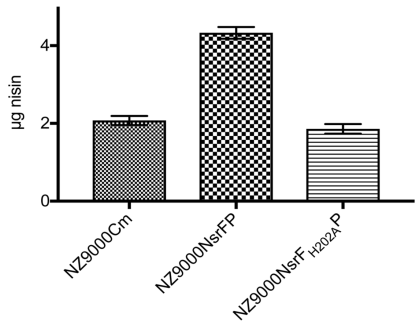

FIGURE 6 | Nisin transport assay. The NZ9000Cm, NZ9000NsrFP, and NZ9000$NsrF_{H202A}$P strain were incubated with 8 µg nisin. After incubation for 30 min the cells were spun down and the amount of nisin in the supernatant was determined using RP-HPLC. The NZ9000Cm and NZ9000$NsrF_{H202A}$P strain showed similar nisin amounts. The NZ9000NsrFP strain revealed a ∼2-fold increased nisin amount, highlighting that NsrFP exports nisin from the *L lactis* membrane.

export nisin from the cellular membrane. Our results are similar to the results found for the NisFEG and SpaFEG transporters leading to the same conclusion that NsrFP is exporting nisin from the cellular membrane as well.

Substrate Specificity of NsrFP

In order to investigate the substrate specificity of NsrFP we used a set of nisin variants. Here, the nisin variants CCCCA, CCCAA, $nisin_{1-28}$, and $nisin_{1-22}$ were used (Khosa et al., 2016a). These variants are lacking the last or last two lanthionine rings or display deletions at the C-terminus of nisin, respectively. CCCCP is a variant, where the cysteine at position 28 (important for ring E formation) is exchanged to a proline (for a schematic view see **Figure 1**).

Expression and purification were performed as previously described (Alkhatib et al., 2014b), resulting in high purity (**Figure 3B**). The activities of these variants were determined against the nisin sensitive NZ9000Cm strain and the strains expressing NsrFP or $NsrF_{H202A}$P, respectively (**Table 1**). By comparing these values the fold of resistance was obtained (**Figure 7**) as determined for the wild type nisin (see above and Materials and Methods).

For CCCCP, the fold of resistance was determined to be 6.0 (IC_{50} against NZ9000Cm was 39.7 ± 1.5 nM and against NZ9000NsrFP was 238.4 ± 11.7 nM). For CCCCA, the fold of resistance was determined to be 31.4 (IC_{50} against NZ9000Cm was 64.4 ± 8.4 nM and against NZ9000NsrFP 2023 ± 143 nM). The CCCAA variants displayed a 130.5-fold of resistance (IC_{50} against NZ9000Cm was 278.6 ± 18.8 nM and against NZ9000NsrFP 36346 ± 3632 nM). The two deletion mutants displayed a 33.4 ($nisin_{1-28}$) and 39.4 ($nisin_{1-22}$) fold

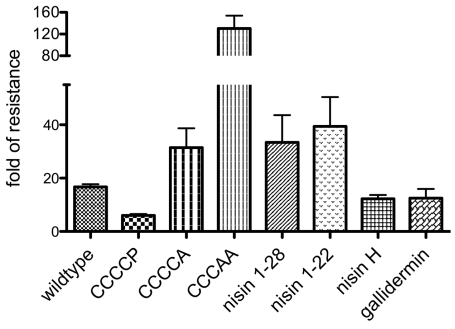

FIGURE 7 | Substrate specificity of NsrFP. Graphical representation of the fold of resistance exhibited by NsrFP with nisin and different nisin variants (CCCCA, CCCAA, CCCCP, $nisin_{1-22}$ and $nisin_{1-28}$) as well as nisin H and gallidermin. The NZ9000Cm and NZ9000NsrFP strains were used to determine the activity of all tested lantibiotics. The error bars indicate the standard error of at least three independent experiments.

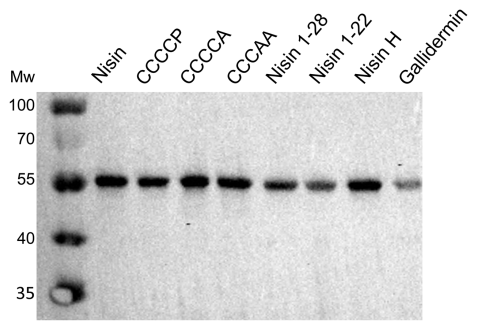

FIGURE 8 | Dependency of nisin variants on induced expression of NsrFP. The expression of NsrFP was monitored by Western blot using a polyclonal antibody against the extracellular domain. As observed, the expression of NsrFP was similar in all cases, independent of the used nisin variant.

of resistance, almost five times higher when compared to wild type. Here IC_{50} were determined to be 157 ± 8.7 nM against NZ9000Cm and 5243 ± 1225 nM against NZ9000NsrFP strain for $nisin_{1-28}$ and 309.9 ± 51.4 nM against NZ9000Cm and 12,220 ± 804 nM against NZ9000NsrFP for $nisin_{1-22}$, respectively.

These results revealed that NsrFP is able to be active as long as the N-terminal region of nisin is present, and since this part is highly conserved in several other lantibiotics, we hypothesized that the NsrFP transporter can besides nisin also recognize other lantibiotics. To test this, we used two other lantibiotics: nisin H (O'Connor et al., 2015) and gallidermin which is produced by *Staphylococcus gallinarum* Tü3928 (Kellner et al., 1988) (schematically shown in **Figure 1**). The latter contains a similar N-terminal part but has in comparison to nisin a structurally non-related C-terminus.

We determined the fold of resistance mediated by NsrFP for these lantibiotics (**Table 1** and **Figure 7**). Here, it was observed that nisin H as well as gallidermin are also recognized and NsrFP confers resistance to these lantibiotics. Our calculated fold of resistance is 12.3 for nisin H and 12.5 for gallidermin (**Table 1**). This strengthens the observation that the N-terminal region plays a predominant role in substrate recognition, since nisin H and gallidermin are recognized as well with similar efficiencies.

We were wondering whether the effect of a higher fold of resistance actually was a result of an increased expression of NsrFP in the membrane. Therefore, we incubated NZ9000NsrFP cells with the corresponding nisin variants (note: the concentration is 1/2 IC_{50} value of each nisin variant) and visualized the expression of NsrFP by Western blot. Here, we observed that the expression levels of NsrFP were similar for each strain and thereby cannot be the reason for the higher increased fold of resistance (**Figure 8**).

DISCUSSION

Lantibiotics possess antimicrobial activity against various bacteria including the well known MRSA, VISA, and VRE strains (Piper et al., 2009). However, various bacteria, especially human pathogens are actually inherently resistant against lantibiotics, which they do not produce themselves. Interestingly, this resistance is often arising from the expression of one or two membrane embedded proteins. Here, one belongs to the BceAB-type ABC transporter family and confer resistance against antimicrobial peptides including lantibiotics (Kallenberg et al., 2013; Kingston et al., 2014). Genetically the BceAB-type transporters are often located next to a TCS in the genome, which regulates the expression of the genes encoded (Dintner et al., 2011). It is thought that especially the ECD, which is a hallmark of BceAB-type ABC transporters is involved in lantibiotic sensing and transferring the signal to the corresponding histidine kinase (Staroń et al., 2011; Kallenberg et al., 2013). We focused on the nisin resistance operon from the *S. agalactiae* COH1, more specifically the BceAB-type ABC transporter NsrFP (Khosa et al., 2013). This transporter is localized on a gene operon together with the membrane associated protease NSR and the TCS NsrR and NsrK (Khosa et al., 2013, 2016b). We heterologously expressed the transporter in *L. lactis*, which lacks the NsrR/NsrK TCS and observed that NsrFP is able to confer resistance by itself. The fold of resistance, which we used as a measure of the activity, revealed that the *L. lactis* cells are able to deal with a 16-fold higher nisin concentration when compared to the same strain lacking NsrFP. The fold of resistance of an ATP hydrolysis deficient mutant of NsrFP is reduced to levels observed for the nisin sensitive NZ9000Cm strain. Like NisFEG (Stein et al., 2003) and SpaFEG (Stein et al., 2005), NsrFP acts as an lantibiotic exporter, which so far has not been conclusively shown for an lantibiotic resistance ABC transporter.

Intriguing is the observation that the N-terminal part of nisin appears to be important for NsrFP. By using C-terminal

variants and deletions of nisin the fold of resistance increased in comparison to the wild type nisin. Only the variant CCCCP displayed a reduced fold of resistance. The recognition of the N-terminal region was further underlined by the observation that nisin H as well as gallidermin were also recognized as substrates. Here, especially the latter is containing a similar N-terminal region but differs structurally completely at the C-terminus (**Figure 1**).

Previously, the recognition of ring A and B was observed for the lantibiotic resistance ABC transporter CprABC from *Clostridium difficile*, which recognizes multiple lantibiotics: for example, nisin, gallidermin, subtilin, and mutacin 1140 (McBride and Sonenshein, 2011; Suárez et al., 2013).

Within the nisin resistance operon in *S. agalactiae* COH1 two proteins, namely the membrane associated protease NSR and NsrFP, are present (Khosa et al., 2013, 2016b). NSR is cleaving off the last six amino acids of nisin resulting in $nisin_{1-28}$, which has a 32-fold lower activity. This product of NSR ($nisin_{1-28}$), however, is still well recognized by NsrFP, as shown by an even increased fold of resistance. This suggests that both proteins are working together to obtain full resistance in *S. agalactiae*. The first line of defense would be NSR and the resulting processed product $nisin_{1-28}$, is transported by NsrFP, once it reaches the membrane with high efficiency. This type of cooperativity would be similar to the natural immunity system observed in the nisin and subtilin (auto)immunity systems from *L. lactis* and *B. subtilis*, respectively. There, a cooperative mode of action of the immunity proteins LanI and LanFEG have been observed by which only full immunity was displayed when both protein are simultaneously expressed (Stein et al., 2003, 2005). Therefore, we suggest that NsrFP and NSR have a similar cooperative mode of action and only when both proteins are expressed simultaneously within the membrane of *S. agalactiae* COH1 full resistance is occurring.

AUTHOR CONTRIBUTIONS

SS conceived and directed this study. JR, ML, SL, and KE conducted the experiments. JZ-K established the mutants of nisin and created the figures. JR, ML, and SS wrote the manuscript with input of all authors. All authors read and approved the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

3.6 Chapter VI – Application of the nisin modification and secretion system

Title Systematic characterization of position one variants within the lantibiotic nisin

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Systematic characterization of position one variants within the lantibiotic nisin

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Lantibiotics are a growing class of natural compounds, which possess antimicrobial activity against a broad range of Gram-positive bacteria. Their high potency against human pathogenic strains such as MRSA and VRE makes them excellent candidates as substitutes for classic antibiotics in times of increasing multidrug resistance of bacterial strains. New lantibiotics are detected in genomes and can be heterologously expressed. The functionality of these novel lantibiotics requires a systematic purification and characterization to benchmark them against for example the well-known lantibiotic nisin. Here, we used a standardized workflow to characterize lantibiotics consisting of six individual steps. The expression and secretion of the lantibiotic was performed employing the promiscuous nisin modification machinery. We mutated the first amino acid of nisin into all proteinaceous amino acids and compared their bactericidal potency against sensitive strains as well as strains expressing nisin resistance proteins. Interestingly, we can highlight four distinct groups based on the residual activity of nisin against sensitive as well as resistant *L. lactis* strains.

Since the last decade the exponential increase of the number of antibiotic resistant strains steadily alarms the world health organization, which is reflected in their annually reports regarding the surveillance of antimicrobial and antibiotic resistance (WHO, GLASS report 2016–2017). Therefore, the urgent need for antimicrobial compounds, which can be used as alternatives to the classic antibiotic treatment, has dramatically increased. Some classes of antibiotic such as cephalosporins, macrolides, carbapenems or penicillin derivatives are vital for human medicine and the treatment of microbial infections. However, observed resistance to important antibiotic classes makes it necessary to explore new classes of natural or synthetic antimicrobial compounds[1].

One possible class are antimicrobial peptides (AMP). Within this class especially lanthipeptides possessing antimicrobial activity, which are called lantibiotics (lanthionine containing antibiotics), are considered as possible lead compounds[2]. Lantibiotics are ribosomally synthesized as a precursor peptide (LanA), between 30–60 amino acids in size and undergo specific post-translational modifications (PTM)[3,4]. They are furthermore dissected into an N-terminal leader peptide and a C-terminal core peptide, in which the PTMs are installed by specialized modification enzymes. Upon leader peptide cleavage, the lantibiotic becomes activated and exhibits antimicrobial activity with efficiencies in the low nanomolar to millimolar range. Lantibiotics like NAI-107 or NVB302 are already subjected to pre-clinical trails and might be good candidates in the treatment of multidrug-resistant strains like MRSA or similar Gram-positive strains[5–7].

Up to now >50 different lantibiotics with a similar set of PTMs have been described (for more details see review[8]). One specific PTM within the core peptide of lantibiotics is the dehydration of serine and threonine residues resulting in the formation of dehydroalanine and dehydrobutyrine residues. This reaction is catalysed by a specific dehydratase called LanB (dehydratase of class I lantibiotics)[9]. The hallmark of lantibiotics is the second modification, which leads to the formation of lanthionine (Lan) and/or methyl-lanthionine (MeLan) rings. The reaction proceeds via a Michael-type condensation of the dehydrated serine or threonine residues with the thiol group of a cysteine residue, which is introduced regio- and stereospecifically by the cyclase LanC (cylcase of class I lantibiotics)[10]. This ring formation results in a thioether bond giving rise to high thermostability and more profound resistance against proteolytic degradation. Furthermore, antimicrobial activity strictly depends on the presence of the Lan/MeLan rings[11].

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In general, lantibiotics exercise their antimicrobial activity through different modes of action. One prominent mode is the binding to the pyrophosphate moiety of lipid II molecules concomitant with the inhibition of peptidoglycan biosynthesis[12]. Another mode of action is the perturbation of bacterial cell membranes. Nisin or subtilin build a pore-forming complex with its receptor lipid II thereby sequestering lipid II within the membrane[13,14] (for a review about lipid II binding peptides see[11]). In the case of nisin, which is active in the low nM range, this process occurs on the ms time scale[15,16]. Some lantibiotics such as for example Pep5 however, directly penetrate the target membrane[17].

In comparison to other cationic AMPs, cytotoxicity against human cells is rarely observed, since lipid II, the main target of lantibiotics, is absent in eukaryotic membranes (except the two-peptide lantibiotic cytolysin S/L[18]). Thus, the efficient activity and the low cytotoxicity combined with few examples of known inherent resistances (see[19–21]) constitute these peptides as excellent lead structures for new antibiotics.

Nevertheless, the bottleneck of lantibiotic research is the identification and characterization of these compounds. With respect to the first, lantibiotics can be detected in genome sequences by data-mining approaches using bioinformatic tools such as BAGEL4[22], antiSMASH[23], RiPPquest[24] and RiPPMiner[25]. Such tools either detect open reading frames encoding lantibiotics within a genome based on neighbouring PTM-enzymes or combine specific sequences of PTM-enzymes and the cleavage motifs within the leader peptides.

However, every newly identified lantibiotic requires a detailed experimental characterization with respect to its antimicrobial properties. Therefore, the isolation of a native sample or heterologously expressed samples in Gram-negative bacteria (*E. coli*)[26,27] or Gram-positive bacteria (*L. lactis* or *B. subtilis*)[28–30] is a prerequisite. Several reports indicate that the nisin modification and secretion system can be employed to modify and secrete other lantibiotics. Apparently, the nisin PTM system provides sufficient promiscuity to produce for example bagelicin from *Streptococcus suis* R61, flavucin from *Corynebacterium lipophiloflavum* and others peptides, if their core peptide is fused to the nisin leader peptide[29,31,32]. Nisin, produced by the Gram-positive *Lactococcus lactis* (*L. lactis*) bacterium and modified by the nisin PTM machinery is one, if not the best characterized lantibiotic and may therefore be used as a standardized lantibiotic for benchmarking.

Since the number of novel lanthipeptides/lantibiotics increases due to genome mining, design of hybrid-peptides (by coupling different lanthipeptides to a certain leader peptide), simple mutations or even chemical synthesis, a general pipeline to characterize the potential antimicrobial activity and thereby potency of a lantibiotic is urgently required to ensure appropriate benchmarking of such lantibiotics[1,33,34].

Here, we used a standardized workflow for the characterization of lantibiotics, containing up to six steps depending on the availability of the lantibiotic (Fig. 1a). We exchanged the isoleucine at position one (I_1) of nisin to all other 20 natural occurring amino acids (aa) (Fig. 1b) and determined the impact on the expression, modification and antimicrobial properties of these nisin variants and benchmarked it against wild type nisin.

Results

General characterisation of nisin I1 mutants. The nisin A core peptide was a target of many former studies aiming to alter the antimicrobial activity of nisin or to broaden its spectrum against microbial targets. Position 1 in the nisin core peptide received our attention since some mutations were described that lacked in our opinion a complete and quantitative characterization. For example, the substitutions of tryptophan-analogues[35] as well as the mutants I_1W, I_1K, I_1D[36], I_1G and I_1Q (described in[37]). Accordingly, we characterized all mutants at I_1 of nisin A using our standardized protocol, which is based on six individual steps (Fig. 1a; I-VI).

The first steps (I-III) concerning expression, secretion and purification of the nisin variants gave no major variation compared to wild type nisin, which were all expressed as a leader-containing variant (pre-nisin). The yield of pre-nisin was 6.0 ± 0.3 mg/ L culture supernatant (Supplementary Fig. S1) with a purity >95% based on Tricine-SDS-PAGE (Supplementary Fig. S2). The yield of most I_1 mutants decreased to 40–60% of wild type nisin (Supplementary Fig. S1). The mutants I_1C and I_1W had an even lower yield of approximately 35%. This lower yield however did not affect our purification protocol and all pre-nisin variants were purified using the same protocol and resulted in comparable purity (Supplementary Fig. S2)[29,32].

Step IV concerns leader peptide cleavage and thereby activation of the purified pre-nisin variants. Here, we used the secreted variant of the natural leader peptidase NisP, by which the leader peptide is cleaved *in vitro* and the cleavage efficiency (%) can be calculated as recently shown[38]. We used an RP-HPLC set-up to monitor product formation, in which the appearance of the leader peptide in the chromatograms was used to determine the concentration of activated nisin (Supplementary Fig. S3). Pre-nisin (Supplementary Fig. S3) had a retention time (RT) between 19 to 21 min, whereas the leader peptide eluted as two peaks corresponding to the variants with and without the N-terminal methionine between 14 to 16 min, respectively. The nisin core peptide eluted at a later retention time of approximately 23 min (Supplementary Fig. S3). In some cases, the nisin I_1 mutants resulted in separated peaks of pre-peptide and core peptide after the activation by NisP (compare mutants I_1M, I_1L, I_1V, I_1W, I_1Y, I_1F and I_1C in Supplementary Fig. S4). However, in the case of the I_1 mutants I_1A, I_1G, I_1T, I_1S, I_1K, I_1R, I_1H, I_1Q, I_1E, I_1N and I_1D) the formation of a core peptide peak in terms of a new and separated peak in the chromatogram was not observed (Supplementary Fig. S4). Rather, the peaks with a retention time between 18–21 min contained both species, the cleaved and non-cleaved lantibiotic, and can therefore not be deconvoluted for proper determination of the amount of activated nisin. Hence, only the two peaks of the leader peptide were used and the area was integrated for the determination of the concentration of activated nisin and its I_1 mutants. The concentration was calculated based on a standard calibration curve (Supplementary Fig. S5), where we precisely determined the concentration of the activated nisin or I_1 mutants. We used this concentration determination instead of the colorimetric protein concentration determination as for example Bradford or the BCA-assay since the latter two assays cannot differentiate between the activated and non-activated form of the secreted variants. Since nisin is not active unless the leader is cleaved off, the determination of the area of the leader peak to calculate the

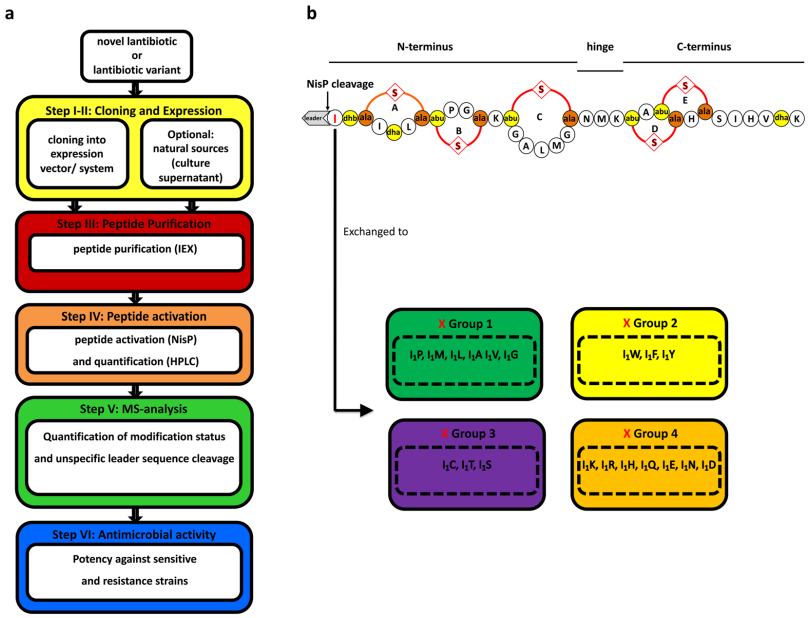

Figure 1. Scheme of lantibiotic characterization (**a**) the lantibiotic nisin and its I_1 mutants (**b**). The characterization of novel lantibiotics or variants is based on a six steps protocol (**a**). I-II are cloning and expression steps (yellow box). Step III covers peptide purification (red box). Step IV represents the activation of the peptide (orange box). Step V is the MS-analysis (green box), while step VI represents the antimicrobial activity of the lantibiotic (blue box). The lantibiotic nisin (NisA) can be dissected into an N-terminal region (with lanthionine ring A and the methyl-lanthionine rings B and C), a hinge region and a C-terminal region (with the intertwined methyl-lanthionine rings D and E) (**b**). The dehydrated amino acids dehydroalanine (dha) and dehydrobutyrine (dhb) (former serines and threonines) are highlighted in yellow. The coupled cysteine residues to dehydrated amino acids are highlighted in orange. The thioether bonds between the (methyl-) lanthionine are marked with a red S. The position one isoleucine is highlighted in red and exchanged to X amino acid from the four different groups of natural amino acids.

amount of the activated lantibiotic directly relates to the antimicrobial activity without need to further purify the peptide after cleavage.

