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Designing and Producing Modified, New-to-Nature Peptides with Antimicrobial Activity by Use of a Combination of Various Lantibiotic Modification Enzymes

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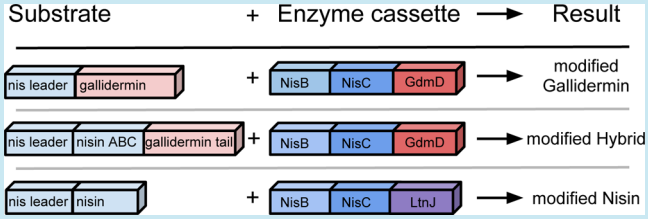
Supporting Information

ABSTRACT: Lanthipeptides are peptides that contain several post-translationally modified amino acid residues and commonly show considerable antimicrobial activity. After translation, the amino acid residues of these peptides are modified by a distinct set of modification enzymes. This process results in peptides containing one or more lanthionine rings and dehydrated Ser and Thr residues. Previously, an *in vivo* lanthipeptide production system based on the modification machinery of the model lantibiotic nisin was reported. Here, we present the addition of the modification enzymes LtnJ and GdmD to this production system. With these enzymes we can now produce lanthipeptides that contain D-alanines or a C-terminal aminovinyl-cysteine. We show experimentally that the decarboxylase GdmD is responsible for the C-terminal decarboxylation. Our results demonstrate that different lanthipeptide modification enzymes can work together in an *in vivo* production system. This yields a plug-and-play system that can be used to select different sets of modification enzymes to work on diverse, specifically designed substrates.



KEYWORDS: *lantibiotic modification, lanthipeptides, GdmD, LtnJ, oxidative decarboxylation, D-Ala, synthetic biology*

Lanthipeptides are peptides that, after ribosomal synthesis, undergo diverse post-translational modifications, leading to dehydrated Ser and Thr residues and (β-methyl) lanthionine formation. These modifications enable the biosynthesis of peptides harboring residues other than the 20 standard amino acids. Lanthipeptides can be subdivided in 4 different classes based on the enzyme(s) that catalyze(s) the reaction to form lanthionine residues.[1] The original term lantibiotic stands only for the two best studied subclasses (namely, I and II) that are well-known for their antimicrobial activity. Lantibiotics provide one of the potential solutions to fight the growing problem of multidrug-resistant pathogens.[2]

Nisin is the best studied lantibiotic to date.[3] It is produced by *Lactococcus lactis* as a precursor peptide containing a leader sequence involved in recognition by the modification enzymes (the dehydratase NisB and the cyclase NisC) and the specific transporter NisT. The mature active nisin molecule is released after translocation and cleavage of the modified precursor by the protease NisP and contains five (methyl)lanthionine rings and three dehydrated residues. The first two rings are important for binding to lipid II, and the last three rings are involved in the insertion into the membrane. After binding to lipid II, nisin undergoes a conformational change due to the so-called hinge region that results in the insertion of the C-terminal part into the membrane to create pores, in a 4 to 8 stoichiometry.[4]

Genes (potentially) involved in the production of lanthionine-containing peptides are commonly found in genomes of Gram-positive prokaryotes.[5−7] Next to the presence of genes encoding the modification enzyme(s) (generally termed *lanB* and *lanC* or *lanM*) responsible for the characteristic lanthionine biosynthesis, other modification enzymes are sometimes also encoded in these gene clusters. These additional modification enzymes have been shown to be responsible for diverse post-translational modifications of amino acid residues.[8] So far, only partial mechanistic information is available for the enzymes LtnJ and EpiD, which play a role in the biosynthesis of lacticin 3147 and epidermin, respectively. LtnJ is a reductase that stereospecifically converts dehydroalanine (Dha) into D-alanine (D-Ala) (see Figure 1) during the biosynthesis of the lantibiotic lacticin 3147.[9] The replacement of D-Ala by L-alanine or several other amino acids in lacticin 3147 caused reductions in both production and bioactivity, demonstrating that not only the presence but also the chirality of the amino acid may play a vital biological role in ribosomally synthesized peptides.[9] EpiD is an example of a flavoprotein that is, together with homologous enzymes, commonly indicated by the designation LanD. LanDs are responsible for the oxidative decarboxylation of the C-terminal cysteine to form aminovinyl-cysteine (see Figure 1). These extra modifications contribute to the specific activity and production levels of the target lantibiotic.[9,10]

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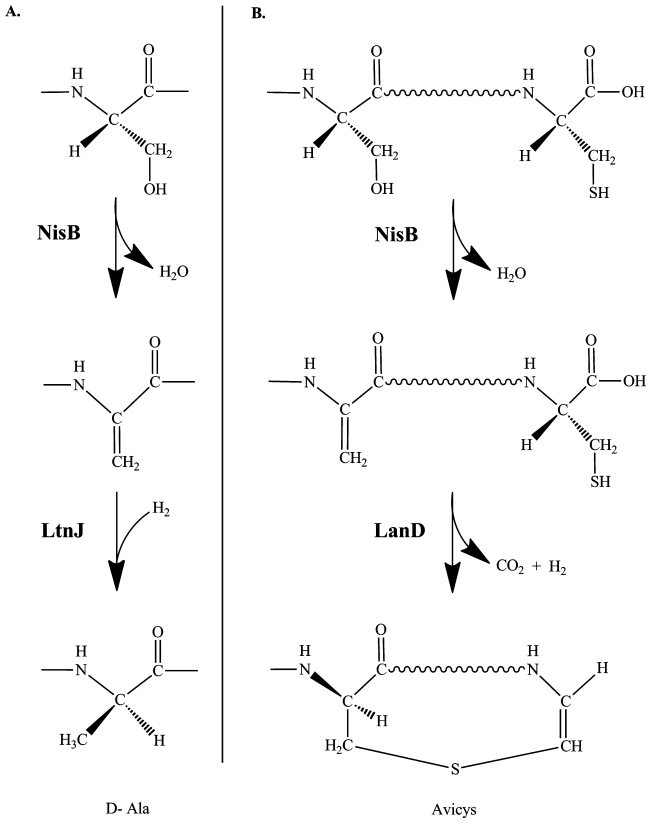

Figure 1. Biosynthesis pathways of D-Ala (A)[9] and aminovinyl-cysteine (B).[18] D-Ala synthesis starts with the dehydration of serine by NisB resulting in Dha. In a second step, the double bond is hydrogenated by LtnJ yielding the D-Ala residue. Aminovinyl-cysteine synthesis starts with the dehydration of serine by NisB resulting in Dha. In a second step GdmD carries out the decarboxylation of the last cysteine residue. Coupling of the thiol group to the double bond in Dha yields the *S*-[(*Z*)-2-aminovinyl]-3-(*S*)-D-cysteine.

It has been shown that the nisin dehydratase (NisB), transporter (NisT), and cyclase (NisC) can be expressed in *Lactococcus lactis* under the control of the nisin inducible promoter and are able to modify and transport diverse substrates fused to the nisin leader peptide both related and unrelated to lanthipeptides.[11−15] The broad substrate tolerance of these enzymes makes this a versatile system. This system has been used *in vivo* to improve medically relevant peptides.[13,14] Other lantibiotic modification systems have also been used for the production of lanthionine-containing peptides.[16] Moreover, the possibility of expressing and purifying type II modification enzymes (LanM) has enabled the *in vitro* modification of synthetic substrates even containing noncanonical amino acids.[17]

We aim to extend the *in vivo* production system containing the modification enzymes of nisin (NisBTC) with additional modification enzymes found in gene clusters of other lantibiotics, enabling us to probe the modularity of the lantibiotic biosynthesis machinery. In this way we will create a library of modification systems that can be used *in vivo* in a modular way to modify a diverse set of substrates (see Figure 2). Here, we report the heterologous expression in *L. lactis*, containing the enzymes NisBTC, of the enzymes LtnJ and GdmD in order to hypermodify and transport diverse peptide substrates harboring several modifiable residues and infer the substrate requirements for those enzymes. Ultimately, we aim to use this system to design and produce new-to-nature molecules with potentially improved antibiotic activity and/or novel physicochemical properties.

RESULTS AND DISCUSSION

Functional Expression of GdmD. Previously NisB and NisC have been successfully used to synthesize novel lanthionine containing peptides in *L. lactis*. To expand the possibilities of this system, we decided to add the C-terminal