All I_1 mutations were subjected to NisP cleavage and most of the variants were cleaved, but a strong influence of the nature of the I_1 substitutions with respect to cleavage efficiency was observed (Supplementary Fig. S6). The cleavage efficiency for wild type pre-nisin A was $94.3 \pm 1.7\%$ but lower for the mutants I_1M, I_1L, I_1A and I_1V ($72.3 \pm 1.4\%$, $72.4 \pm 1.3\%$, $81.0 \pm 1.8\%$ and $58.1 \pm 2.8\%$, respectively). All other variants displayed efficiency below 50% (Supplementary Fig. S6). This holds especially in those cases, where the amino acid at position one was exchanged to a charged amino acid (e.g. I_1K $9.2 \pm 1.3\%$; I_1E $9.5 \pm 0.7\%$) or contained a bulky hydrophobic side chain (e.g. I_1F $12.9 \pm 0.8\%$; I_1W $5.7 \pm 0.2\%$; I_1Y $22.6 \pm 2.3\%$) (Supplementary Fig. S6). To highlight two examples, NisP was only able to cleave $5.7 \pm 0.2\%$ of the I_1W variant, which was significantly lower as the cleavage efficiency previously reported[35,39]. Interestingly, the I_1P mutant was not cleaved at all by NisP (Supplementary Figs S4 and S6). Even after an extensive prolongation of the incubation time, no leader peptide peak was detected in the RP-HPLC chromatogram (Supplementary Fig. S4). It is important to stress in this context that the cleavage efficiencies cannot be determined from Tricine-SDS gels (20%). Here, the leader peptide as well as the core peptide would co-migrate, which obviously will falsify the staining results.

As previously reported[37,38], a prerequisite for high cleavage efficiency by NisP is the presence of at least one (methyl-)lanthionine ring. Therefore, we wondered whether some of the nisin variants with low cleavage efficiencies were altered in their modification status (step V). To detect possible ring formation(s) we incubated the lantibiotic prior to MS analysis with the thiol-reactive agent 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP), which binds to free cysteines and results in a mass shift of 25 Da per covalently attached CDAP. When no mass shift occurs, all cysteine residues are part of thioether rings, while for every mass shift of 25 Da one cysteine is not part of a (Me)Lan thioether ring. As controls, we also used the unmodified version of pre-nisin (Supplementary Fig. S7a), in which no rings are present and five CDAP adducts were identified indicating that five cysteine side

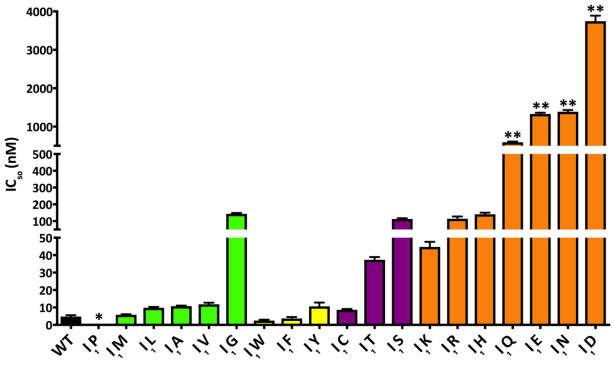

Figure 2. Growth inhibition assay of strain NZ9000-Cm in the presence of nisin and the corresponding I_1 variants. The lantibiotic nisin A (WT) and its I_1 mutants were used for growth inhibition (IC_{50}) against strain NZ9000-Cm. IC_{50} values were grouped in four different sub-groups (group 1: aa M-G, green; group 2: aa W-Y, yellow; group 3: aa C-S, magenta; group 4: aa K-D, orange), Values represents the average of at least five independent measurements and the errors report the standard deviation of the mean (SDM). The nisin variant I_1P was not cleaved by NisP (*) and no growth inhibition assay was conducted. The nisin variants within the forth group marked with (**) showed less antimicrobial activity ($IC_{50} > 500$ nM).

chains were available for a simultaneous labelling reaction and the fully modified pre-nisin, showing no coupling products (Supplementary Fig. S7b). This highlights the quantitative nature of the CDAP assay[31].

For most analysed I_1 mutants no CDAP attachment was observed (Supplementary Figs S8, S9, S10 and Supplementary Table S1). Exceptions of this observation are the mutants I_1L, I_1A, I_1V, I_1G, I_1F, I_1Y, I_1R, I_1H, I_1Q, I_1N, where small amounts of coupling products were observed with variations from 7x dehydrations (dh) to 5x dh with one coupling product (Supplementary Figs S8, S9, S10 and Table S1). These amounts are very small, in comparison to the main species and the MS analysis showed clearly, that the I_1 mutants primary containing all lanthionine rings. In the case of nisin I_1C, where one additional cysteine residue was introduced also one coupling product was observed (Supplementary Fig. S9) In summary, although the analysis of the cleavage reaction revealed I_1 mutants with lower cleavage efficiency, the modifications of the core peptide within these variants were not altered.

Impact of mutations at position I_1 on antimicrobial activity of nisin. Growth inhibition assays (step VI) were used to determine the potency of the activated I_1 mutants. First, this assay was performed against the sensitive strain NZ9000-Cm harbouring an empty plasmid pIL-SV (the strain NZ9000-Erm gave identical results) to determine the value, at which 50% of the cells were inhibited in growth (IC_{50} value).

The nisin A wild type (WT) had an IC_{50} value of 4.8 ± 0.7 nM, which is in a similar range as previous reported values determined with strains NZ9000-Cm/NZ9000-Erm[40–42] (Fig. 2 and Supplementary Table S2). The I_1P mutant was used in very high concentrations (>1 mM) in the growth inhibition assay but displayed no antimicrobial activity (Fig. 2; IC_{50} value is marked with a star symbol). This indicated that this variant was not activated by NisP, in line with our observations described above.

All other variants were used and displayed IC_{50} values ranging from wild type level to 100 nM or even to lower µM values. Based on the measured activities, the variants can be grouped into four classes depending on the amino acid property.

The first group contains mainly aliphatic amino acids (except M)(Fig. 2, green bars and Supplementary Table S2). The mutation to methionine gave similar IC_{50} values to WT (5.8 ± 0.3 nM), but the exchange to the amino acids leucine, alanine and valine lead to a two-fold decrease in activity (9.8 ± 0.5 nM, 10.7 ± 0.4 nM and 11.8 ± 0.9 nM, respectively). The mutation to glycine lead to an even further decrease in activity and the IC_{50} value was determined to 143.0 ± 5.1 nM.

The second group (Fig. 2, yellow bars and Supplementary Table S2) contained aromatic amino acids (except histidine, which belongs to the fourth group) and displayed a high antimicrobial activity against the sensitive strain. Especially, the mutants I_1W and I_1F displayed lower IC_{50} values (2.5 ± 0.2 nM and 3.7 ± 0.8 nM) than WT, indicating an increased antimicrobial activity. The mutant I_1Y showed a lower activity and displayed an IC_{50} value of 10.6 ± 0.9 nM.

The third group (Fig. 2, magenta bars and Supplementary Table S2) displayed IC_{50} values ranging from 8.6 to 112 nM. The mutation I_1C lead to antimicrobial activity of WT (below 10 nM) with an IC_{50} value of 8.6 ± 0.5 nM, whereas the mutation to I_1T or I_1S displayed a considerable reduction of the antimicrobial activity with IC_{50} values of 37.3 ± 1.6 nM and 112.4 ± 5.0 nM, respectively. Interestingly, these substitutions are all amino acids, which are potential targets of the PTM machinery. Since the mutation I_1C introduced an additional cysteine residue, we carefully analysed for the presence of an additional (Me)Lan ring. However, the coupling assay clearly

demonstrated the presence of one accessible cysteine suggesting that this nisin variant still harbours five (Me) Lan rings (Supplementary Fig. S9). Unfortunately, this assay cannot determine which ring(s) are formed. Since the IC_{50} value is not altered, we propose that this mutant follows normal ring formation and the additional introduced cysteine at position one was labelled with CDAP.

The mutations I_1T and I_1S, containing an additional dehydration position. Based on our MS analysis, we saw mainly no additional dehydration, which leads to the conclusion that this position is not well recognized by NisB (Supplementary Fig. S9). But we clearly observed that the possible higher number of dehydrations of the peptide at the N-terminus of the core peptide decreased antimicrobial activity (IC_{50} values > 30 nM). Here, mutation to the polar residue serine had a larger impact on the antimicrobial activity of the core peptide compared to the threonine substitution.

The fourth group (Fig. 2, orange bars and Supplementary Table S2) contained charged amino acids and the amide side chains of glutamate and aspartate. In general, a strong negative effect on the antimicrobial activity was observed within this group. The IC_{50} values were higher compared to the other groups and ranged from 44 nM to 3746 nM. The substitutions at position 1 to the amino acids lysine, arginine or histidine lead to 10-fold or even 20-fold higher IC_{50} values (I_1K: 44.7 ± 3.0 nM, I_1R: 113.9 ± 13.6 nM and I_1H: 140.0 ± 5.0 nM) compared to the WT IC_{50} value. A major alteration in antimicrobial activity was observed in the case of an exchange I_1Q and I_1E, respectively. Here, the IC_{50} values were 592.0 ± 17.8 nM for I_1Q and 1328.0 ± 32.7 nM for I_1E. An even more dramatic effect was observed upon introduction of I_1N or I_1D. Here, IC_{50} values of 1386.0 ± 46.3 nM and 3746.0 ± 144.1 nM were determined. These variants displayed such high IC_{50} values (marked by two star symbols (**)) that these mutations were not analysed in further growth inhibition assays using resistant strains.

In summary, the antimicrobial activity of the different I_1 mutants towards the sensitive strain NZ9000-Cm correlated with the physico-chemical properties of amino acid at position one. Although this substitution was only one amino acid and the modification state of the core peptide was not altered, the active lantibiotics showed drastic variations in antimicrobial activities. Based on the growth inhibition assay, these I_1 mutants were divided into four groups, where aromatic amino acids at position one lead to higher activity but an introduction of polar, charged amino acids or its amidated counterparts decreased the potency of the corresponding variant to the sensitive strain.

The influence of I_1 mutants on immunity and resistance proteins. Lantibiotics are regarded as potential antibiotic candidates, which might have the potential to replace classic antibiotics and thereby overcome the increasing resistances against major antibiotic classes. One potential drawback of lantibiotics is highlighted by the few reported resistance mechanisms against for example nisin[43]. Therefore, it is critical, to screen for strains, which might be resistant against the new lantibiotic, if a new lantibiotic is characterized and its potency is determined. In our protocol, we implemented first the screen against a sensitive strain (see above), but more importantly we included four strains expressing immunity or resistance proteins against nisin. The immunity proteins NisI (lipoprotein) and NisFEG (ABC transporter) from L. *lactis* are the first and second line of defence of the nisin producer strain[44]. Upon expression in the sensitive strain NZ9000, these proteins might provide immunity and one can study the activity of the lantibiotic in the presence of immunity proteins. Additionally, we screened the effect of the I_1 variants on the nisin resistance proteins *Sa*NSR (lipoprotein) and *Sa*NsrFP (ABC transporter) to fully consider the potency of the newly lantibiotic/lantibiotic variant. If these proteins are expressed in the sensitive strain, they confer resistance against the nisin and likely to nisin variants.

The nisin variants were analysed according to the above provided classification of four groups. The first group of I_1 mutants showed a similar tendency in activity towards the strains for immunity and resistance as for the sensitive strain (Supplementary Table S2). The substitutions of isoleucine to methionine, leucine, valine and alanine gave IC_{50} values for strain NZ9000-NisI in a range of 35–65 nM and were comparable to nisin A WT (46.0 ± 6.0 nM) (Fig. 3a). Only the substitution to glycine gave a higher value of 785.7 ± 9.7 nM reflecting the low activity of this variant. The IC_{50} values of the first group for the strain NZ9000-NisFEG were also in the range of 34–50 nM (nisin A WT: 53.0 ± 4.5 nM). Again, a higher IC_{50} value of 557.8 ± 28.3 nM was determined for the I_1G mutant.

When analysing the results of the resistance strains two major changes were observed. In general, the IC_{50} values of the I_1 mutants for strain NZ9000-*Sa*NSR were similar (53–84 nM) to the WT (73.1 ± 3.6 nM). The IC_{50} value for the I_1G mutant was 278.5 ± 13.3 nM and lower in comparison to the IC_{50} values of the other strains (Fig. 3a). The IC_{50} values for strain NZ9000-*Sa*NsrFP changed only for the mutants I_1A (166.1 ± 5.1 nM) and I_1G (2257.0 ± 53.4 nM), respectively, reflecting the lower activity of these variants towards the strain.

The second group of I_1 mutants included the aromatic amino acids, for which a higher activity against the immunity and resistance strains was observed (Supplementary Table S2). The higher activity was determined for all strains ($I_1W > I_1F >$ wild type $> I_1Y$). The exception is the mutant I_1Y and strain NZ9000-NisFEG. Here, the IC_{50} value was lower than the one of nisin A WT (53.0 ± 4.5 nM) (Fig. 3b). Interestingly, the mutant I_1W showed high potency for the strains NZ9000-NisI, NZ9000-NisFEG and NZ9000-*Sa*NSR with IC_{50} values below 25 nM. A higher IC_{50} value of 46.6 ± 1.1 nM was only determined against strain NZ9000-*Sa*NsrFP (Fig. 3b).

The third group of I_1 mutants showed opposing results concerning immunity and resistance (Supplementary Table S2). Here, mutants I_1T and I_1S had lower antimicrobial activity than wild type reflected by IC_{50} value even above 100 nM (Fig. 3c). Especially, the more than five-fold lower activity against strains NZ9000-NisI and NZ9000-*Sa*NsrFP is surprising as the IC_{50} values were determined to be above 500 nM. For the mutant I_1T the values are 653.3 ± 5.1 nM and 716.1 ± 28.6 nM, whereas for the mutant I_1S the values are two-fold higher (1898.0 ± 62.3 nM and 2893.0 ± 34.8 nM) (Fig. 3c). The exception is the mutant I_1C, which showed a similar activity towards the strains as nisin A WT with IC_{50} value in the range of 41 to 101 nM. Based on the MS analysis only one free cysteine was present, we cannot conclude which of the cysteines is not part of a thioether bridge. As this variant showed a similar antimicrobial activity, we however suggest no alteration in ring pattern (Supplementary Fig. S9).

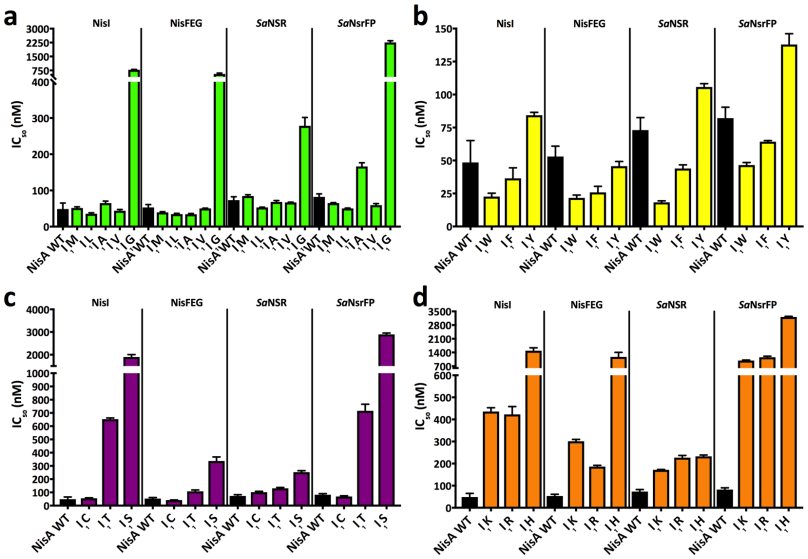

Figure 3. Growth inhibition assay of strains NZ9000-NisI, NZ9000-NisFEG, NZ9000-*Sa*NSR and NZ9000-*Sa*NsrFP in the presence of nisin and the corresponding I_1 variants. The lantibiotic nisin A (WT) and its I_1 mutants were used for growth inhibition (IC_{50}) with strains NZ9000-NisI, NZ9000-NisFEG, NZ9000-*Sa*NSR and NZ9000-*Sa*NsrFP. The IC_{50} values were grouped in sub-groups of the I_1 mutants. The group 1 with the aa M-G (**a**), group 2 with the aa W-Y (**b**), group 3 with the aa C-S (**c**) and group 4 with the aa K-H (**d**). Values represent the average of at least five independent measurements and the errors report the standard deviation of the mean (SDM).

In the last group of I_1 mutants, all variants showed lower antimicrobial activity towards the strains of immunity and resistance (Supplementary Table S2). The IC_{50} values were above 400 nM (Fig. 3d) except for mutants I_1R and I_1K, which had IC_{50} values of 186.4 ± 3.1 and 301.2 ± 4.7 nM, respectively, against strain NZ9000-NisFEG. Similar to this, mutants I_1R, I_1K and I_1H showed a higher antimicrobial activity against strain NZ9000-*Sa*NSR (Fig. 3d).

In summary, I_1 mutations clearly influenced the efficiency of the immunity and resistance proteins. The IC_{50} values of the sensitive strain and resistance strains for the different I_1 mutations can be used to calculate a fold of resistance (Fig. 4). Here, the effect of certain mutations is even less pronounced (e.g. I_1S or I_1T on NZ9000-NisI) but still show the reduced activity. The reasons for this reduction might be that these variants are better ligand and/or substrates for the immunity and resistance proteins. Another explanation might be, that the variants have an altered interaction with the membrane (I_1K; I_1R, I_1W, I_1F). The same is true for the opposite case, in which the mutants are more active. Here, the mutants are either worse substrates or showed a loss in membrane attraction due to charge repulsion (I_1E/D mutants) or reduced hydrophobicity (I_1G mutant).

Discussion

The increased detection of antibiotic resistances of human pathogenic strains urgently calls for the identification of novel lead structures, which can be used to develop long lasting antibiotics. One promising family of candidates are the antimicrobial peptide subfamily of lantibiotics. They are small ribosomally synthesized and post-translational modified peptides[45,46], which possess a potent antimicrobial activity generally in the nM range. Their antimicrobial activity makes them excellent candidates to treat MDR-strains such as MRSA or VRE[47]. Recently, numerous new lantibiotics were discovered by "*in silico* genome mining" approaches, using available bacterial genome sequence data[48]. Here, BAGEL4 and its predecessor BAGEL3 are powerful algorisms to detect lantibiotics sequences within bacterial genome sequences[22]. Novel lantibiotic such as flavucin, bagelicin and agalacticin were found and their antimicrobial properties were determined[29]. In the study by Heel *et al.* 2016 the potency of many new lantibiotics were screen against a set of Gram-positive and Gram-negative strains to show their antimicrobial potencies. Till now, however, it is not possible to deduce the potency solely on sequence information and every lantibiotic needs to be expressed and purified for subsequent characterization of its antimicrobial activity. To ensure comparability of the determined activities and the potencies derived from these experiments a standardized protocol needs to be established, which allows benchmarking novel against already characterized lantibiotics (e.g. nisin). This should also include mutations designed or natural variants of previously characterized lantibiotics.

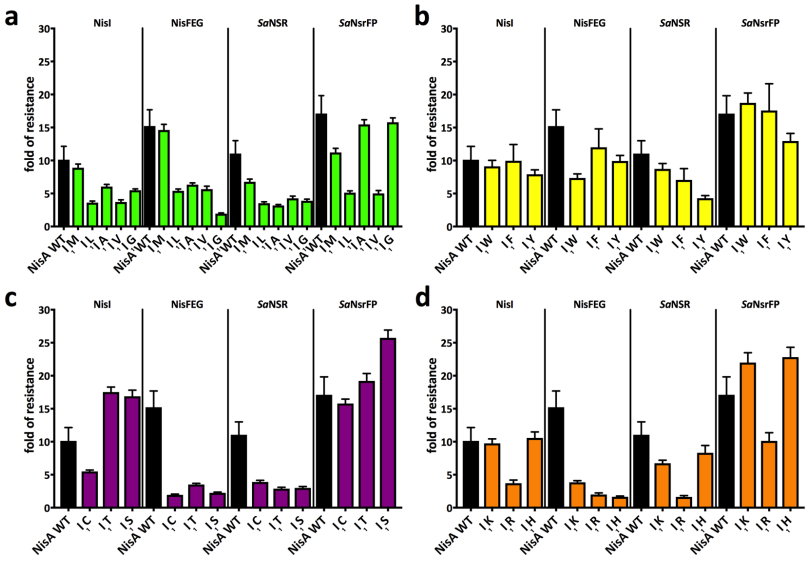

Figure 4. Fold of resistance of strains NZ9000-NisI, NZ9000-NisFEG, NZ9000-*Sa*NSR and NZ9000-*Sa*NsrFP in comparison to NZ9000-Cm. The resistance of the strains NZ9000-NisI, NZ9000-NisFEG, NZ9000-*Sa*NSR and NZ9000-*Sa*NsrFP towards the lantibiotic nisin A (WT) and its I_1 mutants are presented as fold of resistance. The fold of resistance values were grouped in sub-groups of the I_1 mutants. The group 1 with the aa M-G (**a**), group 2 with the aa W-Y (**b**), group 3 with the aa C-S (**c**) and group 4 with the aa K-H (**d**). Values represent the average of at least five independent measurements and the errors report the standard deviation of the mean (SDM).

In this study, we used a standardized protocol based on six individual steps (I-VI). Some of these steps have been previously (partly) published, but not as a combined robust protocol[29,32,40,42,49]. By replacing I_1 of nisin A against any other amino acid, the complete influence of the exchange on expression, purification and/or activity was monitored to provide a quantitative characterisation.

The characterisation of a novel lantibiotic starts with the expression system and the choice of homologous or heterologous expression. Homologous expression of a lantibiotic is often associated with its isolation from supernatants of lantibiotic producer strains (examples are epidermin[50], mutacin 1140[51], NAI-107 and related lantibiotics[52] or pinensins[53]). The yield of this strategy can be limited, especially if the producer is hardly cultivable under lab conditions or induction of the lantibiotic is not trigged (e.g. geobacillin I[54], salivaricin 9[55] and staphylococcin Au-26,[56]).

Therefore, for some lantibiotics such as lichenicidin from *Bacillus licheniformis* ATCCC 14580[26], or prochlorosins from *Prochlorococcus* MIT 9313[57] a heterologous expression in *E. coli* is the preferential strategy to obtain higher yields of the pre-lantibiotic. Alternatively, the expression via the NICE-system in *L. lactis*[58] is a possibility. Here, the pre-lantibiotic is modified after induction with the lantibiotic nisin by the PTM system (NisB/NisC) and secreted by the ABC transporter NisT[32].

The nisin PTM system was shown to be of sufficient promiscuity to modify and secrete lantibiotics as well as non-lantibiotic peptides[29,32]. As an advantage any lantibiotic can be produced as a pre-lantibiotic. This results in higher yields, as the lantibiotic is not antimicrobial active and is not limiting the growth of the producer strain. Furthermore, the pre-lantibiotic (as pre-nisin) shows higher pH stability in contrast to nisin (low solubility at pH > 7)[59]. Normally, we observed yields for the pre-nisin and its variants of 3–6 mg per liter cell culture supernatant after purification (Supplementary Fig. S1). Considering these yields further down-streaming steps are employed to characterize the lantibiotic.

Although NisB and NisC are promiscuous in modifying their substrate, it is crucial that both enzymes fully modify the core peptide similar to pre-nisin. Otherwise, the steps III (cleavage by NisP) and VI (antimicrobial assay) might be less informative. Thus, even new mutants of nisin, like mutants in the hinge region[60,61] or in the leader peptide[37], required a full characterisation with respect to their modification and antimicrobial activity.

The purified pre-lantibiotics need to be analysed by RP-HPLC before and after activation by proteolytic cleavage of the leader peptide. In this study, we activated pre-nisin and the I_1 mutants *in vitro* by purified leader peptidase NisP (Supplementary Figs S3 and S4; described in[38,49]). The cleavage efficiency and also the exact concentration of the active lantibiotic were determined via RP-HPLC (Supplementary Fig. S6). Hence, every activation of the pre-lantibiotic can be monitored and quantified by integrating the leader peptide peaks, which are used for calculation of the amount of activated lantibiotic. We observed for some I_1 mutants, that the properties

during the RP-HPLC runs of the pre-lantibiotics and activated lantibiotics made it impossible to distinguish between both peaks (Supplementary Fig. S4). Consequently, the areas of leader peptide peaks were used for quantification. One other aspect that has to be taken in consideration is the fact that for nisin mutants, such as I_1A or I_1K, the core peptide cannot be purified by means of preparative RP-HPLC from the supernatant. In general, the analytic RP-HPLC is needed to quantify the lantibiotic and as a quality control for the purification and the cleavage reaction.

The major advantage of RP-HPLC compared to other technics is the more accurate determination of the concentration of the activated lantibiotic, which is a prerequisite for a reliable determination of the biological activity of the lantibiotic. In comparison to methods determining the total amount of peptide/protein (e.g. BCA-assay and other colorimetric assays) the determination via HPLC minimizes the error. For example, if only 15% of a pre-lantibiotic is cleaved, but the total amount of pre-lantibiotic is used for the calculations, the determined IC_{50} value would be six to seven-fold larger, which obviously indicates a falsely lower antimicrobial activity.

Sometimes a lower cleavage efficiency of the leader peptidase and later a lower antimicrobial activity might reflect incomplete modification of the peptide (e.g. dehydrations or the lack of lanthionine rings) or disrupted recognition by the leader peptidase. As an example, the mutation of I_1 in nisin Z to tryptophan leads to the production of two variants. The I_1W mutant as a main product and a small amount of I1W/Dh2T, where the threonine residue escape the dehydration and an altered activity is proposed[62]. Therefore, a proper determination of dehydration and the formation of lanthionine rings by MS-analysis is required. MS-analysis provides information about the dehydration reaction of NisB[63] by the loss of water (−18 Da), but cannot indicate the formation of the (methyl-)lanthionine rings. Furthermore, it is not possible to distinguish between the position of the dehydration within the nisin core peptide, which might have escaped modification like threonine position 2 or serine position 33[64]. Thus, it is inevitable to use at least one orthogonal method to determine the presence of lanthionine ring(s). Either the use of an alkylation agent (e.g. CDAP to couple the free thiol group from cysteine residues) or tandem MS-MS analysis to determine the alternate fragmentation pattern between WT pre-lantibiotic and potential mutant variants is necessary.

In our protocol we choose the CDAP-coupling assay to detect nisin I_1 mutants, which might still possess free thiol groups. The advantage of the CDAP-coupling assay is the additional mass shift in the MS spectra. Here, a mass shift of 25 Da per thiol-conjugated easily indicates incomplete ring-formation.

The activated lantibiotic was used for antimicrobial activity assays (step VI), in which different indicator strains were employed. Here, the assay for antimicrobial activity needs to be suitable for the characterisation of the lantibiotic. The broth dilution with 2-fold dilution series of lantibiotics in a MIC assay is generally used to determine the antimicrobial activity[65]. However, if only one stock concentration is used, one obvious limitation is the factor two in serial dilutions, which will detect only changes larger than two. Therefore, we suggest to use the IC_{50} assay with a high diversity of stock concentrations to screen every data point. Still, both methods have their limit as no information about the mode of action of the lantibiotic can be acquired.

The active lantibiotic were screened against a sensitive strain to determine the IC_{50} value by growth inhibition assay (described with modifications in[41]). We used the *L. lactis* strain NZ9000 with the corresponding empty vector systems pNZ-SV or pIL-SV, termed NZ9000-Erm and NZ9000-Cm, respectively. Generally, we would like to propose that the strain *L. lactis* NZ9000 is used as a standard strain, in order to have comparable values from antimicrobial activity assay.

The full potential of a lantibiotic or lantibiotic variants can be shrouded if the screen is only performed against sensitive strains. Therefore, based on the IC_{50} value from the sensitive strains, a second screen against strains, which express immunity or resistance proteins should be performed[66]. We used the strains NZ9000-NisI and NZ9000-NisFEG to study the effect of the immunity proteins from *L. lactis* as they confer immunity via different mechanisms[67,68]. To analyse the effect of a lantibiotic on resistance potency, strains NZ9000-*Sa*NSR and NZ9000-*Sa*NsrFP expressing proteins involved the nisin resistance from *Streptococcus agalactiae* were used[42,66]. Importantly, the ratio of the IC_{50} values (fold of resistance) of these strains to the sensitive strain describes the alteration of substrate specificity on the immunity or resistance proteins (described in[40]).

As final remark, the production of lantibiotics, especially if they are heterologously expressed, has to be benchmarked against a standardized lantibiotic. Here, the well-studied lantibiotic nisin can be used again to benchmark against novel and unknown lantibiotics.

In summary, our analysis and the suggested roadmap demonstrate that a detailed and multifaceted investigation of the antimicrobial potency of lantibiotic is necessary to uncover the full potential of novel lantibiotics, but also to quantitatively compare the efficiency of different members of this promising family of antimicrobial peptides.

Materials and Methods

Microorganisms and culture conditions. Strains and the plasmids used in this study are listed in Table S3. Cultures of *L. lactis* NZ9000 were grown in M17 medium at 30 °C supplemented with 0.5% glucose (GM17 and the appropriate antibiotics (final concentrations 5 µg/ml). In the case of pre-lantibiotic secretion, the *L. lactis* strain NZ9000 was grown in minimal medium at 30 °C supplemented with 0.5% glucose and the appropriate antibiotics (final concentrations 5 µg/ml).

The cultures of the *E. coli* strain DH5α were grown in Luria-Bertani (LB) medium at 37 °C under aerobic conditions with the appropriate antibiotic (100 µg/ml ampicillin or 30 µg/ml kanamycin final concentration).

Cloning of nisin variants. The substitution of amino acids in the *nisA* gene was performed by standard site-directed mutagenesis. Here, the vector pNZ-SV-nisA was used as a template to introduce mutations by Pfu-DNA polymerase (Thermo-Scientific) following standard protocol of the manufacture. Used oligonucleotides are listed elsewhere (Supplementary Table S4). Sequence analysis verified the correctness of the nisin variants

and plasmids were transformed into electro-competent *L. lactis* NZ9000 strain, already containing the pIL3-BTC vector for expression.

Expression and purification of NisP. The soluble variant of NisP was expressed by *L. lactis* NZ9000 harbouring the plasmid pNGnisP8His and subsequently purified as previously described[38].

Expression purification and activation of pre-nisin variants. Pre-nisin and its variants were expressed and purified as previously described[41,49,69]. The purity of pre-nisin and its variants was controlled by Tricine-SDS-PAGE (20%)[70]. The activation of all variants was performed by NisP cleavage overnight at 8 °C. Pre-nisin cleavage and its variants was monitored by RP-HPLC[49,69].

Determination of active nisin by HPLC analysis. Pre-nisin and the activated nisin variants were analysed by RP-HPLC (Agilent Technologies 1260 Infinity II) with a LiChrospher WP 300 RP-18 end-capped column and an acetonitrile/water solvent system. The cleavage efficiency and the concentration of active lantibiotic was calculated as described previously[49,69].

Cloning and expression of immunity and resistance proteins. The cloning of plasmids encoding for immunity and resistance proteins used in this study were previously described[40–42,71]. A nomenclature of the strains used for antimicrobial activity assay is shown elsewhere (Supplementary Table S3).

Determination antimicrobial activity by growth inhibition assay. The growth inhibition assay with the NZ9000 strains was conducted as described in[41]. There, the inhibitory concentration (IC_{50}), where only 50% of the cells survive can be calculated for the different lantibiotics.

The fold of immunity/resistance was calculated by dividing the IC_{50} values of strains expressing the immunity or resistance proteins from the IC_{50} value of the control strain harbouring the empty plasmid[40,42].

MALD-TOF analysis: Dehydration and lanthionine ring analysis. Prior to MS analysis the activated nisin variant were desalted via C_{18} ZipTip purification according to the manufactory manual (Merk-Millipore). The vacuum dried pellet was directly used for MALDI-TOF analysis or coupled with an organic coupling agent for free cysteine residues[72]. For coupling, the pellet was dissolved in 9 µl citrate buffer (25 mM, pH 3) and incubated with 2 µl TCEP (Tris[2- carboxyethyl]phosphine) (100 nmol) for 20 min at 29 °C. After the incubation, the coupling agent CDAP (1-cyano-4 dimethylaminopyridinium tetrafluoroborate) (140 nmol) was added and incubated for 15 min at 29 °C.

For MALDI-TOF analysis the vacuum dried pellet was dissolved with 50 µL 50% acetonitrile solution containing 0.1% TFA. From the soluble sample, 1 µl was mixed with 10 µl matrix solution (10 mg/ml alpha-cyano-4-hydroxycinnamic acid, dissolved in 50% acetonitrile containing 0.1% (v/v) trifluoroacetic acid) and 1 µl of the mixture was spotted on the target. The sample was analysed with MALDI-TOF (UltrafleXtreme, Bruker Daltonics, Bremen, Software: Compass 1.4) in positive mode.

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Author Contributions

L.S. and S.H.J.S. conceived and directed this study. M.L. and J.R. conducted the experiments. M.L., J.R., S.H.J.S. and L.S. wrote the manuscript. All authors read and approved the manuscript.

Additional Information

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Supplemental information

Systematic characterization of position one variants within the lantibiotic nisin

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Key words: lantibiotic, nisin, MS analysis, antimicrobial activity

Figures of supplemental information:

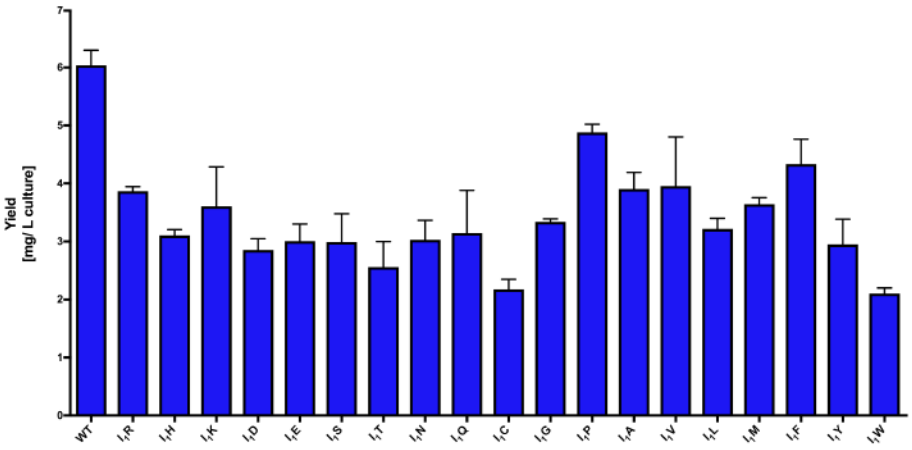

Figure S1: The yield per liter cell culture of pre-nisin variants
Summary of the yields per liter cell culture supernatant after cation-exchange chromatography of nisin A and their corresponding I_1 mutants. The purified peptides were quantified via RP-HPLC. Error bars represent the standard deviation of at least three biological replicates.

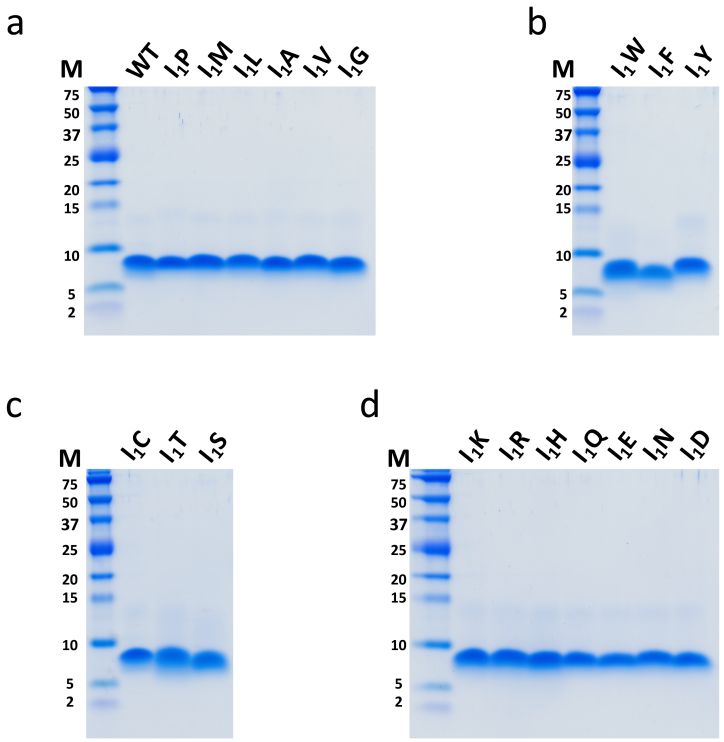

Figure S2: Tricine-SDS-Gels of pre-nisin, I_1 mutants.
Group 1 contained amino acids M-G (**a**), group 2 amino acids W-Y (**a**), group 3 with the amino acids C-S (**a**) and group 4 the amino acids K-H (**a**).

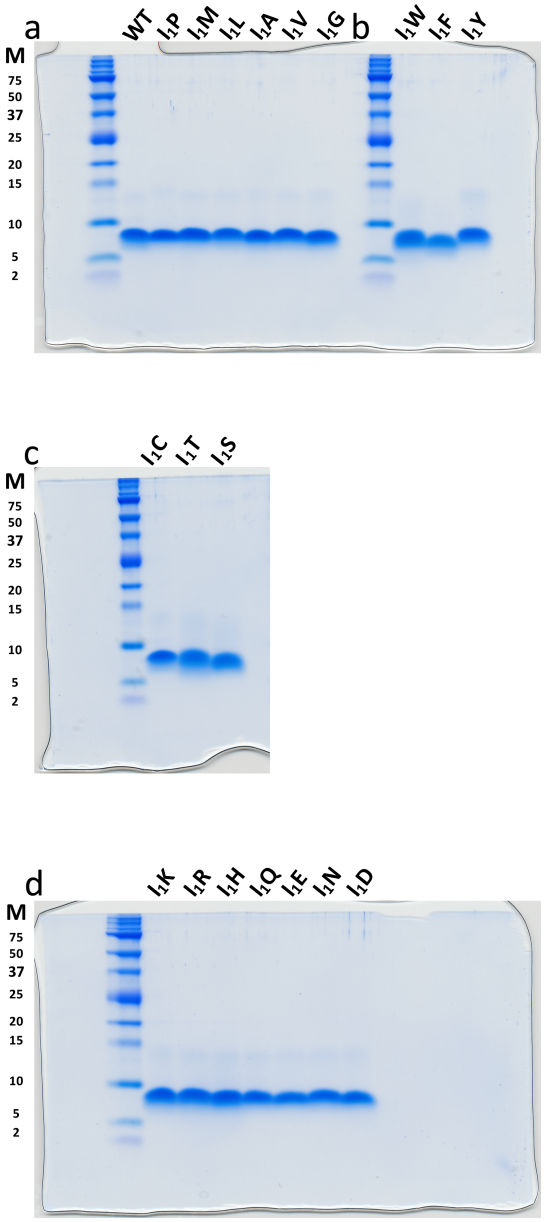

Figure S2: Original Tricine-SDS-Gels of pre-nisin, I_1 mutants.

Group 1 contained amino acids M-G (**a**), group 2 amino acids W-Y (**a**), group 3 with the amino acids C-S (**a**) and group 4 the amino acids K-H (**d**).

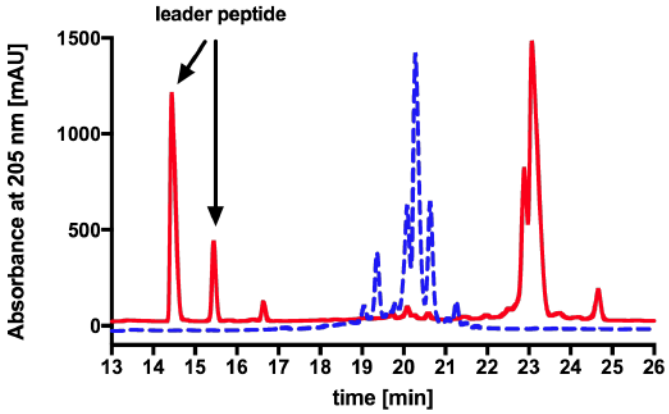

Figure S3: RP-HPLC chromatogram of pre-nisin and nisin.
The retention profile (min) of pre-nisin WT is shown in blue and the product of the cleavage reaction is shown in red. The black arrows indicate the leader peptide peaks (± N-terminal methionine) of cleaved pre-nisin, which is used for quantification.