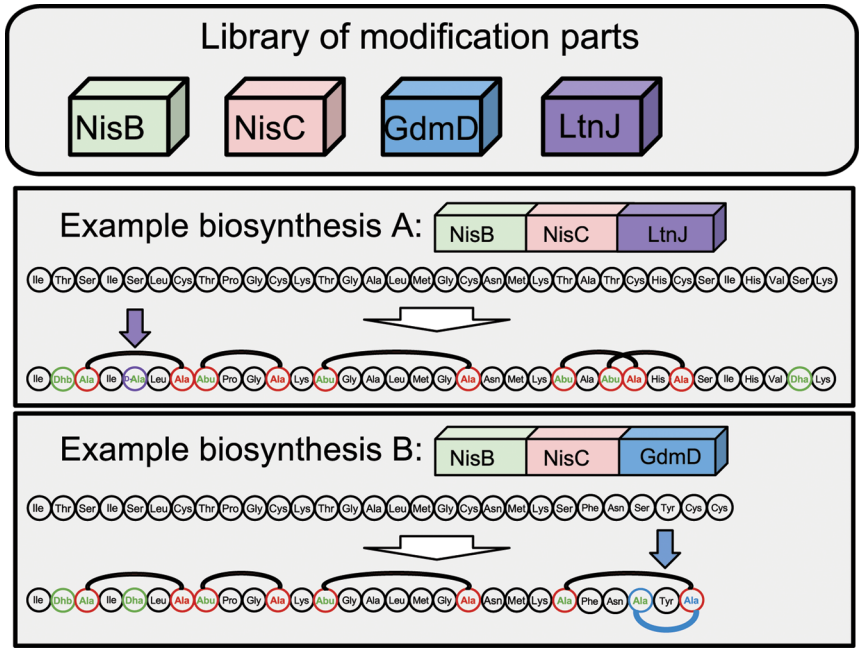

Figure 2. Graphical representation of the library of modification enzymes with two examples of their use. The enzymes can be combined in a modular way to create engineered peptides with modified amino acids. Example A shows the production of nisin with D-Ala at position 5. Example B shows the production of the hybrid lantibiotic NisA-(Δ23-34)-SFNSYCC with a C-terminal aminovinyl-cysteine.

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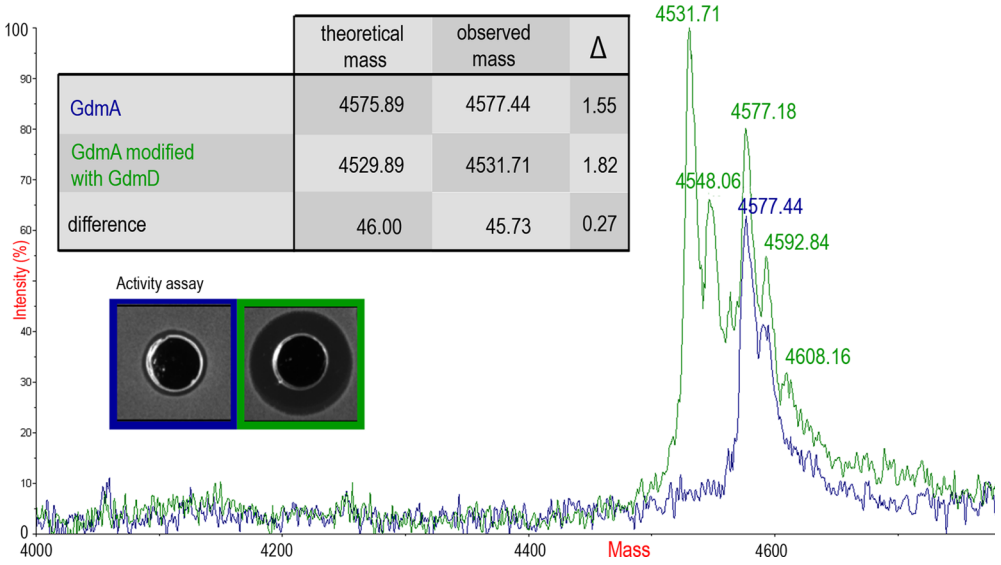

Figure 3. MS spectrum and activity assay comparing leaderless gallidermin fused to the nisin leader produced with (green) and without (blue) GdmD. TCA-precipitated supernatant of an induced culture of *L. lactis* NZ9000 harboring the plasmids pNZE-gdmA-gdmD and pIL3BTC (green) or pNZE-gdmA and pIL3BTC (blue). A clear mass shift of approximately 46 Da is visible, indicating that GdmD has partially modified the substrate as also peaks are visible corresponding to the unmodified substrate. The activity assay displays growth inhibition of *M. flavus* after *in vitro* cleavage of the leader peptide.