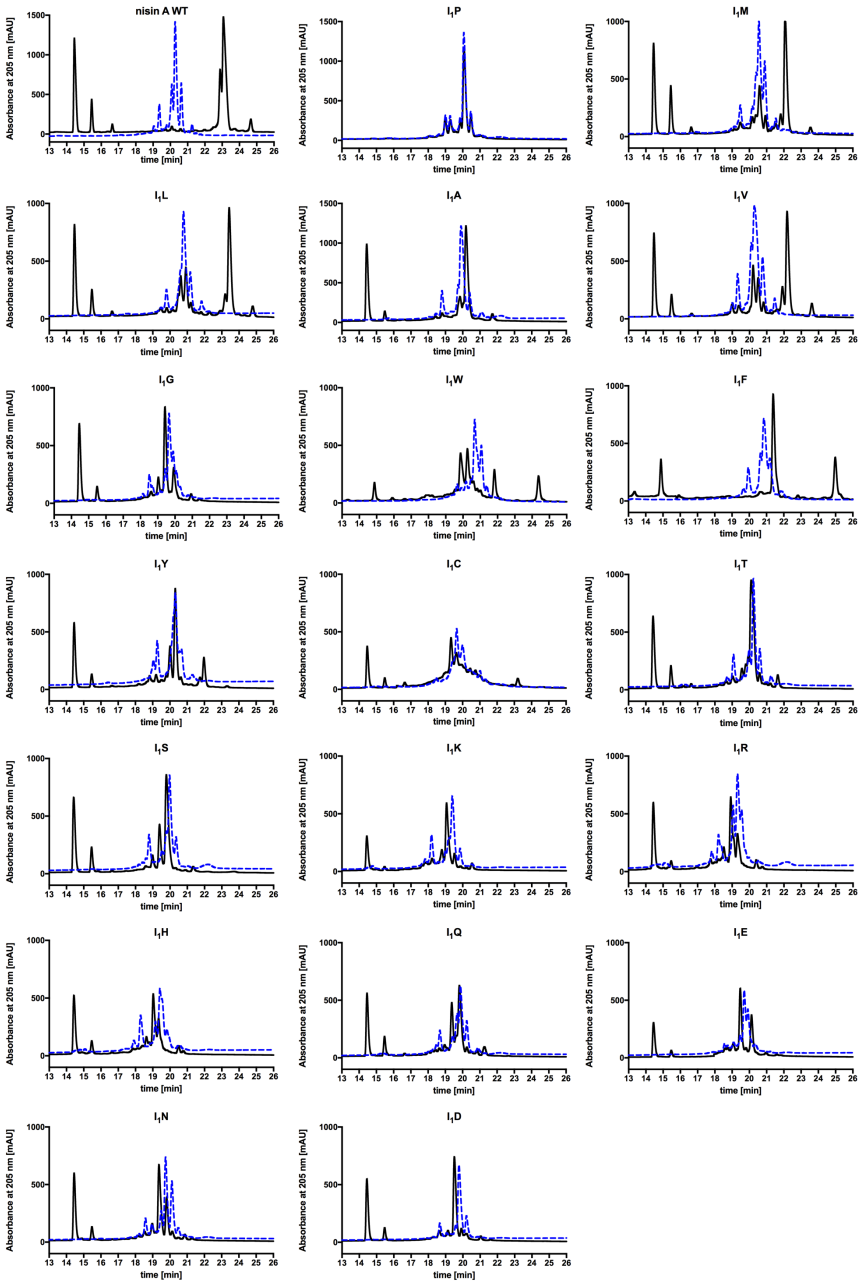

Figure S4: RP-HPLC chromatograms of the cleaved pre-nisin and I_1 mutants
The RP-HPLC chromatogram summaries all pre-nisin and I_1 mutants, which were used in this study. The pre-nisin variants were analysed before cleavage (blue dotted line) and after NisP cleavage (black line). Please note that the scaling of the y axis is different for nisin A WT, I_1P and I_1A.

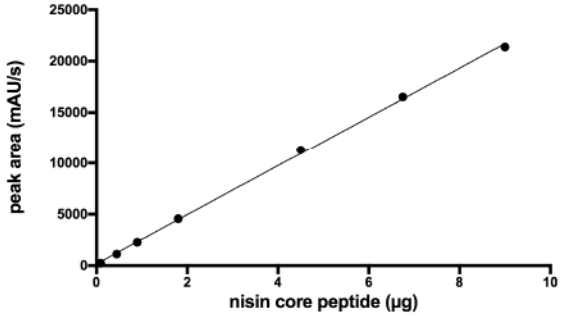

Figure S5: Calibration line of the nisin core peptide.
The slope of the calibration line makes it possible to quantify the total yield and the cleavage efficiency of NisP. Slope: 2392 ± 30.16, R^2: 0.9992

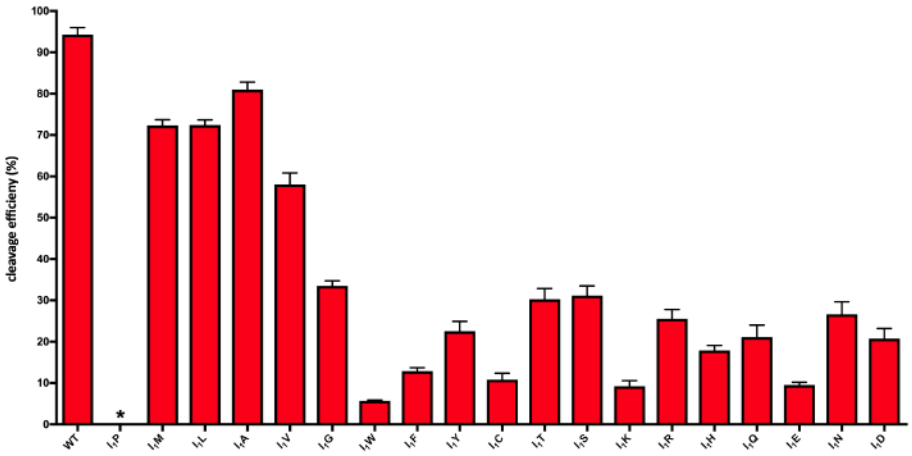

Figure S6: Quantification of the cleavage efficiency of NisP by RP-HPLC.
The leader peptide peak areas were used to determine the final concentration of the activated species and to calculate the efficiency (example see Supplementary Fig. S3, red line). The nisin mutant I_1P was not cleaved by NisP (*). Error bars represent the standard deviation of at least three biological replicates.

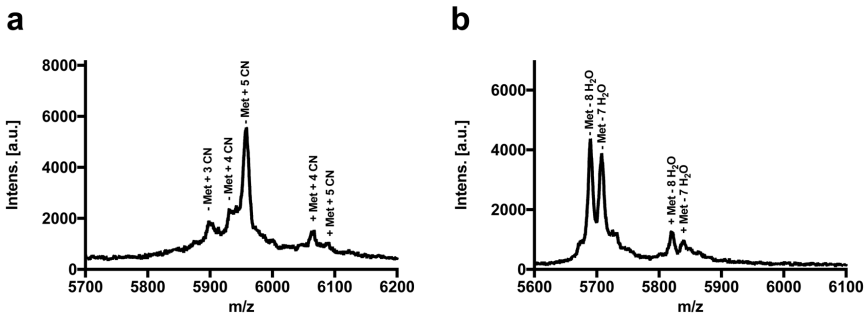

Figure S7: MALDI-TOF analysis to determine the level of dehydrations and ring formations

To demonstrate the specificity of the CDAP coupling, we threated unmodified pre-nisin and fully modified pre-nisin. **a**: unmodified pre-nisin with a maximum number of five coupling products, which demonstrated the accessibility of all five cysteine residues. The fully modified pre-nisin showed no coupling products indicating that all cysteine residues were involved in lanthionine rings (**b**).

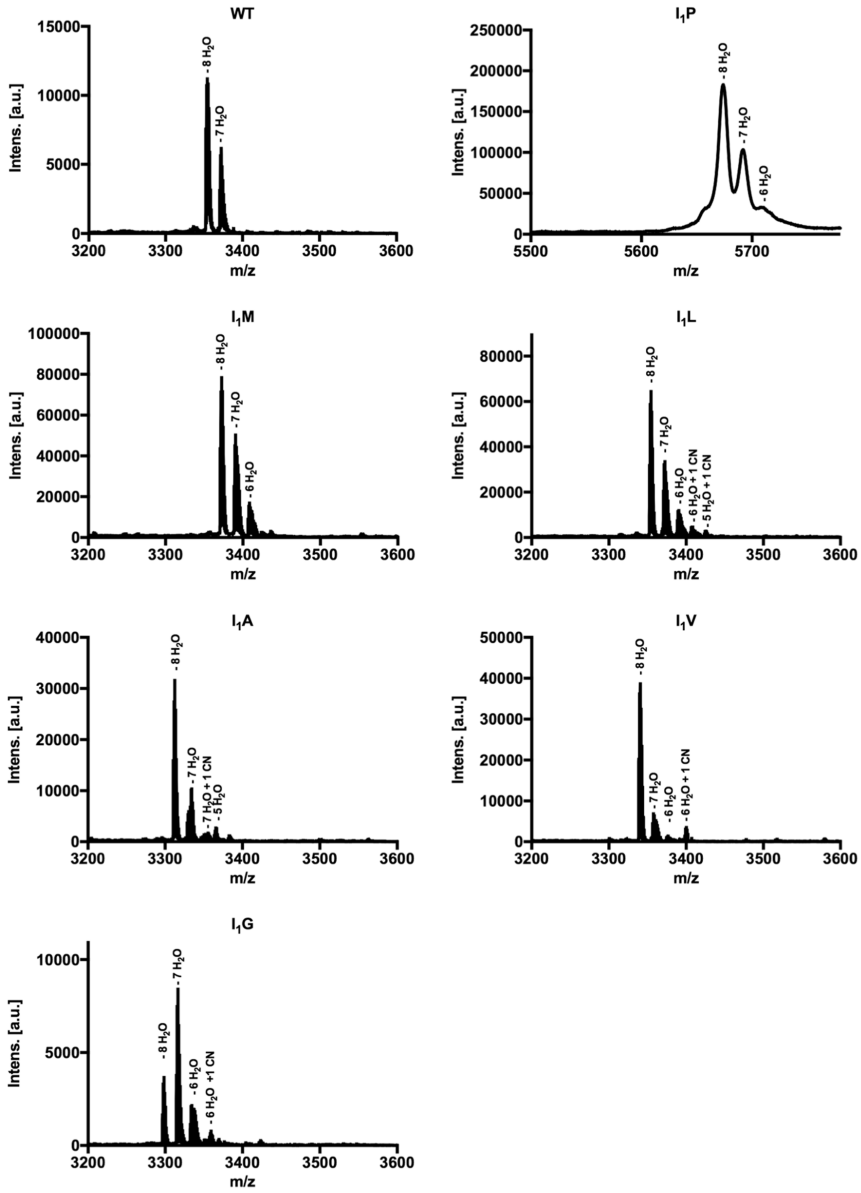

Figure S8: MALDI-TOF analysis to determine the level of dehydrations and ring formations for the nisin A variant group 1.

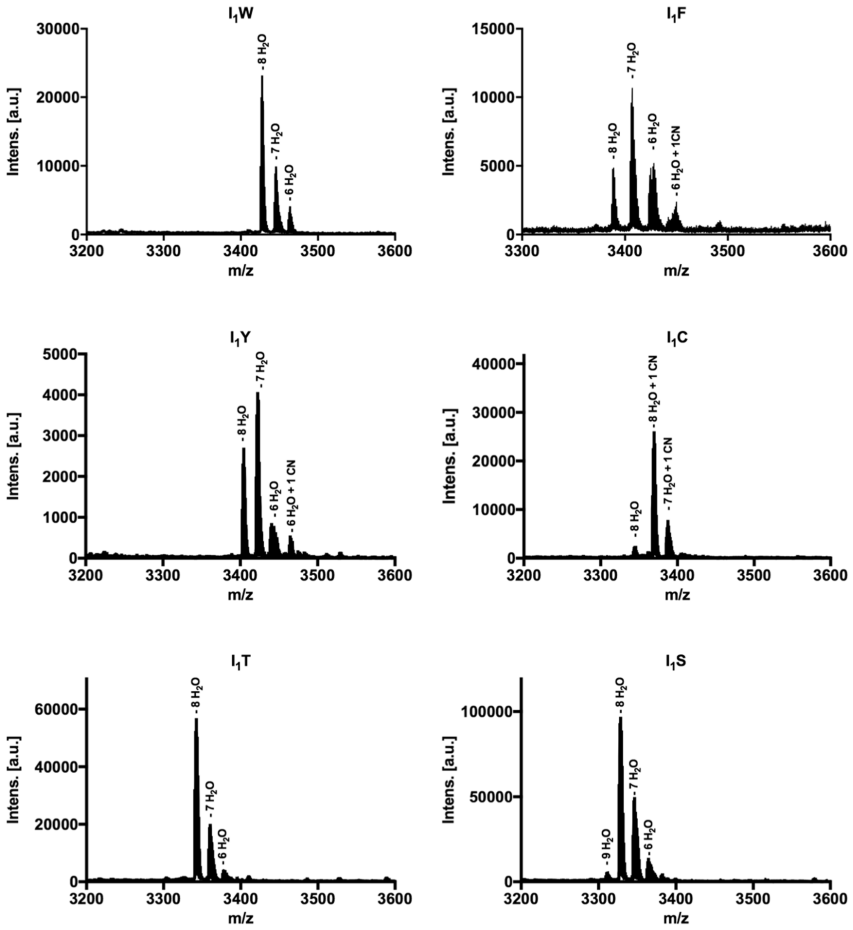

Figure S9: MALDI-TOF analysis to determine the level of dehydrations and ring formations for the nisin A variant group 2 and 3.

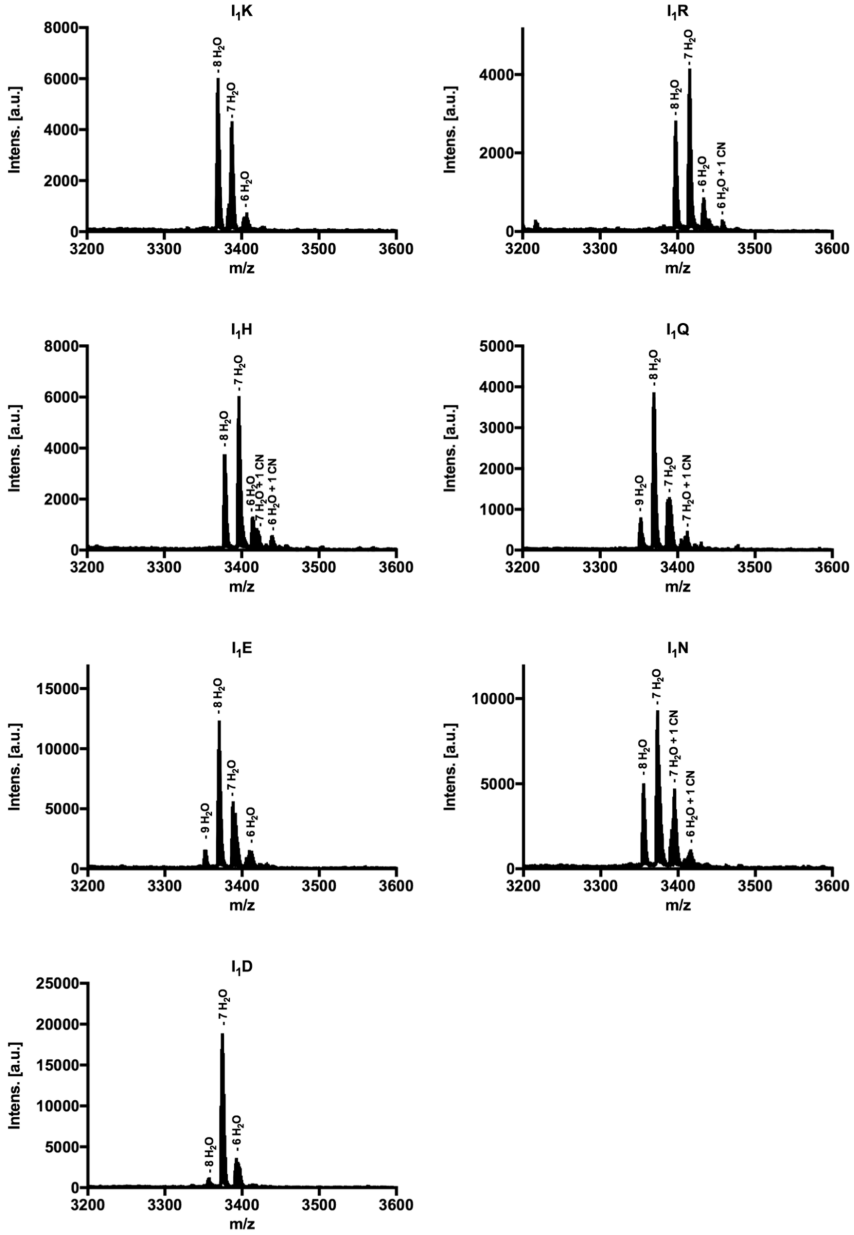

Figure S10: MALDI-TOF analysis to determine the level of dehydrations and ring formations for the nisin A variant group 4.

Tables of supplemental information:

Table S1: Overall data from the MS analysis of nisin A variants

	Variant	Observed masses [Da]	Modification
Group 1	WT	3354	- 8 H_2O
		3372	- 7 H_2O
	I_1P	5672	Uncleaved + Met - 8 H_2O
		5690	Uncleaved + Met - 7 H_2O
		5708	Uncleaved + Met - 6 H_2O
	I_1M	3372	- 8 H_2O
		3390	- 7 H_2O
		3408	- 6 H_2O
	I_1L	3354	- 8 H_2O
		3372	- 7 H_2O
		3390	- 6 H_2O
		3415	- 6 H_2O + 1 CN
		3433	- 5 H_2O + 1 CN
	I_1A	3312	- 8 H_2O
		3330	- 7 H_2O
		3355	- 7 H_2O + 1 CN
		3366	- 5 H_2O
	I_1V	3340	- 8 H_2O
		3358	- 7 H_2O
		3376	- 6 H_2O
		3401	- 6 H_2O + 1 CN
	I_1G	3298	- 8 H_2O
		3316	- 7 H_2O
		3334	- 6 H_2O
		3359	- 6 H_2O + 1 CN
Group 2	I_1W	3427	- 8 H_2O
		3445	- 7 H_2O
		3463	- 6 H_2O
	I_1F	3388	- 8 H_2O
		3406	- 7 H_2O
		3424	- 6 H_2O
		3449	- 6 H_2O + 1 CN
	I_1Y	3404	- 8 H_2O
		3422	- 7 H_2O
		3440	- 6 H_2O
		3465	- 6 H_2O + 1 CN
Group 3	I_1C	3344	- 8 H_2O
		3369	- 8 H_2O + 1 CN
		3387	- 7 H_2O + 1 CN
	I_1T	3342	- 8 H_2O
		3360	- 7 H_2O
		3378	- 6 H_2O
	I_1S	3310	- 9 H_2O
		3328	- 8 H_2O
		3346	- 7 H_2O
		3364	- 6 H_2O
Group 4	I_1K	3369	- 8 H_2O
		3387	- 7 H_2O
		3405	- 6 H_2O
	I_1R	3397	- 8 H_2O
		3415	- 7 H_2O
		3433	- 6 H_2O
		3458	- 6 H_2O + 1 CN
	I_1H	3378	- 8 H_2O
		3396	- 7 H_2O

		3414	**- 6 H_2O**
		3421	**- 7 H_2O + 1 CN**
		3439	**- 6 H_2O + 1 CN**
	I_1Q	**3351**	**- 9 H_2O**
		3369	**- 8 H_2O**
		3387	**- 7 H_2O**
		3412	**- 7 H_2O + 1 CN**
	I_1E	**3352**	**- 9 H_2O**
		3370	**- 8 H_2O**
		3388	**- 7 H_2O**
		3406	**- 6 H_2O**
	I_1N	**3355**	**- 8 H_2O**
		3373	**- 7 H_2O**
		3398	**- 7 H_2O + 1 CN**
		3416	**- 6 H_2O + 1 CN**
	I_1D	**3356**	**- 8 H_2O**
		3374	**- 7 H_2O**
		3392	**- 6 H_2O**

Table S2: IC_{50} values of nisin A and variants

IC_{50} values were determined against the sensitive strain NZ9000-Cm and against strain NZ9000 expressing the immunity/resistance proteins NisI, NisFEG, *Sa*NSR and *Sa*NsrFP.

	NZ9000-Cm	NZ9000-NisI	NZ9000-NisFEG	NZ9000-*Sa*NSR	NZ9000-*Sa*NsrFP
	IC_{50} [nM]	IC_{50} [nM]	IC_{50} [nM]	IC_{50} [nM]	IC_{50} [nM]
WT	4.8 ± 0.7	46.0 ± 6.0	53.0 ± 4.5	73.1 ± 3.6	82.1 ± 3.7
I_1P			not cleavable		
I_1M	5.8 ± 0.3	51.5 ± 1.6	39.2 ± 1.0	84.5 ± 1.7	64.9 ± 0.7
I_1L	9.8 ± 0.5	35.5 ± 1.5	34.6 ± 1.2	53.0 ± 0.4	50.4 ± 0.6
I_1A	10.7 ± 0.4	65.0 ± 2.8	34.2 ± 1.0	68.0 ± 1.9	166.1 ± 5.1
I_1V	11.8 ± 0.9	43.8 ± 1.7	50.4 ± 0.3	66.8 ± 0.34	59.2 ± 2.2
I_1G	143.0 ± 5.1	785.7 ± 9.7	557.8 ± 28.3	278.5 ± 13.3	2257.0 ± 53.4
I_1W	2.5 ± 0.2	22.7 ± 1.5	21.7 ± 1.2	18.3 ± 0.7	46.6 ± 1.1
I_1F	3.7 ± 0.8	36.5 ± 3.3	25.9 ± 1.9	43.9 ± 1.2	64.3 ± 0.4
I_1Y	10.6 ± 0.9	84.3 ± 1.1	45.7 ± 1.8	105.7 ± 1.3	137.9 ± 4.1
I_1C	8.6 ± 0.5	55.1 ± 2.3	41.7 ± 0.7	101.4 ± 3.8	68.8 ± 3.2
I_1T	37.3 ± 1.6	653.3 ± 5.1	107.7 ± 6.2	130.8 ± 3.4	716.1 ± 28.6
I_1S	112.4 ± 5.0	1898.0 ± 62.3	337.3 ± 17.5	253.1 ± 6.2	2893.0 ± 34.8
I_1K	44.7 ± 3.0	435.7 ± 9.9	301.2 ± 4.7	171.7 ± 0.8	983.6 ± 16.2
I_1R	113.9 ± 13.6	422.9 ± 20.4	186.4 ± 3.1	227.0 ± 5.6	1153.0 ± 31.0
I_1H	140.0 ± 5.0	1488.0 ± 85.1	1172.0 ± 134.3	232.9 ± 3.4	3213.0 ± 19.1
I_1Q	592.0 ± 17.8				
I_1E	1328.0 ± 32.7			n.d.	
I_1N	1386.0 ± 46.3				
I_1D	3746.0 ± 144.1				

n.d.: not determined.

Table S3: Nomenclature of the strain NZ9000 expressing immunity (NisI and NisFEG) and resistance proteins (*Sa*NSR and *Sa*NsrFP)

Strain name	plasmid	Expressed protein	properties	Ref.
NZ9000	-	-	sensitive *Lactococcus lactis* strain lacking the gens for *NisABTCPIFEG*	1
NZ9000-Erm	pNZ-SV Erm	-	erythromycin resistance; sensitiv strain	2
NZ9000-Cm	pIL-SV Cm	-	chloramphenicol resistance; sensitiv strain	3
NZ9000-NisP	pNG-nisP8His	NisP	chloramphenicol resistance; sensitive strain expressing the peptidase NisP from *Lactococcus lactis*	4
NZ9000-NisBTC	pIL3-BTC	NisBTC	chloramphenicol resistance; sensitive strain expressing the modification proteins NisB, NisC and the ABC transporter NisT from *Lactococcus lactis*	5
NZ9000-NisI	pNZ-SV-nisI	NisI	erythromycin resistance; immunity strain expressing the lipoprotein NisI from *Lactococcus lactis*	2
NZ9000-NisFEG	pIL-SV-nisFEG	NisFEG	chloramphenicol resistance; immunity strain expressing the ABC transporter NisFEG from *Lactococcus lactis*	3
NZ9000-*Sa*NSR	pNZ-SV-nsr	*Sa*NSR	erythromycin resistance; resistance strain expressing the nisin peptidase NSR from *Streptococcus agalactiae*	6
NZ9000-*Sa*NsrFP	pIL-SV-nsrFP	*Sa*NsrFP	chloramphenicol resistance; resistance strain expressing the BceAB-type ABC transporter NsrFP from *Streptococcus agalactiae*	7

Table S4: Primer sequences used for site-directed mutagenesis
The primer pairs were used for the point mutations at position 1 in the core peptide of nisin A. The exchanged codon is labelled red within the forward (fw) and reversed (rw) primer sequences.

Oligonucleotide name	Sequence (5'-3')
I-K fw	GTGCATCACCACGCAAAACAAGTATTTCGC
I-K rw	GCGAAATACTTGTTTTGCGTGGTGATGCAC
I-M fw	GTGCATCACCACGCATGACAAGTATTTCGC
I-M rw	GCGAAATACTTGTCATGCGTGGTGATGCAC
I-N fw	GTGCATCACCACGCAATACAAGTATTTCGC
I-N rw	GCGAAATACTTGTATTGCGTGGTGATGCAC
I-R fw	GTGCATCACCACGCAGAACAAGTATTTCGC
I-R rw	GCGAAATACTTGTTCTGCGTGGTGATGCAC
I-S fw	GTGCATCACCACGCAGTACAAGTATTTCGC
I-S rw	GCGAAATACTTGTACTGCGTGGTGATGCAC
I-T fw	GTGCATCACCACGCACAACAAGTATTTCGC
I-T rw	GCGAAATACTTGTTGTGCGTGGTGATGCAC
I-V fw	GTGCATCACCACGCGTTACAAGTATTTCGC
I-V rw	GCGAAATACTTGTAACGCGTGGTGATGCAC
I-A fw	GTGCATCACCACGCGCTACAAGTATTTCGC
I-A rw	GCGAAATACTTGTAGCGCGTGGTGATGCAC
I-D fw	GTGCATCACCACGCGATACAAGTATTTCGC
I-D rw	GCGAAATACTTGTATCGCGTGGTGATGCAC
I-E fw	GTGCATCACCACGCGAAACAAGTATTTCGC
I-E rw	GCGAAATACTTGTTTCGCGTGGTGATGCAC
I-G fw	GTGCATCACCACGCGGTACAAGTATTTCGC
I-G rw	GCGAAATACTTGTACCGCGTGGTGATGCAC
I-L fw	GTGCATCACCACGCTTAACAAGTATTTCGC
I-L rw	GCGAAATACTTGTTAAGCGTGGTGATGCAC
I-Y fw	GTGCATCACCACGCTATACAAGTATTTCGC
I-Y rw	GCGAAATACTTGTATAGCGTGGTGATGCAC

I-C fw	GTGCATCACCACGCTGTACAAGTATTTCGC
I-C rw	GCGAAATACTTGTACAGCGTGGTGATGCAC
I-W fw	GTGCATCACCACGCTGGACAAGTATTTCGC
I-W rw	GCGAAATACTTGTCCAGCGTGGTGATGCAC
I-P fw	GTGCATCACCACGCCCAACAAGTATTTCGC
I-P rw	GCGAAATACTTGTTGGGCGTGGTGATGCAC
I-H fw	GTGCATCACCACGCCATACAAGTATTTCGC
I-H rw	GCGAAATACTTGTATGGCGTGGTGATGCAC
I-Q fw	GTGCATCACCACGCCAAACAAGTATTTCGC
I-Q rw	GCGAAATACTTGTTTGGCGTGGTGATGCAC
I-F fw	CATCACCACGCTTTACAAGTATTTCGC
I-F rw	GCGAAATACTTGTAAAGCGTGGTGATG

References:

1. de Ruyter, P.G., Kuipers, O.P. & de Vos, W.M. Controlled gene expression systems for Lactococcus lactis with the food-grade inducer nisin. *Appl Environ Microbiol* **62**, 3662-7 (1996).
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3. AlKhatib, Z. et al. The C-terminus of nisin is important for the ABC transporter NisFEG to confer immunity in Lactococcus lactis. *Microbiologyopen* **3**, 752-63 (2014).
4. Abts, A., Montalban-Lopez, M., Kuipers, O.P., Smits, S.H. & Schmitt, L. NisC binds the FxLx motif of the nisin leader peptide. *Biochemistry* **52**, 5387-95 (2013).
5. Rink, R. et al. Lantibiotic structures as guidelines for the design of peptides that can be modified by lantibiotic enzymes. *Biochemistry* **44**, 8873-82 (2005).
6. Khosa, S. et al. Structural basis of lantibiotic recognition by the nisin resistance protein from Streptococcus agalactiae. *Sci Rep* **6**, 18679 (2016).
7. Reiners, J., Abts, A., Clemens, R., Smits, S.H. & Schmitt, L. Stoichiometry and structure of a lantibiotic maturation complex. *Sci Rep* **7**, 42163 (2017).

4 Discussion and concluding remarks

In this thesis the translocation and proteolytic processing of the lanthipeptide nisin was addressed to obtain a deeper understanding of these processes and placed in context with the nisin biosynthesis. At the beginning, the introduction (1) and chapter I (3.1) gave an overview about the biosynthesis, secretion and maturation of the ribosomally synthesized and post-translationally modified lanthipeptides. Additionally, these sections provided background information about the ABC transporter NisT (1.5.2), the leader peptidase NisP (1.5.3) and the nisin immunity and resistance proteins (1.5.4 and 1.5.5). Subsequently, the chapters II-IV (3.2, 3.3 and 3.4) present the results regarding the secretion and maturation of nisin, whereas chapters V (3.5) and VI (3.6) deal with the nisin immunity and resistance proteins as well as the characterization of NisA variants produced by the nisin system.

Now, the discussion will outline five topics with the focus on the secretion and maturation of nisin. First, the discussion will recapitulate the characterization of the ABC transporter NisT, which is the first *in vitro* characterization of the nisin exporter (4.1). Secondly, the role of the leader peptide and putative recognition motifs in the translocation process are discussed (4.2). Afterwards, the secretion of nisin is extended to the interplay of NisT with the modification enzymes NisB and NisC (4.3). A hypothetic model of the transport cycle of NisT will then merge the proposed modification mechanism with the secretion process (4.4 and 4.5). Afterwards, the characterization of NisA maturation will close the nisin biosynthesis (4.6 and 4.7). Finally, the achieved objects and open questions in the field are summarized and future perspectives are highlighted to conclude the thesis (4.8).

4.1 The ABC transporter NisT

ABC transporters are a large superfamily of MPs, which are ubiquitous found all kingdoms of life and hydrolyse ATP to provide energy for substrate translocation across a membrane (Anderson, Berger et al. 1991, Higgins 1992, Davidson, Dassa et al. 2008, Holland 2011). Generally, ABC transporters can be dissect into the group of exporters and importers and translocate various substrates with different size such as ions, polysaccharides, amino acids, siderophores, drugs and polypeptides (Higgins 1992, Martinoia, Klein et al. 2002, Raetz, Reynolds et al. 2007, Rea 2007, Davidson, Dassa et al. 2008, Ruiz, Gronenberg et al. 2008, Seeger and van Veen 2009, Cuthbertson, Kos et al. 2010, Leprohon, Legare et al. 2011,

Wong, Ma et al. 2014). Within the group of exporters, specialized ABC transporters translocate peptides such as lanthipeptides, thiopeptides, sactipeptides, bottromycins or unmodified bacteriocins independent of the Sec translocon pathway (Fath and Kolter 1993, Nes, Diep et al. 1996, Michiels, Dirix et al. 2001, Gebhard 2012, Arnison, Bibb et al. 2013).

In the BGC of the lanthipeptide nisin the ABC transporter, which exports the precursor peptide NisA is NisT (Kuipers, Beerthuyzen et al. 1993). Similar to other LanT-type ABC transporters NisT secretes its substrate in a LP-dependent manner and disruption of the gene encoding for the protein leads to accumulation of NisA inside the cell (3.2) (Quiao and Saris 1996, Kuipers, de Boef et al. 2004). NisT is a half-size ABC transporter and is supposed to be a functional homodimer (3.2) (Siegers, Heinzmann et al. 1996). One monomer has a two-domain organization comprising of a TMD and a NBD and a molecular weight of 69 kDa. Many studies investigated the secretion of NisA *in vivo* by the nisin modification and secretion system (NisBTC), but seldom was NisT the topic of *in vivo* and none *in vitr*o studies of the nisin exporter are available (Quiao and Saris 1996, Kuipers, de Boef et al. 2004, Rink, Kuipers et al. 2005, Rink, Wierenga et al. 2007, Kluskens, Nelemans et al. 2009, Lubelski, Khusainov et al. 2009, Plat, Kluskens et al. 2011, Plat, Kuipers et al. 2017). Therefore, NisT was characterized *in vitro* by establishing the purification of the detergent-purified MP from *L. lactis* membranes (3.2 and 3.3). NisT was solubilised with different detergents such as DDM, LMNG, FC-16 and purified in different buffer system (e.g. Tris, HEPES, Phosphate and CAPS) supplemented with detergent. The best results, regarding the stability and yielded were obtained in a Tris buffer system supplemented with 2m DTT, 0.5 mM AEBSF and 0.0015% FC-16 (194 µg membrane protein/ 1l cell culture). Additionally, a predominantly monodisperse protein in SEC was observed under the aforementioned condition (3.3). The influence of the detergent was subject of many studies, where starting with the solubilisation of ABC transporter up to purification and biochemical assays, their impact on the MP was investigated (Seddon, Curnow et al. 2004, Gutmann, Mizohata et al. 2007, Infed, Hanekop et al. 2011, Ellinger, Kluth et al. 2013, Yang, Wang et al. 2014). For example, the ABC transporter ABCB1 and ABCA4 were solubilised with various, ionic, non-ionic and zwitterionic detergents and screened for stability and homogeneity (Pollock, McDevitt et al. 2014). Another study showed, that the choice of the detergent has also an impact on the purity of the MP (Wiseman, Kilburg et al. 2014). In the case of NisT a high purity protein sample was obtained with the Tris buffer-based purification protocol (3.3). Interestingly, the

ATPase activity of NisT was restored to a higher basal activity of NisT by changing the detergent to the non-ionic detergent CYMAL5. Here, the basal ATPase activity has a maximal V_{max} value of 79.9 ± 2.9 nmol•min^{-1}•mg^{-1} with a K_m value of 0.37 ± 0.04 mM. This basal activity is similar to other purified ABC transporters (Choudhury, Tong et al. 2014, Lin, Huang et al. 2015, Reimann, Poschmann et al. 2016, Zheng, Nagao et al. 2017). In summary, the first *in vitro* isolation and biochemical characterization of the nisin exporter NisT was successful.

To get further insights into the mechanism of a peptide exporter, a structural investigation of NisT using X-ray crystallography was initiated (3.2). Therefore, the detergent purified ABC transporter was subjected to crystallization trails with various conditions (variations of salt, pH, precipitant, additives and temperature). Unfortunately, the detergent based approach to obtain crystals was not successful and therefore a bilayer mimicking approach was chosen. The bicelle reconstitution of MPs was proven to be an alternative to crystalize different MP in a lipidic environment (Poulos, Morgan et al. 2015). Bicelles are formed in aqueous solution by the mixture of an amphiphile like dihexanoyl phosphatidylcholine (DHPC) or 3-([3-Cholamidopropyl] dimethylammonio)-2-hydroxy-1-propanesulfonate (CHAPSO) with dimyristoyl phosphatidylcholine (DMPC). In these disc-like aggregates the long chain lipids are surrounded by the amphiphiles and enables the reconstitution of an MP in the bilayer structure (Ujwal and Bowie 2011). Two conditions of the bicelles approach gave needle shaped crystals, from which one condition was used for further optimization. Moreover, the protein stability was strongly improved by the lipid environment, which was shown for other MPs and MP oligomers (Gupta, Donlan et al. 2017, Montenegro, Cantero et al. 2017, Neumann, Rose-Sperling et al. 2017). Although, the size of the needle was improved by the addition of the nucleotide ADP the obtained crystals did not diffract.

To get a structural glimpse on NisT, modeling tools such as SWISS-MODEL (Arnold, Bordoli et al. 2006) or Phyre2 (Kelley, Mezulis et al. 2015) can be used. Here, a homology model of NisT was generated with Phyre2, which is based on the sequence of NisT and on six known structures of ABC transporters (Figure 25) (3.2). The general architecture of the NisT homology model is similar to other ABC transporter type I exporters, which are symmetrical homodimers. One TMD consists of six transmembrane helices (TMH), which form the translocation pathway with the opposite TMD (Figure 25C). All TMHs are spanning through

the membrane and the cytosolic NBD follows after TMH 6. In the homology model NisT adapts an inward-facing conformation, where the extracellular gate is closed. Here, the rotation of TMH 1 and 6 would allow the transformation to the outward-closed/outward-opened conformation as observed in the structure of Sav1866 by X-ray crystallography and DEER spectroscopy experiments (Xu, Seelig et al. 2017).

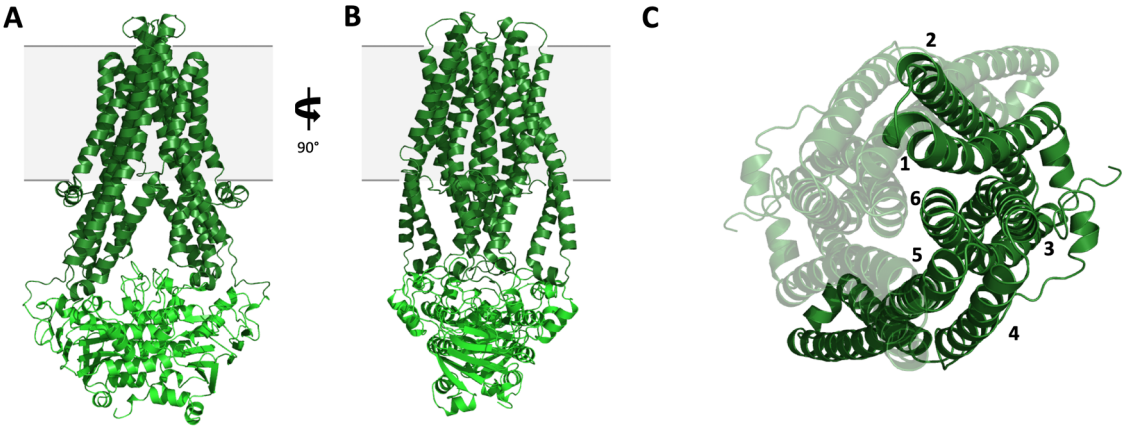

Figure 25: Homology model of the ABC transporter NisT.
A) Homology model of NisT generated with Phyre2 (Kelley, Mezulis et al. 2015) based on six known ABC transporter structures (Chapter II). **B**) Rotation of model by 90° for side view. **C**) Top view of the transmembrane helices (TMH) from extracellular side. The TMHs of one monomer are numbered (1-6). Colour code: TMDs are displayed in dark green and NBDs in light green. The program PyMOL 2.1.1 was used to display the homology models in a cartoon representation.

Furthermore, the TMH 3 and 4 would move together with TMH 1 and 6. The coupling helices, which are located at the interface to the NBDs, lie between TMH 4 and 5. Thus, any conformational changes in the NBD are transferred via these helices to the TMD (Locher 2009). The binding of ATP and its hydrolysis in the NBDs enables the movement of the TMDs and thereby the ABC transporter can switch from the IFC to the OFC via transition states such as the occluded conformation (Choudhury, Tong et al. 2014, Husada, Bountra et al. 2018). Within the homology model the NBDs are in close contact and dimerized like in a nucleotide bound state. In summary, the homology model of NisT, which was generated by known structures of ABC transporters, provides only a model based on homology. But as a protein structure prediction based on only a sequence is error prone, the final structure of NisT might be diverge from the finial homology model (Kelley, Mezulis et al. 2015). Additionally, a model based on templates with higher sequence similarity (> 60%) such as closer homologs (NshT and NsuT) or related LanT-type transporter (SlvT) would result in a better homology model. Unfortunately, no crystal structures of RiPP exporters, except McjD (20% sequence identity to NisT) the exporter of the lasso peptide microcin J25, are available (Choudhury, Tong et al. 2014).

4.2 The role of the leader peptide in the translocation process

Signal peptides are important factors, which define the localisation of peptides, oligopeptides or proteins. In bacteria, two transport pathways allow the translocation of substrates out of the cytosol to the target location. One route is the general secretion (Sec) pathway, where the cargo is exported in an unfolded and thereby transport competent state by the Sec translocon (du Plessis, Nouwen et al. 2011, Denks, Vogt et al. 2014). The alternative route is via the twin-arginine translocation (Tat) complex, that export folded substrates (Palmer, Sargent et al. 2010). Both pathways are strongly depended on their specific signal peptides, which further define alternative routes within their export pathway (e.g. co- or post-translational export via the Sec translocon).

Similarly, the leader peptide of lanthipeptides or other RiPPs function as a signal peptide for the translocation process (Kuipers, de Boef et al. 2004, Arnison, Bibb et al. 2013). Moreover, the LP facilitates further functions such as an enzyme recognition site (e.g. modification enzymes and peptidase), distance marker for the PTM sites in the CP and "chaperon" for the modified CP (van der Meer, Rollema et al. 1994, Chatterjee, Patton et al. 2006, Li, Yu et al. 2006, Mavaro, Abts et al. 2011, Muller, Ensle et al. 2011, Abts, Montalban-Lopez et al. 2013, Ortega, Velasquez et al. 2014, Ortega, Hao et al. 2015). Hitherto, the secretion of the lanthipeptides was often investigated in presence of their corresponding modification enzymes (Ekkelenkamp, Hanssen et al. 2005, Uguen, Hindre et al. 2005, Kuipers, Meijer-Wierenga et al. 2008, Dischinger, Josten et al. 2009, Fuchs, Jaskolla et al. 2011, Wescombe, Upton et al. 2011, Zhang, Yang et al. 2014, Mohr, Volz et al. 2015). Although, it was shown for the nisin modification and secretion system, that these enzymes could function independent from each other (Kuipers, de Boef et al. 2004). Furthermore, nothing is known about the recognition site of the exporter within the LP, even though the FNLD-box might be important for the secretion process (Plat, Kluskens et al. 2011).