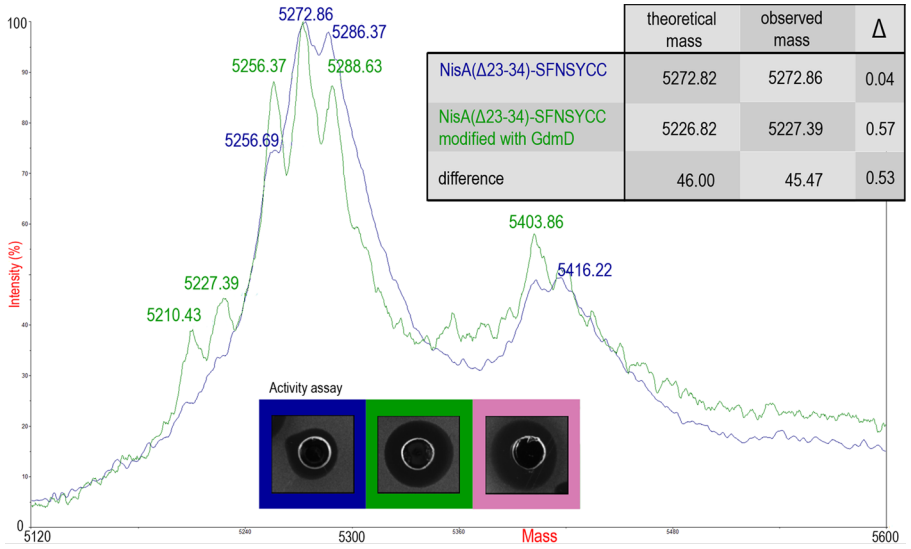

Figure 4. MS spectrum and activity assay comparing NisA(Δ23-34)-SFNSYCC produced with (green) and without (blue) GdmD. TCA-precipitated supernatant of an induced culture of *L. lactis* NZ9000 harboring the plasmids pNZE-NisA(Δ23-34)-SFNSYCC-gdmD and pIL3BTC (green) or pNZE-NisA(Δ23-34)-SFNSYCC and pIL3BTC (blue) were analyzed. A clear mass shift of approximately 46 Da is visible indicating that gdmD has partially modified the substrate. Peaks corresponding to the unmodified substrate are also visible. The activity assay displays growth inhibition of *M. flavus* after *in vitro* cleavage of the leader peptide (blue = NisA(Δ23-34)-SFNSYCC; green = NisA(Δ23-34)-SFNSYCC modified with GdmD; pink = NisA(Δ23-34)).

decarboxylase activity of GdmD from *Staphylococcus gallinarum* Tü3928.[19] GdmD consists of 181 amino acids that share 87% sequence homology with the 181 amino acids of the well studied decarboxylase EpiD. Kupke et al. showed that purified EpiD possesses a relatively broad substrate specificity in *in vitro* experiments.[20] Furthermore a mutation in the *epiD* gene resulted in loss of activity, which could be restored by introducing a plasmid containing just the *epiD* gene, thus showing that *epiD* is essential for the production of active epidermin.[21] The addition of this LanD modification module to an *in vivo* lanthipeptide production system based on the nisin modification machinery allows for increased structural and functional variability.

First we checked whether the enzyme was able to modify its native substrate gallidermin (encoded by *gdmA*) in which the original leader was replaced by the nisin leader (Supplementary Table 2). We investigated this with *Micrococcus flavus* growth inhibition assays and with MS (MALDI-TOF). *L. lactis* NZ9000 harboring pIL3BTC and pNZE-gdmA-gdmD yielded a gallidermin peptide of the expected mass corresponding to 5 dehydrations and decarboxylation of the C-terminus (see green graph in Figure 3). An additional peak is observed, corresponding to fully dehydrated peptide without the C-

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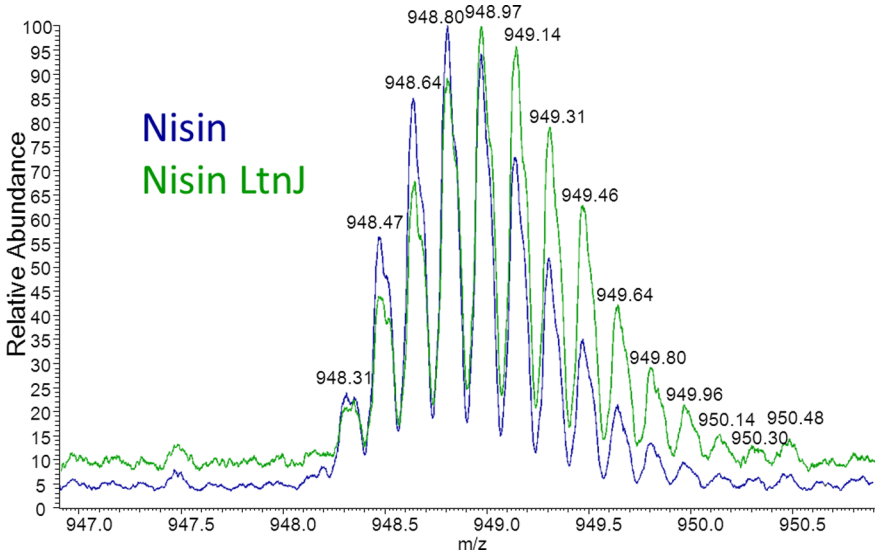