In order to determine the recognition motif of the exporter and to investigate NisA secretion *in vivo*, a quantitative secretion assay was development based on the nisin secretion and modification system (3.3) (Rink, Kuipers et al. 2005, van den Berg van Saparoea, Bakkes et al. 2008). In contrast to the published results, the focus was on the exporter and thereby the modification enzymes are absent in the assay to determine the effect of LP mutants on NisT. The developed *in vivo* secretion assay allows the quantitative analysis of the secreted peptides in the supernatant via RP-HPLC, in which UV absorption at

205 nm is used to determine the peptide amount (3.3). First, the secretion of FNLD-box mutants was analysed and showed similar results to the described mutants in the presence of the modification enzymes NisB and NisC (Plat, Kluskens et al. 2011). All analysed mutants ($NisA_{F-18A}$, $NisA_{N-17A,}$ $NisA_{L-A}$ and $NisA_{D-15A}$) display around 7-20% of the WT secretion level, where the single alanine substitution at position F-18 and L-16 showed the strongest reduction (10 and 7%). The secretion was strongly impaired with the quadruple mutant ($_{-18}AAAA_{-15}$) and was at background level, which is defined by the H-loop mutant of the NisT NBD. This mutant of NisT is reassigned as a control for a non-secreting transporter as the ATP hydrolysis is abolished (3.3). Hence, the FLND-box is a recognition site for NisT and not only for NisB as well as NisC.

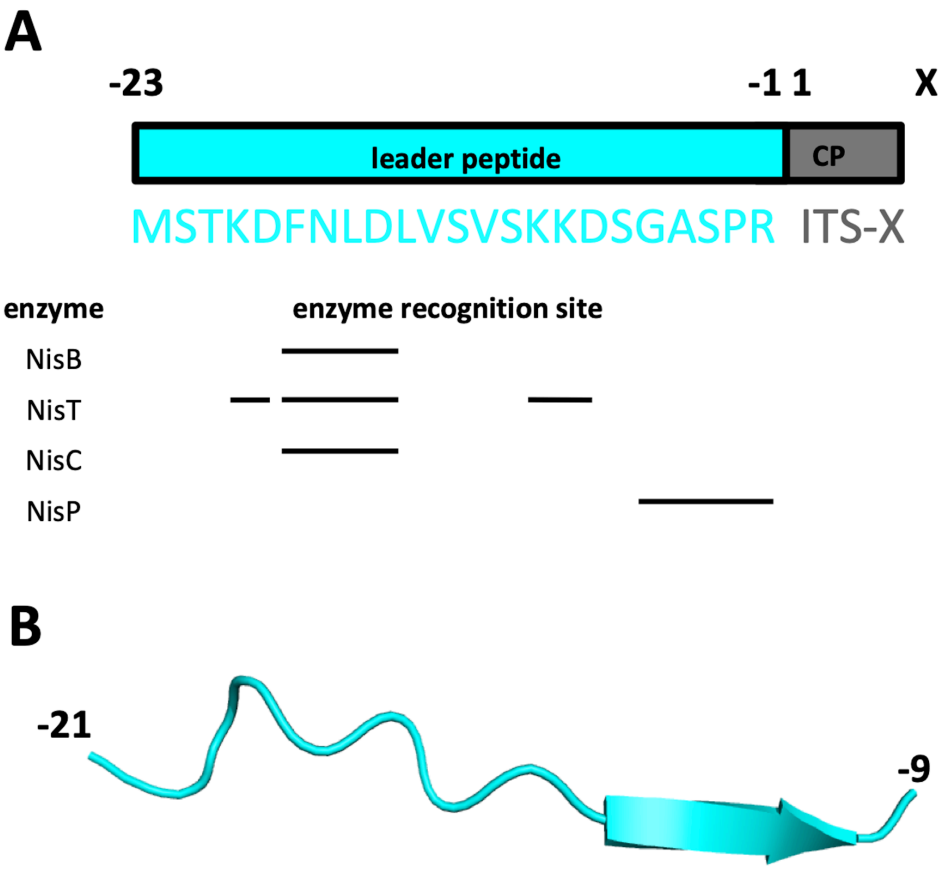

Figure 26: Enzyme recognition sites in the leader peptide.
A) The enzyme recognition site of the transporter NisT, the peptidase NisP and the modification enzymes NisB as well as NisC. **B**) Structure of $NisA_{LP}$ (aa -21 till -9) from a co-crystal with the dehydratase NisB (PDB ID: 4WD9). Colour code: The leader peptide is displayed in cyan and the core peptide in dark grey. The program PyMOL 2.1.1 was used to display the homology models in a cartoon representation.

To find additional ERS, a sequence alignment of various lanthipeptide LPs was performed, which are classified based on the group of the full-length lanthipeptide (nisin-group, epidermin-group etc.). The alignment indicated, that besides the FNLD-box other aa at certain positions are conserved and were selected for mutagenesis in the $NisA_{LP}$ (3.3). One

motif is in front of the ERS (FNLD) and consists of the charged aa lysine and aspartate (K-20 and D-19). A second region in located in the spacer region of the LP downstream of the ERS (K-9, K-8 and D-7). The secretion levels of all mutants were decreased in comparison to WT NisA at least by 50% (3.3). Interestingly, the addition of opposite charges at these positions strongly reduced the secretion levels (e.g. mutation K-20E, K-9E and K-8E). Therefore, the aa at positions K-20, D-19, K-9, K-8 and D-7 are important for an electrostatic interaction with the binding site of NisT. Similarly, in the case of the AMS transporter EnkT from *Enterococcus faecium* NKR-5-3 charged aa within the LP were important for secretion of the peptide (Sushida, Ishibashi et al. 2018). Additionally, the hypothesis is supported by published results of $NisA_{LP}$ mutants in presence of NisB and NisC. There, the aa sequence $_{22}STKD_{-19}$ within the $NisA_{LP}$ was indispensible for NisB binding to NisC, but it did not influence the dehydration in the CP (Khusainov, Moll et al. 2013). The authors speculated, that the complex formation of the modification enzymes and the transporter was hampered. Additionally, mutation in the LP of nisin Z and nisin A of the positions K-9 and D-7 (D-7A) displayed a reduced secretion level (van der Meer, Rollema et al. 1994, Plat, Kuipers et al. 2017). In sum, with the determined secretion levels of the $NisA_{LP}$ mutants it can be concluded that the reduction is mainly depended on alternate substrate recognition by NisT. Accordingly, the recognition sites of NisT within the LP of NisA are defined (Figure 26A).

In the context of secondary structure motifs of some lanthipeptide LPs (e.g. epidermin, Pep5, nukacin ISK-1 and lacticin 481 (Schnell, Entian et al. 1988, Weil, Beck-Sickinger et al. 1990, Furgerson Ihnken, Chatterjee et al. 2008, Nagao, Morinaga et al. 2009), the recognition of α-helical structures by the modification enzymes, peptidases and exporters is discussed. For example, the peptidase domain of the ABC transporter LctT recognizes the α-helical structure within lacticin 481 LP and the introduction of an proline residue reduced the proteolytic activity (Furgerson Ihnken, Chatterjee et al. 2008). In the case of the $NisA_{LP}$ no distinct secondary structure motif was observed. On one hand, there is a random coil NMR structure of the LP in aqueous solution (van den Hooven, Rollema et al. 1997) and on the other hand the LP displays a β-strand motif in in the co-crystal structure of NisB and NisA (Figure 26B) (Ortega, Hao et al. 2015). It is tempting to speculate, that a secondary structure motif is only formed after ligand binding to the ERS, but if this hold true for $NisA_{LP}$ additional structural information of NisA with the enzymes of modification, export and processing are necessary.

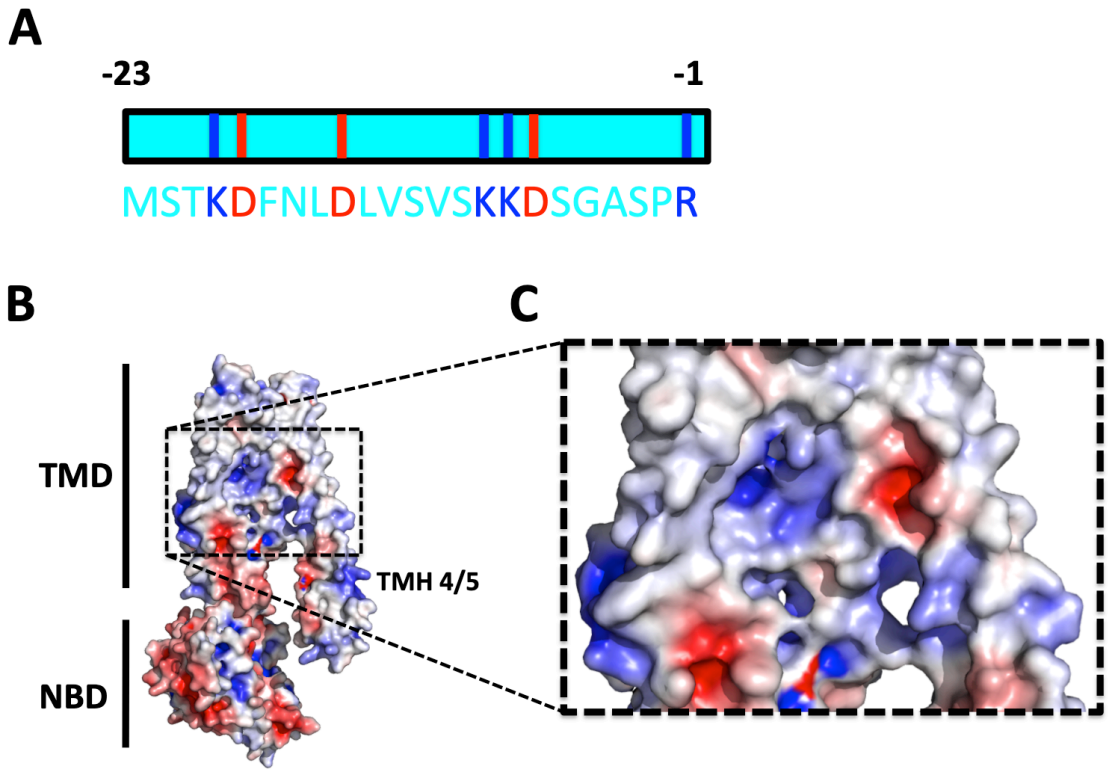

Figure 27: Electrostatic surface potential of NisT homology model.
A) Charge amino acids in the leader peptide of NisA. Positively charge residues are highlighted in blue and negatively charge residues in red. **B**) Homology model of NisT generated with Phyre2 (Kelley, Mezulis et al. 2015) based on six known structures of ABC transporter. Here, only one monomer of the homodimer NisT is displayed. The PDB2PQR server (Dolinsky, Nielsen et al. 2004) was used to calculate the Poisson-Boltzmann electrostatics and the APBS electrostatic plugin for PYMOL was used to display the electrostatic surface potential. For the calculation a PARSE force field was chosen. The protonation state was calculated with PROPKA. Further parameters were: temperature of 298.2 K (25°C), salt concentration 0.15 M and pH of 7. **C**) Zoom in to putative substrate binding site in close proximity to the extracellular gate of the TMHs 1 and 6. The electrostatic property of the surface is displayed in red (negative; -5 kT/e), blue (positive; +5 kT/e) and white (neutral; 0 kT/e). The program PyMOL 2.1.1 was used to display the homology models in a surface representation.

The importance of charged residues within the $NisA_{LP}$ (Figure 27A) for the secretion activity of NisT would insinuate, that a hydrophilic environment is found in the translocation pathway formed by the TMDs. Unfortunately, no structural information of the exporter and its substrate NisA are available and therefore homology models of NisT enable to study the structure of the exporter (3.2). Here, the calculation of the electrostatic surface potential with the PDB2PQR server (Dolinsky, Nielsen et al. 2004) allows to identify regions, which are strongly positively or negatively charged (Figure 27B). Generally, the charged residues and regions are equally distributed but there are some exceptions. First, the NBD is mainly negatively charge at the exterior site. To the interior and thereby to the other NBD (when dimerized), the charge is equally distributed. Secondly, the exterior sites of the TMD have positively to neutral charged regions, whereas the interior sites have stronger charged regions. Especially, in close proximity to the extracellular gate (Figure 27C) a positively and

negatively charge patch would provide a matching hydrophilic environment for the conserved recognition motifs of the $NisA_{LP}$. Noteworthy, the LP binding to this location would provide a signal for extracellular gate opening after which the peptide is translocated either with N- to C- or C- to N-terminus directionality. The current models would allow both possibilities (Lubelski, Khusainov et al. 2009, Plat, Kuipers et al. 2017).

The importance of the LP for the translocation process was further investigated by the analysis of LP hybrids (3.3). Exchanging the $NisA_{LP}$ to six LP from other class I and class II lanthipeptides created these hybrids. Already described are the hybrids from subtilin LP with nisin A/nisin Z CP ($SpaS_{LP}$-NisA /$SpaS_{LP}$-NisZ), that were secreted and processed by the *L. lactis* and *B. subtilis* 6633 strains via their natural modification and secretion systems (NisBTC; SpaTBC) (Kuipers, Rollema et al. 1993, Rintala, Graeffe et al. 1993). The lanthipeptide exporter NisT is highly specific for the natural LP and only one hybrid ($Salv_{LP}$-NisA), where the LP has the highest sequence identity with $NisA_{LP}$, was secreted (3.3). In presence of the modification enzymes NisB and NisC, the secretion was enhanced and secretion of the hybrids $SpaS_{LP}$-/$Salv_{LP}$- and Gal_{LP}-NisA was observed. Interestingly, all tested LPs embody the FN/DLD-box, but they exhibit a higher diversity regarding the position of the charged aa. For example, $Salv_{LP}$ has an additional charged residue after the FNLD-box (E-12 instead of S-12), $SpaS_{LP}$ has $_{-20}DD_{-19}$ in front of the FNLD-box, but lacks the Lys residue and within the $GdmA_{LP}$ a four aa (KNEL) insertion shifts the critical Lys residue (K-20). Hence, the correct position of the recognition motifs for NisT are prerequisite for the LP specificity, where the electrostatic interaction and the hydrophobic interaction with the FNLD-box are the main contributions (Figure 28A). If, any alternation in the LP occurs the secretion efficiency is reduced by an impaired substrate recognition at the substrate binding site (Figure 28B). Thus, the probability of extracellular gate opening after substrate binding is reduced and unnatural substrates might have higher K_D values. The specificity of a peptide transporter for its natural substrate was shown for McjD with its substrate MccJ25 (Romano, Fusco et al. 2018). There, other post-translationally modified peptides were tested to stimulate ATPase activity of McjD, but for all substrates it displayed a reduced activity. Similarly, the LP hybrids of subtilin ($SpaS_{LP}$-NisA) and salivaricin D ($Salv_{LP}$-NisA) reduced the *in vitro* ATPase activity of NisT (3.3). Consequently, a false LP association might lead to a lower secretion level by hampering the transport cycle reset, which leaves NisT in a secretion in-competed state (Figure 28B).

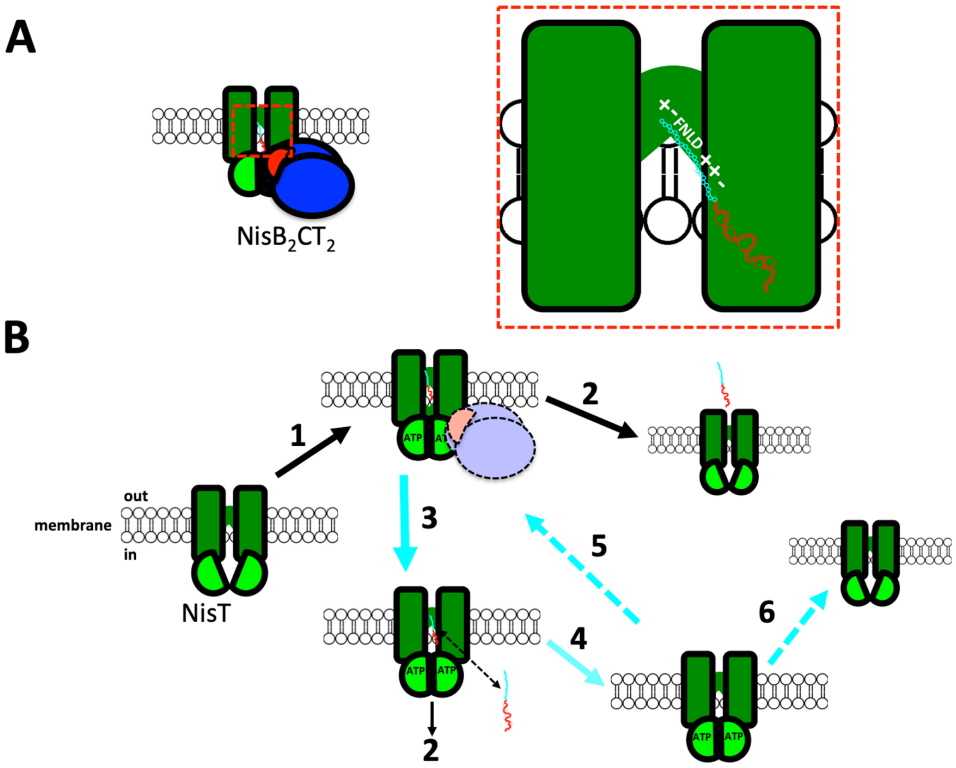

Figure 28: Influence of leader peptide on the translocation process.
A) Scheme of leader peptide binding at NisT substrate binding site. **B**) The translocation of any substrate by NisT starts with the formation (1) of the postulated, multimeric enzyme complex ($NisB_2CT_2$) at the membrane. After transfer of the substrate (mNisA) from the modification complex ($NisB_2C$) the correct recognition of the leader peptide by NisT triggers the conformational changes and the opening of the extracellular gate. The substrate is released to the exterior and ATP hydrolysis resets the transport to the ground state (2). The transfer of an unnatural substrate (e.g. leader peptide mutants or leader peptide hybrids) leads to false recognition (3). Subsequently, some substrates are released (2), but other dissociate from NisT (4). Next, the transporter either interacts with the modification complex again (5) or ATP hydrolysis reset the transport cycle (6). Colour code: NisT TMD is displayed in dark green and NisT NBD in light green. NisB is displayed in blue and NisC in red.

A phylogenetic analysis of the lanthipeptide exporters and their LPs showed that both dissect into the similar clades (3.3). Presumably, this indicates the correlation of the exporter to its substrate (the leader peptide) resulting in substrate specificity. However, a LP with high sequence identity to the natural LP is still recognized, if the position of the recognition motifs is correct, and translocated by the exporter.

4.3 Interplay of NisT with the modification enzymes NisB and NisC

Early studies on the nisin modification system indicated the membrane association of the dehydratase NisB (Engelke, Gutowski-Eckel et al. 1992), which was later extended by the membrane localized multimeric enzyme complex consisting of NisBTC (Siegers, Heinzmann et al. 1996). Similarly, the same observations were made for the modification and secretion systems of class I lanthipeptide subtilin and class II lanthipeptide nukacin ISK-1 (Kiesau,

Eikmanns et al. 1997, Nagao, Aso et al. 2005). The localization of modification and secretion at one place would provide an efficient targeting and transfer of modified precursor/ mature peptide to the lanthipeptide exporter. This further would allow the secretion of only fully modified peptide without intermediates, which might be unmodified or only partly modified. In the case of the nisin system, the secretion of only mNisA and neither uNisA nor dNisA was shown (3.3) (van den Berg van Saparoea, Bakkes et al. 2008, Lubelski, Khusainov et al. 2009)

In 2008, van den Berg van Saparoea *et al.* proposed a channelling mechanism of mNisA by NisBTC (van den Berg van Saparoea, Bakkes et al. 2008). They observed, that mNisA is quickly secreted after induction and the deletion of the modification components especially NisB strongly reduced the secretion. The channelling mechanism was further supported by *in vivo* studies, in which the modification mechanism and interaction of NisB and NisC were investigated (Lubelski, Khusainov et al. 2009, Khusainov, Heils et al. 2011, Khusainov and Kuipers 2013). The quantitative analysis of the *in vivo* secretion (3.3) by the nisin modification and secretion system is also in line with the pervious published results. The determined kinetic parameter of the maximal velocity and the apparent secretion rate demonstrate the enhancement of NisA secretion by the modification enzymes (3.3, Table 2).