Figure 5. Isotopic ESI-MS spectrum comparing the 6 times charged prenisin produced without (blue) and with (green) LtnJ. Mass spectra were recorded during 7 min. Prenisin was isolated from the supernatant of an induced culture of *L. lactis* NZ9000 harboring the plasmids pNZ-nisA and pIL3EryBTC (blue) or pNZ-nisA-ltnJ and pIL3EryBTC (green) using cation exchange chromatography. The difference in relative abundance of the isotope peaks strongly indicates that the LtnJ-modified peptide has an additional mass of more than 1 Da when compared to wild-type nisin. The fact that both spectra show the same first peak (of 948.31) indicates that the biosynthesis with LtnJ yields a mix of nisin with and without D-Ala.

terminal decarboxylation. Next to these peaks, oxidized versions are observed, corresponding to the expected masses with an additional oxygen. The strain NZ9000 harboring pIL3BTC and pNZE-gdmA, which does not contain a decarboxylase, only produced the fully dehydrated fusion peptide without the decarboxylated C-terminus (see blue graph in Figure 3). After cleavage of the nisin leader peptide using trypsin the active leaderless peptide was released and used to investigate growth inhibition of *M. flavus*. Only the sample containing the decarboxylated peptide showed significant inhibition of growth (see Figure 3). The observed growth inhibition suggests that NisB and NisC were able to insert the wild-type ring pattern into gallidermin precursor peptide fused to the nisin leader. This could be expected since GdmBC and NisBC belong to the same evolutionary group.[22] These results prove experimentally what has been inferred on the basis of homology before, namely, that GdmD is a decarboxylase that can modify GdmA. The inhibition assays demonstrate that C-terminal decarboxylation is essential for activity of gallidermin against *M. flavus*. Previously it was shown that in the wild-type producer mutations in the C-terminal part of gallidermin that prevent aminovinyl-cysteine formation abolished the production or secretion of gallidermin.[23] However, in our system secretion of non-decarboxylated gallidermin takes place.

Next we repeated the experiment using a designed hybrid peptide (NisA(Δ23-34)-SFNSYCC) as substrate for GdmD. The peptide consists of the first three lanthionine rings of nisin and its so-called hinge region (NMK) followed by the C-terminal motive of gallidermin (SFNSYCC). From the MS results in Figure 4 we can conclude that the substrate is fully modified by NisBTC and partly by GdmD. In the activity assay we test the peptide with (NisA(Δ23-34)-SFNSYCC) and without (NisA-(Δ23-34)) the C-terminal gallidermin motive. The hybrid peptide is active with and without GdmD. This shows that the additional tail of SFNSYCC is not abolishing the activity of the first part of nisin (NisA(Δ23-34)), which is active by itself (pink square in Figure 4 and ref 14). Although the test is only qualitative, the radius of the growth inhibition zone suggests that the decarboxylated tail improves the activity when compared to the non-decarboxylated tail.

In this study we used a modified nisin leader (Supplementary Table 2). Our results appear to indicate that the C-terminal decarboxylation by GdmD occurs independently of the leader sequence that is used. This is in line with *in vitro* experiments performed previously that showed that EpiD can modify leaderless epidermin.[24] An interesting question that arises from these results is how the export of gallidermin is regulated. In the *in vivo* system employed here we observed (with MS) the export of not fully modified gallidermin and hybrid peptide (NisA-(Δ23-34)-SFNSYCC). Unfortunately, we could not find any literature describing the presence of non-decarboxylated peptides in the native system. In the native system this is not expected to happen as this would be a waste of resources. Since multiple modifications have to take place in the correct order it would be interesting to identify the factor(s) ensuring complete biosynthesis and transport in the wild-type system.

Functional Expression of LtnJ. The presence of D-amino acids can lead to increased stability and improved biological properties of biologically active peptides.[25,26] LtnJ is a reductase that stereospecifically converts Dha into D-Ala during the biosynthesis of lacticin 3147, a two-component class II lantibiotic. Thus, LtnJ is an interesting enzyme to add to the *in vivo* lanthipeptide production system.[14] First we decided to probe its specificity with the model lantibiotic nisin. In nisin there are two Dha residues (positions 5 and 33 in the propeptide) that could serve as a substrate for LtnJ. The MALDI-TOF data of the peptides purified from NZ9000 (pIL3EryBTC, pNZ-nisA-ltnJ) revealed a positive mass difference of approximately ∼1.8 Da when compared to nisin produced by NZ9000 (pIL3EryBTC, pNZ-nisA) (see Supplementary Figure 1). This observed mass difference was confirmed in three independent experiments. If the biosynthesis would have yielded fully converted Dha, we would expect to see a difference of 2 or 4 Da. Therefore we speculate that the biosynthesis produced a mix of nisin molecules containing Dha and D-Ala. To confirm this we performed ESI-MS (see Figure 5

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Table 1. Modification of Prenisin Mutants by LtnJ, Analysed by MALDI-ToF

name	dehydrations	hydrogenation	theor value	obsd value	obsd mass diff
NisA	8	0	5687.31	5687.52	1.87
NisA-LtnJ	8	1	5689.31	5689.39	
NisA(Δ23-34)	5	0	4472.87	4474.35	1.59
NisA(Δ23-34)-LtnJ	5	1	4474.87	4475.94	

Table 2. Strains and Vectors Used in This Work[a]

	characteristic	purpose	refs
		Strains	
L. lactis NZ9000	*pepN::nisRK*	expression host and sensitive strain	33
L. lactis MG1363	pMRC01	lacticin 3147 producing strain	9
S. gallinarum Tü3928	gallidermin producing strain	GdmD source	19
Micrococcus flavus B423		sensitive strain	NIZO food research
		Vectors	
pNZE-empty	EryR	expression vector	this work
pNZ8048	CmR	expression vector	34
pNZnisA-E3	EryR *nisA*	nisin expression	35
pIL3BTC	*nisBTC* under the control of P*nisA* CmR	modification and transport of lantibiotics	14
pNZE-gdmD	EryR, *gdmD*	expression of GdmD	this work
pNZE-gdmA-gdmD	EryR, nisinleader-*gdmA*, *gdmD*	expression of leaderless gallidermin fused to the nisin leader and GdmD	this work
pNZE-nisA(Δ23-34)-SFNSYCC	EryR, *nisA*(Δ23-34)-SFNSYCC	expression of hybrid peptide	this work
pNZE-nisA(Δ23-34)-SFNSYCC-gdmD	EryR, *nisA*(Δ23-34)-SFNSYCC	expression of hybrid peptide with GdmD	this work
pMRC01	lacticin 3147 gene cluster	used for cloning of *ltnJ*	9
pNZ-nisA	CmR, *nisA*	expression of nisin	this work
pIL3EryBTC	EryR, *nisBTC*	modification and transport of lantibiotics	this work
pORI28	EryR	used for cloning	36
pNZ-nisA-ltnJ	CmR, *nisA*, *ltnJ*	expression of nisin and LtnJ	this work
pNZ-nisA(Δ23-34)-ltnJ	CmR, *nisA*(Δ23-34), *ltnJ*	expression of truncated nisin and LtnJ	this work
pNZ-nisA-Cless-ltnJ	CmR, *nisA*-Cless, *ltnJ*	expression of nisin without Cys and LtnJ	this work
pNZE-nisA(Δ23-34)	EryR, *nisA*(Δ23-34)	expression of truncated nisin	37
pNZ-nisA(Δ23-34)	CmR, *nisA*(Δ23-34)	expression of truncated nisin	this work
pNZ-nisA-Cless	CmR, *nisA*-Cless	expression of nisin without Cys	this work
pNZE-nisA-Cless	EryR, *nisA*-Cless	expression of nisin without Cys	Manuel Montalbán-López unpublished

[a]CmR: chloramphenicol resistance. EryR: erythromycin resistance.

and Supplementary Figure 4) focusing on the 6 times charged ion. The relative abundance of the isotope peaks of nisin produced with (green graph) and without (blue graph) LtnJ is clearly different confirming the mass difference observed with MALDI-ToF. The fact that both spectra show the same first peak (of 948.31) indicates that indeed the biosynthesis with LtnJ yields a mix of nisin with and without D-Ala.