Table 2: Kinetic parameter of the *in vivo* secretion assay.
The supernatant of NisA secreting *L. lactis* strains was analysed by RP-HPLC. The amount of NisA (nmol) plotted against the time (min) was fitted with an allosteric sigmoidal fit and kinetic parameter were determined. V_{max} (nmol) is the maximal amount of secreted NisA and $V_{s\ app}$ the apparent secretion rate ($NisA \bullet NisT^{-1} \bullet min^{-1}$). The Table was taken from chapter III (3.3).

strain	V_{max} (nmol)	$V_{s\ app}$ ($NisA \bullet NisT^{-1} \bullet min^{-1}$)
NZ9000BTC	534 ± 44	100.3 ± 35.2
NZ9000$BT_{H551A}C$	n.s.	n.d.
NZ9000BTC_{H331A}	168 ± 16	n.d.
NZ9000BT	247 ± 15	n.d.
NZ9000T	137 ± 30	7.4 ± 1.6
NZ9000TC	38 ± 4	n.d.
NZ9000BC	n.s.	n.d.

n.d.: not determined ; n.s.: not secreted

A complete nisin modification and secretion system consisting of NisBTC has the highest V_{max} value (534 ± 44 nmol) in comparison to NisBT (247 ± 15 nmol), NisTC (38 ± 4

nmol) or NisT (137 ± 30 nmol), which reflects its high secretion efficiency. Furthermore, the apparent secretion rate ($V_{S\ app.;}$ NisA•NisT^{-1}•min^{-1}) of NisBTC is 14 fold higher than of NisT and shows how NisB and NisC enhance the secretion efficiency (3.3, Table 2). Interestingly, the expression of a catalytic-inactive NisC (H331A mutant) in the NisBTC system (strain NZ9000BTC$_{H331A}$) did not result in WT secretion levels (3.3, Table 2) as observed in a previous study (Lubelski, Khusainov et al. 2009). Presumably, early kinetics of the secretion process might better display differences between the used strains (also compare (van den Berg van Saparoea, Bakkes et al. 2008)).

Another explanation is that the export of NisA is disturbed by an impaired channelling via the modification enzymes or by a false substrate recognition by NisT. The impaired channelling would indicate, that the presence of inactive NisC does not enhance the secretion of NisA. This was observed by Kuipers *at al.* (Kuipers, Meijer-Wierenga et al. 2008), but not by Lubelski *et al*., where the secretion was increased to WT level. Additionally, an *in vivo* pull-down experiment demonstrated that the inactive NisC variant (H331A) stabilized a complex of NisB and NisC, which was co-eluted with His-tagged NisA (Khusainov, Heils et al. 2011). A higher stabilization in comparison to the WT system would suggest, that the complex is not released as modification is not completed. Moreover, the *in vitro* assembly of NisB and NisC showed that the modification complex is not formed after the ring E is installed (Reiners, Abts et al. 2017). In summary, the data suggest that after completed modification, the NisB/NisC complex transfers NisA to NisT, but the incomplete modification stalls the modification complex and thereby slows secretion process.

Alternatively, the exporter NisT has different transport kinetics for the different substrates depending on the modification state. Here, Kuipers *et al.* studied the secretion of peptides and their Cys-less variants, which were secreted by NisBT or NisBTC with and without inactive NisC. They did not observe any enhancement of the secretion due to the active or inactive variant of NisC and therefore they stated, that the conformational aberrations between linear (only dehydrated) and fully modified NisA ((methyl-)lanthionine containing) is causing the difference (Kuipers, Meijer-Wierenga et al. 2008). Hence, the high specificity of NisT to mNisA might explain the different secretion efficiencies, but then the V_{max} value for the strain NZ9000BT and NZ9000BTC$_{H331A}$ should be the same. However, the value for the inactive NisC is 32% lower and the latter explanation for the differences is less likely (3.3). In conclusion, the differences in the secretion level origin from an impaired

channelling of the substrate from the modification enzymes to the transporter (van den Berg van Saparoea, Bakkes et al. 2008).

In respect of the modification complex, the main interaction surface comes from the NisB dimer, which is commonly accepted as the main component of the modification/secretion complex (van den Berg van Saparoea, Bakkes et al. 2008, Lubelski, Khusainov et al. 2009). Additionally, a modification/secretion complex of NisBT is enough to achieve higher secretion level of NisA or LP-fused peptides (Kluskens, Kuipers et al. 2005, Rink, Wierenga et al. 2007). The cyclase NisC seems to be dispensable for secretion, but higher secretion efficiency is only reached with NisC (3.3) (Rink, Kluskens et al. 2007, Kuipers, Meijer-Wierenga et al. 2008, van den Berg van Saparoea, Bakkes et al. 2008, Lubelski, Khusainov et al. 2009). Here, NisC seem to contribute to a correct channelling and targeting of the substrate mNisA after complete modification (3.3) (Kuipers, Meijer-Wierenga et al. 2008). Hence, the interplay of NisB, NisC and NisT is very important for an efficient NisA secretion.

The interaction of NisT, NisB and NisC was first investigated by Siegers *et al*. and lead to the proposed multimeric enzyme complex, where NisB interacts with NisC and NisT with NisC (Siegers, Heinzmann et al. 1996). But any interaction of NisT with NisB was not shown, although NisB is assumed to be the central component for the modification and secretion complex (van den Berg van Saparoea, Bakkes et al. 2008). The specific interaction of NisT with NisB and NisC with and without NisA was shown by a pull-down assays (3.3) and support the proposed multimeric enzyme complex of NisBTC (Siegers, Heinzmann et al. 1996), where all components of the complex are membrane located and interacting with NisT. The formation of a transport competent modification and secretion complex comprising of $NisB_2CT_2$ might be achieved via four models (Figure 29). In model 1 the dimer of NisT ($NisT_2$) interacts loose with the pre-assembled complex of $NisB_2CA$. Here, the modified peptide is targeted after modification to the translocation pathway, where the LP is interacting with $NisT_2$ and translocation is initiated.

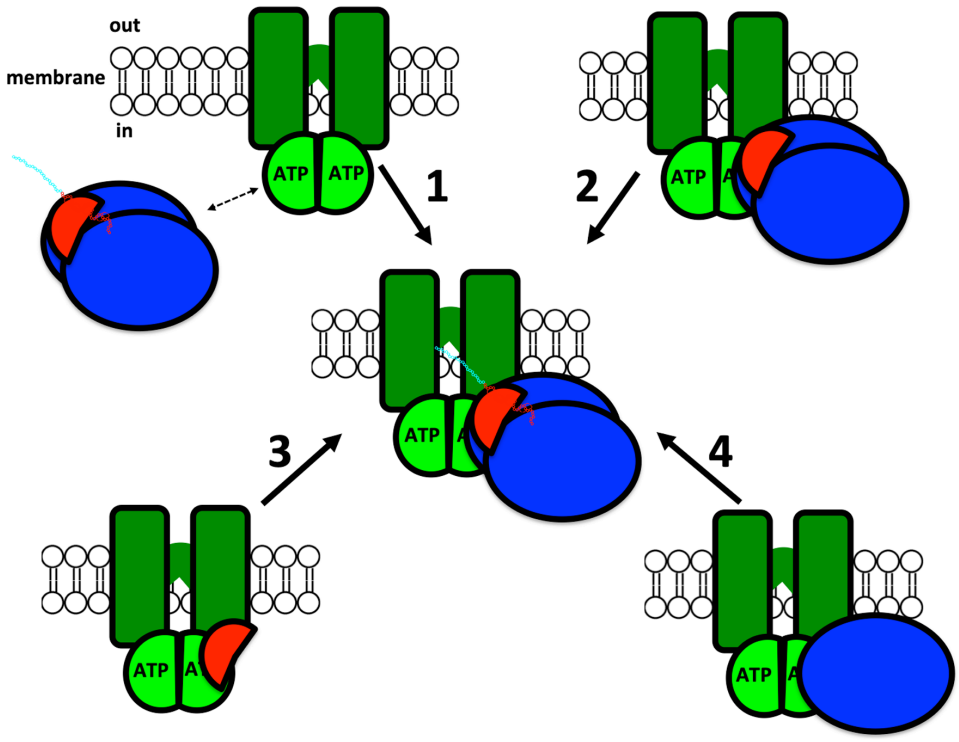

Figure 29: Complex formation prior to substrate translocation.
The translocation process starts with the complex formation of the postulated, multimeric enzymes complex ($NisB_2CT_2$) at the membrane. There, different possibilities of complex association prior to substrate translocation are conceivable. (**1**) The pre-assembled complex of $NisB_2CA$ interacts with NisT during or after the modification process. (**2**) The membrane located, multimeric enzyme complex consisting of $NisB_2CT_2$ is pre-assembled at the membrane without substrate (inactive state). The interaction of NisA with NisB initiates the modification and consequently the translocation. (**3**) NisT interacts with NisC and the pre-assembled complex $NisB_2A$ initiates the modification and consequently the translocation. (**4**) NisT interacts with NisB and the pre-assembled complex NisBCA initiates the modification and consequently the translocation. Colour code: NisT TMD is displayed in dark green and NisT NBD in light green. NisB is displayed in blue and NisC in red. The precursor peptide mNisA is illustrated in red, where the leader peptide is highlighted in cyan.

In model 2 a complex of $NisB_2CT_2$ is located at the membrane in an inactive state. The accumulation of ribosomally synthesized NisA in the cytoplasm leads to complex activation via LP binding at NisB. Following, NisB and NisC modify the CP, before NisT secretes mNisA. In model 3 NisC interacts with a NisT dimer first. The pre-assembled complex of $NisB_2A$ binds to NisC and $NisT_2$. Then, the modification starts and consequently the modified peptide is secreted. In the model 4 first NisB or a NisB dimer interacts with $NisT_2$, before the pre-assembled complex of NisBCA /NisCA initiates the assembly of the whole complex. Based on various *in vivo* and *in vitro* studies, the favour is for model 1, where the modification complex is pre-assembled after substrate binding and then a transient complex of $NisB_2CT_2A$ is formed (Engelke, Gutowski-Eckel et al. 1992, Siegers, Heinzmann et al. 1996, Lubelski, Khusainov et al. 2009, Khusainov, Heils et al. 2011, Reiners, Abts et al. 2017). Nevertheless, all components of the multimeric complex are interacting with each other and partly assembled complexes of NisCA, $NisB_2A$/$NisB_2A_2$, NisTC, NisTB and NisTBC were observed and

also model 2 would be possible (3.3) (Abts, Montalban-Lopez et al. 2013, Ortega, Hao et al. 2015).

Prior, to translocation the binding of NisA (or LanA) to a helix-turn-helix motif presumably leads to the assembly and activation of the modification complex. Here, this helix-turn-helix motif for substrate binding is found in NisB and is a common substrate binding site of various RiPP PTM enzymes (Montalban-Lopez and Kuipers 2016). In general, the activation of the modification complex is achieved by two mechanisms.

The first mechanism is the conformational selection (Yang and van der Donk 2013, Repka, Chekan et al. 2017). Here, the modification enzymes are in an equilibrium between inactive and active form. The binding of the LP at one binding site via the FNLD-box (NisB) (Mavaro, Abts et al. 2011, Ortega, Hao et al. 2015, Repka, Hetrick et al. 2018) stabilises the active form. Then, the CP samples between the NisB and NisC, where either the dehydration or the cyclisation event is favoured by an energetically preferred intermediate (thermodynamic control) or transition states with lower energy (kinetic control). In the later case either the binding of the substrate or the constant rate of the reaction determines the order of the PTM (dehydration-cyclisation or cyclisation-dehydration). Interestingly, the formation of methyl-lanthionine ring E prevents the dehydration of the CP at position 29 (S29), thus the cyclisation reaction seem to be faster (Lubelski, Khusainov et al. 2009).

In the second mechanism (conformational activation) the LP (or NisA) binding to NisB induces a conformation change, which actives the enzyme. By comparing the crystal structure of NisB (co-crystal with NisA; halo-enzyme) with MibB (apo-enzyme), a movement of the elimination domain is observed. Thus, the LanB adopts a more compact structure in comparison to the apo structure (3.1) (Ortega, Hao et al. 2015, Ortega, Hao et al. 2016). This conformational difference in the overall structure might indicate the difference between an inactive and active form. However, some observations disfavour the conformational activation as some dehydratases (e.g. NisB, LctM and HalM2) catalyse the PTM reaction in the CP without the LP (Khusainov and Kuipers 2012, Oman, Knerr et al. 2012, Thibodeaux, McClerren et al. 2015, Thibodeaux, Wagoner et al. 2016). Therefore, the activation of the modification complex follows the mechanism of conformational selection. After completed modification the substrate NisA is transferred to NisT and thereby to the translocation pathway.

4.4 The enhancement of lanthipeptide secretion

ABC Transporters are primary active transporter, which facilitate the ATP-dependent export of substrates across a membrane against a concentration gradient (Higgins 1992). NisT, the exporter of the lanthipeptide nisin, translocates its substrate via a proposed channelling mechanism in presence of the PTM enzymes NisB and NisC (van den Berg van Saparoea, Bakkes et al. 2008). Generally, the transporter is able to export NisA without the associated PTM enzymes (Kuipers, de Boef et al. 2004), but the secretion rate is strongly enhanced by a factor of 14 in presence of the modification machinery (3.3). The question is now, why the presence of the PTM enzymes does enhance the secretion rate? To answer this question, all possible causes underlie more or less two effects.

The first is the conformational effect, where the interaction of the transporter with the substrate and/or interaction partner alters the conformation. Similarly to the modification complex, the binding of the LP (NisA) enables a conformational change in NisT and shifts the transporter from an inactive to an active form. Here, the later form is transport component. Additionally, not only the binding of the substrate but the interaction with the assembled modification complex $NisB_2C$ shifts NisT to a transport component form. The latter postulation is more likely as the interaction with $NisB_2$ and $NisB_2C$ leads to higher secretion rates (3.3) (van den Berg van Saparoea, Bakkes et al. 2008).

The second is kinetic effect, in which i.a. the rate constants of the different enzyme/substrate (ES) complexes contribute to the secretion rate. To simplify the different factors, three systems are compared (Figure 30). The lanthipeptide export dependent and independent of the modification enzymes are compared, where the ATP hydrolysis and thereby the reset of the transporter are neglected. Furthermore, the number of transporter is set as one and in presence of the complex a 1:1 stoichiometry is assumed.

In the first system, the transporter NisT is translocating NisA (e.g. uNisA) without the PTM enzymes (Figure 30A). Thus, the diffusion rate (τ_1) of the substrate through the cytoplasm to the transporter and the substrate association rate (k) to the transporter contribute to the secretion rate (s_1). At low NisA concentrations the probability of secretion is low, but increases with the increasing NisA concentration in the cytoplasm. However, a free diffusion of NisA would only allow the selection of mNisA over uNisA or dNisA, when the transporter has a higher substrate specificity to mNisA, to ensure the secretion of only modified NisA.

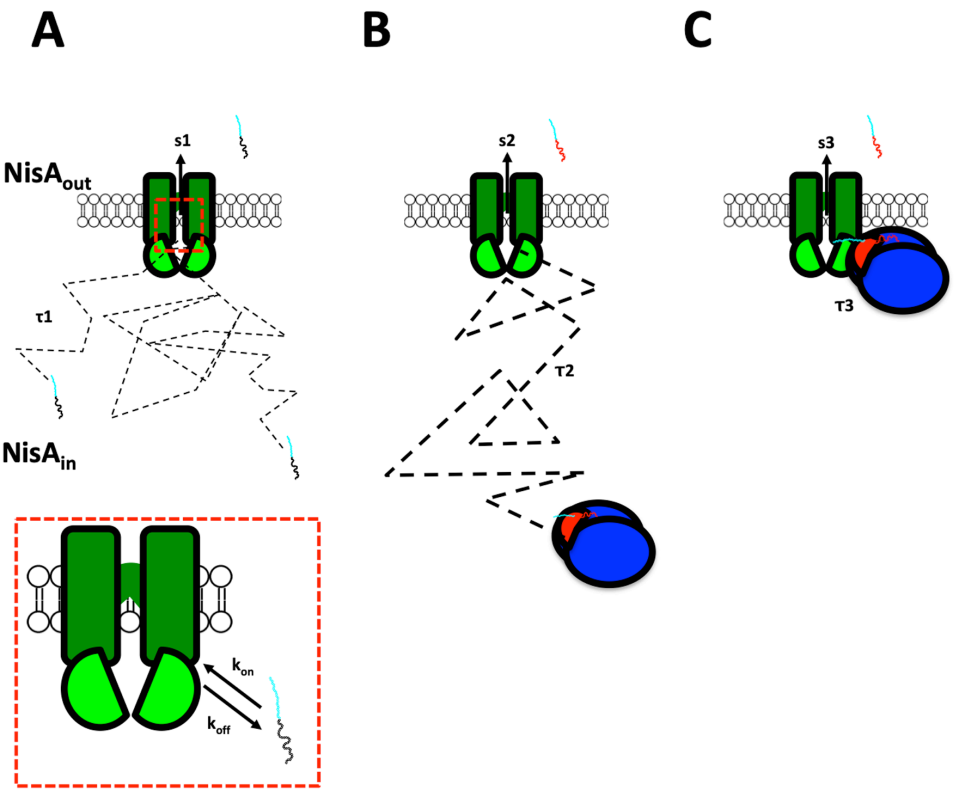

Figure 30: Kinetic effect models of lanthipeptide export.
A) NisA secretion by the ABC transporter NisT without the direct interaction with the PTM enzymes NisB and NisC. **B**) NisA secretion by NisT with interaction of the pre-assembled complex of $NisB_2C$ after diffusion though the cytoplasm. **C**) NisA secretion by NisT with interaction of the pre-assembled complex of $NisB_2C$, which is membrane localized. Colour code: NisT TMD is displayed in dark green and NisT NBD in light green. NisB is displayed in blue and NisC in red. The precursor peptide uNisA and mNisA are illustrated in black and red, respectively. There, the leader peptide is highlighted in cyan.
s: secretion rate; τ: diffusion rate; k: rate constant

In the second system, the transporter NisT is translocating mNisA in a complex with the PTM enzymes, which are located in the cytoplasm (Figure 30B). Thus, the diffusion rate (τ_2) of the pre-assembled complex through the cytoplasm to the transporter, the modification reaction, the complex (NisT to $NisB_2C$) formation rate, and the NisA transfer rate contribute to the secretion rate (s_2). As the radius of the $NisB_2C$ complex is much higher than of a NisA molecule, the diffusion rate would be magnitudes higher due to the inverse proportionality of the diffusion constant (D). In addition, the contributions of the different rate constants (modification rate and NisA transfer rate) would result in a lower secretion rate of system B in comparison to system A, which gives the correlation: s2<s1.

In the third system, the transporter NisT is translocating mNisA in a complex with the PTM enzymes, which is located at the membrane (Figure 30C). Thus, the diffusion rate (τ_3) of the pre-assemble membrane-located complex to the transporter, the modification reaction, the complex (NisT to $NisB_2C$) formation rate, and the NisA transfer rate contribute to the secretion rate (s_3). The direct interaction and membrane localization of the modification

complex reduces a term for the diffusion rate and it can be combined with a term for the complex formation (complex formation is equal to $\tau 3$). Hence, the complex formation rate, the modification reaction, the transfer of NisA to NisT and the binding to NisT contribute to the secretion rate. Based on the *in vivo* data (Kuipers, Meijer-Wierenga et al. 2008, van den Berg van Saparoea, Bakkes et al. 2008, Lubelski, Khusainov et al. 2009, Khusainov, Heils et al. 2011, Khusainov and Kuipers 2013, Khusainov, Moll et al. 2013) and chapter III (3.3), the following correlation of the secretion rates can be made: The secretion rate of system A is higher in comparison to system B (s1>s2) and additional the secretion rate of system B is lower than of system C (s2<s3). Finally, the secretion rate of system A is lower than system C (s1<s3). Although, on the first view the secretion rate of the transporter (s1) alone would be higher. However, the secretion rate of NisT (system A) alone is lower due to the enhancing effect of the PTM enzymes NisB and NisC. The observed enhancement effect is similar to ABC importers and their corresponding substrate binding proteins (Bosdriesz, Magnusdottir et al. 2015). There, the excess of SBPs over the transporter and especially the substrate (nutrient) increases the binding events of loaded SBP and transporter at low substrate concentrations. Hence, this enhances the uptake rate of the importer/SBP complex in comparison to an importer alone. In the case of the nisin modification and secretion system, the amount of modification enzymes must exceed the amount of transporter to maximize the export of substrate per transporter, especially at low substrate concentrations. There, the additional substrate binding sites of modification complexes plus transporter exceed the number of only transporter. Thus, the effective substrate secretion rate is increased, if the complex is additionally membrane located. The export of mNisA within minutes after induction and at presumably low cytosolic substrate concentration supports this hypothesis (van den Berg van Saparoea, Bakkes et al. 2008). Moreover, the binding affinity of NisB and NisC to NisA at a low micromolar range facilitates a sufficient encounter rate (Mavaro, Abts et al. 2011, Abts, Montalban-Lopez et al. 2013). Then, the complex formation increases the effective substrate concentration at the transporter and enhances the secretion rate after modification.

In the end, a combination of the conformational effect and the kinetic effect might contribute to the enhanced secretion of NisA. The binding of $NisB_2CA$ to NisT would induce a conformational change and make the exporter transport competent. Furthermore, the

encounter rate and the substrate concentration at the transporter are increased by the modification complex, which leads to a higher effective secretion rate.