As an additional confirmation we decided to use a nisin mutant in which all cysteines were replaced by alanines (NisA-Cless), so that it possesses 4 Dha residues after modification by NisB. NZ9000 (pIL3EryBTC, pNZ-nisA-Cless-ltnJ) produces a fully dehydrated peptide with a positive mass difference of 4 Da compared to the control lacking LtnJ (see Supplementary Figure 2). The data strongly supports conversion of Dha to D-Ala by LtnJ in NisA-Cless. Considering all the residues flanking D-Ala in lacticin 3147(Phe, Leu, Ile, and Ala) and lactocin S (Ala, Val, and Leu) are hydrophobic,[27] it is likely that Dha33, of which one flanking residue is lysine, is the residue escaping the conversion of Dha into D-Ala in both mutants. To confirm this we repeated the experiment with pNZ-nisA(Δ23-34) and pNZ-nisA(Δ23-34)-ltnJ that lack Dha33. The peptide produced by NZ9000 (pIL3EryBTC, pNZ-nisA(Δ23-34)-ltnJ) has a positive mass shift compared to the one produced by NZ9000 (pIL3EryBTC, pNZ-nisA(Δ23-34)) (see Table 1 and Supplementary Figure 3). The difference observed in this experiment is very similar to the difference observed for the full length nisin, suggesting that Dha5 (present in both substrates) rather than Dha33 (only present in full length nisin) was modified by LtnJ.

LtnJ is the only known hydrogenase involved in the stereospecific formation of D-Ala during the biosynthesis of a lantibiotic (Lacticin 3147).[9] The enzyme responsible for this reaction during Lactocin S biosynthesis has not been detected yet, and no protein in the gene cluster has significant homology with LtnJ.[9] In the native system, the leader peptides of LtnA1 and LtnA2 do not share high similarity, therefore hinting at a leader-independent interaction of LtnJ with its substrate, similar to EpiD[24] and GdmD. In this study, LtnJ was found to catalyze the D-Ala formation in prenisin, prenisin-Cless, and nisin-(Δ23-34), indicating that the leader of LtnA1 or LtnA2 is not required for the substrate recognition by LtnJ and showing broad substrate tolerance. In line with our results, it has been reported that PenN (40.4% identity with LtnJ)[28] can also modify both LtnA1 and LtnA2 with their original leader

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peptide attached.[29] To our knowledge, this is the first report demonstrating that LtnJ can modify peptides other than lacticin 3147. Moreover, we show that LtnJ can be combined with a dehydratase other than the original LtnM enzyme.

The modifications of one of the two Dha's in nisin and the only Dha in nisin-(Δ23-34) indicate that Dha33 was most likely not modified by LtnJ. This may be caused by the positive charge of the C-flanking Lys, although further investigation is required to confirm this hypothesis. The modification on Ser5 of nisin also indicates that the neighboring lanthionine ring has no effect on the function of LtnJ even though in lacticin 3147 and lactocin S the D-Ala residues are located only in the linear part of the peptides. Moreover, the nisin-Cless peptide demonstrates that lanthionine bridges are not necessary for substrate recognition.

Overall, these results show that it is possible to extend the *in vivo* lanthipeptide production system consisting of NisBTC with the enzymes LtnJ and GdmD, enabling the introduction of D-alanines and aminovinyl-cysteines into lanthipeptides. With this extensive range of post-translational modifications it is now possible, *in vivo*, to generate peptides with greater diversity.

METHODS

Bacterial Strains, Plasmids, and Growth Conditions. Strains and vectors used in this work are given in Table 2. *L. lactis* strains were cultured in M17 medium supplemented with 0.5% glucose at 30 °C for genetic manipulation or a minimal expression medium (MEM) for protein expression and purification assays.[14] *Micrococcus flavus* was grown in LB medium with shaking at 30 °C. Chloramphenicol and/or erythromycin were used at 5 μg/mL when necessary.

Molecular Cloning. Molecular cloning techniques were performed according to Sambrook and Russell.[30] Preparation of competent cells and transformation was performed as described previously.[31] Fast digest restriction enzymes and ligase were supplied by Fermentas and used according to the manufacturer.

Construction of Expression Vectors. *ltnJ* gene was amplified from pMRC01 using the primers P-ltnj-for (5′ *Hin*dIII) and P-ltnj-rev (5′ *Xho*I). After amplification and digestion using *Hin*dIII and *Xho*I, it was ligated into pNZ-nisA digested with the same enzymes, resulting in pNZ-nisA-ltnJ. The plasmid was transformed into NZ9000, isolated, and sequenced to check the integrity of the sequence. pNZ-nisA-Cless and pNZ-nisA-Cless-ltnJ were constructed from pNZE-nisA-Cless (Khusainov and Montalbán-López, unpublished data) by digestion with *Bgl*II and *Hin*dIII and were ligated into pNZ-nisA and pNZ-nisA-ltnJ digested with the same restriction enzymes, respectively. For the construction of the mutants lacking the C-terminus of nisin pNZ-nisA(Δ23-34) and pNZ-nisA(Δ23-34)-ltnJ, we used the primers P-for-(Δ23-34)/P-rev-(Δ23-34) and P-for-(Δ23-34)-ltnJ/P-rev-(Δ23-34) with pNZ-nisA and pNZ-nisA-ltnJ as the template, respectively.

Similarly, *gdmA* (without its leader sequence) and *gdmD* were amplified from genomic DNA from *S. gallinarum* Tü3928 with primers (see Supplementary Table 1) and cloned between *Eag*I and *Hin*dIII (for *gdmA*) or *Hin*dIII and *Sty*I (for *gdmD*) of pNZE-empty, generating pNZE-gdmA and pNZE-gdmD.

pIL3EryBTC was constructed by replacing the chloramphenicol resistance gene in pIL3BTC by the erythromycin resistance gene from pORI28. For this purpose, both vectors were cleaved with *Ava*I and *Stu*I.

pNZE-empty was created by round PCR using pNZE-nisA as template. The primers (see Supplementary Table 1) were designed to replace the sequence between the restriction sites *Bgl*II and *Hin*dIII to facilitate future cloning.

pNZnisA-E3 was digested with *Bgl*II and *Hin*dIII, and the fragment containing *nisA* was then ligated into the backbone of pNZ8048 cut with the same enzymes to yield pNZ-nisA.

Protein Expression and Purification. The vectors containing the structural gene and the additional modification enzyme, either derived from pNZE-empty or pNZ8048, were transformed into NZ9000 (pIL3BTC) or NZ9000 (pIL3EryBTC), respectively. MEM medium was inoculated at 2% from an overnight culture of the producer strain grown in GM17. When the fresh culture reached an OD(600 nm) of 0.4−0.6, nisin was added at a final concentration of 5 ng/mL. Cells were harvested after 2 h of induction by centrifugation at 4 °C for 10 min at 5000 rpm, and the supernatant was kept for the isolation of the peptides. To concentrate protein from supernatant, trichloroacetic acid (TCA) precipitation was performed according to Sambrook et al.[30] Alternatively, when higher amounts of prepeptide were required, larger volumes of supernatant were concentrated by fast flow chromatography. Cell-free supernatant was mixed 1:1 with a 100 mM lactic acid solution and applied to a 5 mL HiTrap SP-Sepharose (GE Healthcare) column for cation exchange chromatography. Peptides were washed with 50 mM lactic acid and eluted with 50 mM lactic acid, 1 M NaCl.[32] Subsequently a PD-10 Desalting Column (GE Healthcare) was used to desalt the sample. The desalted peptides were freeze-dried.

Mass Spectrometry. *MALDI-TOF.* A 1-μL sample of TCA-precipitated supernatant was spotted, dried, and subsequently washed with Milli-Q water on the target. Subsequently, 1 μL of matrix solution (5 mg/mL α-cyano-4-hydroxycinnamic acid from Sigma-Aldrich dissolved in 50% acetonitrile and 0.1% trifluoroacetic acid) was spotted on top of the washed sample. A Voyager DE PRO matrix-assisted laser desorption ionization−time-of-flight (MALDI-TOF) mass spectrometer (Applied Biosystems) was used to obtain mass spectra. Data were analyzed with "Data Explorer" software version 4.0.0.0 (Applied Biosystems).

ESI-MS. A 1-mg freeze-dried sample was solved in 100 μL of 70% acetonitrile and 0.1% formic acid. Before being applied on ESI-MS the solution was diluted 100 times by Milli-Q water. Mass spectra were measured on a TSQ Quantum AM (ThermoFinnigan) triple quadrupole mass spectrometer using an electrospray source in positive ion mode. The quadrupole resolution was set at 0.01, and a mass range m/z 946.9−950.9 was measured. Averaged spectra were smoothed with a boxcar of 15 points.

Antimicrobial Activity Assay. A culture of *M. flavus* or *L. lactis* NZ9000 OD(600 nm) = 0.5 was added at 1% (v/v) into melted LB-agar at 45 °C and poured in plates. Once the agar was solid, wells of 8 mm were created and filled with 50 μL of the lantibiotic solution. Lantibiotics were activated with 1 μL of 1 mg/mL trypsin stock solution added directly to the well.

ASSOCIATED CONTENT

Supporting Information

Two tables with the primers and peptide substrates used for this study. Two figures with MALDI-TOF spectra of prenisin and truncated prenisin with or without LtnJ. Two figures describing ESI-MS of nisin and NisA-Cless. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

†Shared first authors. A.J.v.H., M.M.-L., and O.P.K. conceived the study and designed the experiments. A.J.v.H., D.M., D.H., and M.M.-L. performed the experiments. A.J.v.H., M.M.-L., D.M., and O.P.K. analyzed data and wrote and corrected the manuscript.

Notes

The authors declare no competing financial interest.

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