4.5 Model of a NisT transport cycle

Next, a model for the entire modification and transport cycle of NisA can be proposed (Figure 31). In the beginning, NisA is synthesized at the ribosome as an unmodified precursor peptide, which is than modified by the PTM complex $NisB_2C$ to mNisA. Here, the PTM enzymes are presumably located at membrane with NisT to enable efficient translocation. In the ground state, the ABC transporter NisT is adopting an inward-facing conformation (IFC) with closed extracellular gates. The intercellular ATP level in *L. lactis* (1 mM) (Palmfeldt, Paese et al. 2004) induces the NBD dimerization following the ATP switch model, after which the substrate binding site at the TMDs becomes accessible for NisA. In a next step, mNisA is transferred from $NisB_2C$ after complete modification, where the last lanthionine-ring is initiating the complex release of $NisB_2CA$ (Reiners, Abts et al. 2017). Interestingly, the transfer of the substrate to the transporter NisT is less efficient, when NisC is affect through an inactive mutation or deletion (3.3). Thus, the substrate mNisA is only channelled to the translocation pathway properly with fully functional modification enzymes (van den Berg van Saparoea, Bakkes et al. 2008, Khusainov, Heils et al. 2011, Khusainov and Kuipers 2013, Khusainov, van Heel et al. 2015). Subsequently, the binding of mNisA and/or $NisB_2C$ facilitate the transition of NisT to a transport competent conformation. This includes the transition from the IFC to an occulted conformation (OC) as observed for McjD, PtrD or PCAT1 (Choudhury, Tong et al. 2014, Lin, Huang et al. 2015, Morgan, Acheson et al. 2017). Now, the extracellular gate is only opening after correct interaction of LP with the recognition site in the cavity. If the substrate, especially the LP has less strong interactions with the binding site a transition to the outward-facing conformation (OFC) is less likely. This is especially the case for the FNLD-box mutant F-18A as well as the K-20E, D-19K, K-9E, K-8E and D-7K mutants. Additionally, a false interaction such as with other $LanA_{LP}$ hinders or reduces secretion by NisT/NisBTC (3.3).

Upon mNisA release ATP hydrolysis resets the transporter to the ground state and enables a new transport cycle, similarly to other ABC transporters (Husada, Bountra et al. 2018). Here, the secretion rate is not coupled to the ATPase rate but to the interaction with the lanthipeptide modification complex of $NisB_2C$ and the substrate mNisA (3.3). In the

channelling mechanism ATP hydrolysis might be needed for the reset of the transporter and does not initiate the translocation itself. However, a false initiation hampers the reset, which leads to a lower secretion level, as NisT is not transport competent.

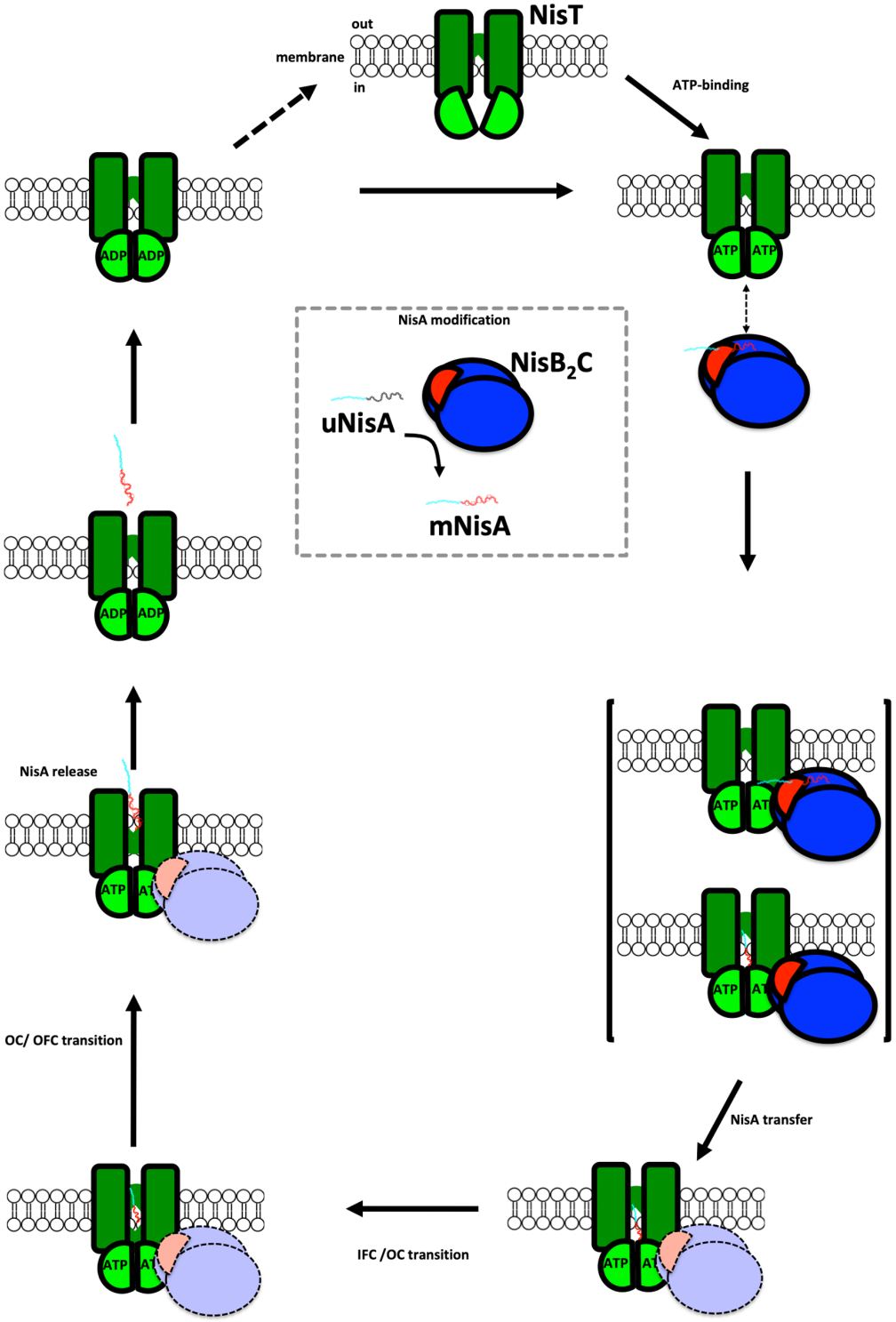

Figure 31: Transport cycle of NisT.
The scheme illustrates the secretion of mNisA by the modification and secretion complex. Colour code: NisB is displayed in blue, NisC in red and NisT in green (TMD dark green, NBD light green). The precursor peptide uNisA and mNisA are illustrated in black and red, respectively. There, the leader peptide is highlighted in cyan. The figure was taken from Chapter III.
IFC: inwards-facing conformation; OC: occluded state; OFC: outward-facing conformation

4.6 The finial maturation of NisA

After translocation, the LP of the modified lanthipeptide (e.g. mNisA) is cleaved by specific leader peptidases (LanP) with either endopeptidase or endo- and aminopeptidase activity (Schnell, Engelke et al. 1992, van der Meer, Polman et al. 1993, Dischinger, Josten et al. 2009, Ortega, Velasquez et al. 2014, Escano, Stauffer et al. 2015, Chen, Xu et al. 2019). The removal of the LP is often the finial maturation of a lanthipeptide yielding the mature lanthipeptide, after which lantibiotics display their antimicrobial activity (van der Meer, Polman et al. 1993).

The precursor of nisin (mNisA) is processed by the subtilisin-like serine peptidase NisP, which cleaves specifically after the cleavage site (GASPR) yielding the mature nisin and the LP (van der Meer, Polman et al. 1993, Plat, Kluskens et al. 2011, Montalban-Lopez, Deng et al. 2018). In addition, the processing of mNisA is independent from the translocation process and thereby the specific activity of NisP can be studied (Kuipers, de Boef et al. 2004). Although, the activity of many LanPs was investigated by *in vivo* and *in vitro* studies (van der Meer, Polman et al. 1993, Geissler, Götz et al. 1996, Plat, Kluskens et al. 2011, Ortega, Velasquez et al. 2014, Escano, Stauffer et al. 2015, Tang, Dong et al. 2015) only little is known about the kinetic parameter of the reaction (Ortega, Velasquez et al. 2014). Additionally, the substrate specificity remains elusive as a strong lanthionine ring dependence (Kuipers, de Boef et al. 2004, Nagao, Morinaga et al. 2009, Voller, Krawczyk et al. 2013) as well as an independence (Geissler, Götz et al. 1996, Ortega, Velasquez et al. 2014, Tang, Dong et al. 2015, Montalban-Lopez, Deng et al. 2018) of the cleavage reaction was observed.

4.7 Substrate specificity of NisP

In the case of NisP, the substrate specificity was investigated by employing various NisA substrates to the leader peptidase, which either had an altered cleavage site (CS) (Plat, Kluskens et al. 2011, Montalban-Lopez, Deng et al. 2018), different modification states (3.4) or altered MeLan/Lan ring pattern (3.4) (Montalban-Lopez, Deng et al. 2018).

The activity of NisP is generally strongly dependence on the proper cleavage site and any alternation of the aa properties of P1 to P4 (Schlechter-Berger nomenclature) hampers the cleavage reaction. Thus, the exchange of the CS to enterokinase CS (DDDK) (Montalban-Lopez, Deng et al. 2018) or the mutation of P1 or P4 abolished NisP activity (van der Meer,

Rollema et al. 1994). However, the exchange to the NisP auto-cleavage site (VSLR), trombine CS (AVPR) or Factor Xa CS (IEGR) results in the cleavage of precursor peptide (Montalban-Lopez, Deng et al. 2018). Noteworthy, the position P1´ seems also to be important as charged and bulky hydrophobic side chains influenced the cleavage efficiency of NisP (3.6). This is inline with a proposed substrate binding model of NisP, where the position P1´ and P2´ favour small hydrophobic amino acids (Siezen, Rollema et al. 1995).

The presence of MeLan/Lan rings are very important for an efficient LP cleavage by NisP, which is observed for substrates with at least one MeLan/Lan ring (e.g. the first lanthionine ring between aa position 3− 7) (3.4) (Montalban-Lopez, Deng et al. 2018). No difference of the cleavage reaction was observed with different ring mutants of NisA, except if all rings are missing (3.4). Thus, the MeLan/lan rings are important for the correct positioning and interaction of the precursor peptide with the substrate binding pocket. The low k_{cat} values and the increased K_m values of unmodified and dehydrated NisA in comparison to mNisA demonstrate an impaired cleavage reaction, where the catalytic efficiency of NisP is at least one magnitude higher with the fully modified NisA (2.5×10^6 $M^{-1}s^{-1}$) (3.4).

In summary, NisA recognition via the binding of the LP to the substrate binding pocket in NisP (S1−S4) contributes to a basal proteolytic activity. The presence of MeLan/Lan rings increases the turnover rates, decreases the apparent K_m value, which leads to a higher catalytic efficiency cleavage. The correct orientation of the CS (GASPR) in the active centre represents a rate-limiting step of the reaction, where the probability of cleavage is reduced when substrates lack MeLan/Lan rings. The leader peptidase NisP can be purified as a soluble variant (3.4) (Abts, Montalban-Lopez et al. 2013, Montalban-Lopez, Deng et al. 2018), which is a usable peptidase with a high specificity for MeLan/Lan ring containing peptides but with lower specificity for linear peptides. This emphasises the importance of NisP as a tool to cleave peptide pharmaceuticals, which contain thioether rings (Kluskens, Nelemans et al. 2009). Additionally, NisP cleavages after Arg and leaves no additional aa at the CS in comparison to the TEV protease (Parks, Leuther et al. 1994). Therefore, a potential biotechnological application for this peptidase is conceivable.

4.8 Conclusions and further perspectives

In the past decades, a huge progress was made to achieve a deeper understanding of the nisin modification, secretion and maturation. We have detailed knowledge about the modification mechanism, after which the PTMs are installed with high region- and stereospecificity (Li, Yu et al. 2006, Lubelski, Khusainov et al. 2009, Mavaro, Abts et al. 2011, Khusainov and Kuipers 2012, Abts, Montalban-Lopez et al. 2013, Garg, Salazar-Ocampo et al. 2013, Ortega, Hao et al. 2015, Repka, Hetrick et al. 2018). Furthermore, previous studies elucidate the importance of the interplay between NisT and the PTM enzymes for NisA secretion (Kuipers, de Boef et al. 2004, Kuipers, Meijer-Wierenga et al. 2008, van den Berg van Saparoea, Bakkes et al. 2008). However, we lack information's about the molecular mechanism of the secretion process and especially the nisin exporter NisT.

Hence, the present thesis set the foundation for the characterization of the secretion process on a molecular level. The first biochemical characterization of NisT *in vitro* provides a glance on mode of action of a lanthipeptide exporter. Additionally, the determined kinetic parameter of the secretion process with the apparent secretion rate of NisT with and without the PTM enzymes, support the proposed channelling mechanism of NisA translocation (van den Berg van Saparoea, Bakkes et al. 2008). Frist crystallization trials with bicelle reconstituted NisT gave promising results to obtain diffracting NisT crystals for X-ray crystallography. A high resolution crystal structure of NisT in different conformations (ATP/AMP-PNP-bound; with/without interaction partner) would allow to study the binding and translocation process of NisA. The importance of recognition site within the LP of NisA was shown in the present thesis by a quantitative secretion assay. Moreover, the conducted interaction study of NisT with the modification enzymes in the present thesis demonstrated, that NisT is interacting with NisB as well as with NisC. Thus, the interplay of NisT with the modification enzymes NisB and NisC could be studied in the future by structural biology methods such as small-angle X-ray scattering (SAXS) (Putnam, Hammel et al. 2007, Schindler, de Vries et al. 2016) or electron microscopy (3DEM) (Binshtein and Ohi 2015, Takizawa, Binshtein et al. 2017) to obtain structural information about the partial or the whole NisBTC complex. Noteworthy, the modification complex comprising of $NisB_2CA$ was already investigated by SAXS (Reiners, Abts et al. 2017). Thus, the major object in the future would be to study the secretion and modification complex *in vitro* to answer the open questions of the NisA transfer after modification and how the assembly and disassembly of the complex

affect the translocation process. In addition, an *in vitro* secretion assay of reconstituted NisT in a lipid bilayer (e.g. liposomes) would further enlighten, how the modification enzymes NisB and NisC enhance the transport rate of NisA and thereby the activity of NisT. Furthermore, this assay would answer the question, if the substrate specificity (e.g. uNisA, dNisA or mNisA) of NisT leads to an altered transport rate or if the enhancement is seldom depended on the modification enzymes.

The finial maturation of lanthipeptides is the cleavage of the LP by the dedicated leader peptidase (van der Meer, Polman et al. 1993, Furgerson Ihnken, Chatterjee et al. 2008, Dischinger, Josten et al. 2009, Ortega, Velasquez et al. 2014, Escano, Stauffer et al. 2015, Chen, Xu et al. 2019). Thus, it is prerequisite to yield a biological active lanthipeptide and a deeper knowledge of the reaction is important for future applications. The genome mining approaches (Agrawal, Khater et al. 2017, Blin, Medema et al. 2017, van Heel, de Jong et al. 2018) for secondary metabolites such as lanthipeptides proceed to increasing numbers of novel lanthipeptide with desired biological activity such as antimicrobial or antiviral activities. However, for these peptides, which are post-translational modified in a LP-dependent manner, the LP has to be removed by a leader peptidase with a specificity for a correct and efficient cleavage. The determination of kinetic parameter and the substrate specificity of NisP in the present thesis permit to increase the knowledge about the processing of NisA and LanAs in general. In the case of NisP a basal proteolytic activity even for peptides lacking the MeLan/Lan rings was observed. However, the cleavage efficiency is strongly increased by the presence of at least one MeLan/Lan ring. Therefore, potential pharmaceutical peptides with MeLan/Lan rings are highly processed by NisP.

The nisin system is a model system to study lanthipeptides and especially lantibiotics. Hence, any progress in understanding of the basic principles is important to facilitate the development of new applications. Exemplary are the discovery and screening of novel lanthipeptides or the study of protein-protein/ peptide-protein interaction via the nisin system (Montalban-Lopez, van Heel et al. 2017, Urban, Moosmeier et al. 2017, Hetrick, Walker et al. 2018, Schmitt, Montalban-Lopez et al. 2019). In respect of the obstacles of increasing resistances against antibiotics, the discovery and the screening of novel lantibiotics is greatly relevant to overcome the limited, highly potent antibiotics.

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A) Publications list

1) **Lagedroste, M.**, Reiners, J., Smits, S. H. J.; Schmitt, L. (2019). "Systematic characterization of position one variants within the lantibiotic nisin." Sci Rep **9**(1): 935.

2) **Lagedroste, M**.; Smits, S. H. J.; Schmitt, L. (2017). "Substrate Specificity of the Secreted Nisin Leader Peptidase NisP." Biochemistry.

3) Reiners, J.; **Lagedroste, M.**; Ehlen, K.; Leusch, S.; Zaschke-Kriesche, J.: Smits, S. H. J. (2017). "The N-terminal Region of Nisin Is Important for the BceAB-Type ABC Transporter NsrFP from Streptococcus agalactiae COH1." Front Microbiol **8**: 1643.

4) Khosa, S.; **Lagedroste, M.**; Smits, S. H. J. (2016). "Protein Defense Systems against the Lantibiotic Nisin: Function of the Immunity Protein NisI and the Resistance Protein NSR." Front Microbiol **7**: 504.

5) AlKhatib, Z.; **Lagedroste, M.**; Zaschke,J.; Wagner, M.; Abts, A.; Fey, I.; Kleinschrodt, D.; Smits, S. H. J. (2014). "The C-terminus of nisin is important for the ABC transporter NisFEG to confer immunity in Lactococcus lactis." Microbiologyopen **3**(5): 752-763.

6) AlKhatib, Z.; **Lagedroste, M.**; Fey, I.; Kleinschrodt, D.; Abts, A.; Smits, S. H. J. (2014). "Lantibiotic immunity: inhibition of nisin mediated pore formation by NisI." PLoS One **9**(7): e102246.

B) *Curriculum vitae*

Personal details

Name:	Marcel, Lagedroste
Date of birth:	06.10.1987 (Ochtrup)
Family status:	unmarried

Education: University

10.2013 – 09.2019	Doctorate at Institute of Biochemistry Heinrich-Heine-University Duesseldorf Supervisor: Prof. Dr. L. Schmitt Title:" Investigation on translocation and proteolytic processing of the lanthipeptide nisin"
10.2011 – 09.2013	Master of Science: Biochemistry (grade 1.1) Heinrich-Heine-University Duesseldorf Master thesis at Institute of Biochemistry, Heinrich-Heine-University Duesseldorf Supervisor: Prof. Dr. L. Schmitt Title: "Charakterisierung des Nisin Immunitätsenzyms NisI"
10.2008 – 09.2011	Bachelor of Science: Biochemistry (grade 1.6) Heinrich-Heine-University Duesseldorf Bachelor thesis at Institute of Molecular Physical Chemistry, Heinrich-Heine-University Duesseldorf Supervisor: Prof. Dr. C. A. M. Seidel Title: "Untersuchung der Dynamik und Struktur von Lin-7 aus *Drosophila melanogaster* mittels Fluoreszenzspektroskopie"

Civilian service

09.2007 – 06.2008	Caritaswerkstätten Langenhorst

Education: School

08.1998 – 06.2007	Städtisches Gymnasium Ochtrup Degree: university-entrance diploma; grade: 1.7

Conference attendances

03.2018	7th FEBS ABC Transporter conference short talk/ poster presentation
09.2017	Dynamics of Membrane Systems; CRC 1208
03.2017	VAAM Annual Meeting 2017 poster presentation
04.2016	10th Transporter Colloquium GBM poster presentation
09.2015	5th International Meeting on Antimicrobial Peptides poster presentation
03.2014	5th FEBS ABC Transporter conference poster presentation

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Wo das Stichwort Whiskey fällt, da bleibt es nicht aus Tobias Beer, mein Laborpartner aus Labor 29, zu huldigen. Ich danke dir für die gemeinsame Zeit am Institut und die vielen Stunden im Labor, bei denen wir uns über so manches hochpolitische Thema unterhalten haben, so dass viele aus dem Labor geflüchtet sind ;). Ich werde schmunzelnd an jede Flasche Milchpulverlösung zurückdenken, die ich, besonders in der letzten Phase meiner Laborzeit, im Kühlschrank mit einem letzten Schluck, aber noch ausreichendem Schluck (wie ich bemerken möchte), zurückgelassen habe. Ich habe dabei nur versucht, das Andenken an den „Kaiser“ hochzuhalten. Du bist und warst nicht nur im Labor, sondern auch bei allen anderen Gelegenheiten, jemand den man nicht missen möchte. Auch wenn du manchmal

etwas zu viel in Frage gestellst hast :D . Vielen Dank Tobi, mein Labor wird immer das Labor 29 bleiben!

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D) Declaration/Erklärung

Hiermit versichere ich an Eides Statt, dass die Dissertation von mir selbständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf“ erstellt worden ist. Darüber hinaus ist die Dissertation keiner anderen Fakultät vorgelegt worden, noch hat es einen weiteren erfloglosen oder erfolgreichen Promotionsversuch gegeben.

_____ _____

Ort, Datum Unterschrift