Homologues and Bioengineered Derivatives of LtnJ Vary in Ability to Form D-Alanine in the Lantibiotic Lactacin 3147

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Ltn α and Ltn β are individual components of the two-peptide lantibiotic lactacin 3147 and are unusual in that, although ribosomally synthesized, they contain D-amino acids. These result from the dehydration of L-serine to dehydroalanine by LtnM and subsequent stereospecific hydrogenation to D-alanine by LtnJ. Homologues of LtnJ are rare but have been identified *in silico* in *Staphylococcus aureus* C55 (SacJ), *Pediococcus pentosaceus* FBB61 (PenN), and *Nostoc punctiforme* PCC73102 (NpnJ), previously called NpunJ [P. D. Cotter et al., Proc. Natl. Acad. Sci. U. S. A. 102:18584–18589, 2005]. Here, the ability of these enzymes to catalyze D-alanine formation in the lactacin 3147 system was assessed through heterologous enzyme production in a Δ LtnJ mutant. PenN successfully incorporated D-alanines in both peptides, and SacJ modified Ltn α only, while NpnJ was unable to modify either peptide. Site-directed mutagenesis was also employed to identify residues of key importance in LtnJ. The most surprising outcome from these investigations was the generation of peptides by specific LtnJ mutants which exhibited less bioactivity than those generated by the Δ LtnJ strain. We have established that the reduced activity of these peptides is due to the inability of the associated LtnJ enzymes to generate D-alanine residues in a stereospecific manner, resulting in the presence of both D- and L-alanines at the relevant locations in the lactacin 3147 peptides.

The presence of D-amino acids in ribosomally synthesized peptides is extremely rare, but examples of this phenomenon have been reported in peptides produced by microorganisms, invertebrates, and animals (8, 14, 16, 27). This includes the presence of D-alanines in ω -agatoxin IVb, which is produced by the funnel web spider (12), as well as other D-amino acids in peptides isolated from frog skin secretions, such as dermorphin (22) and a variety of bombinins (13, 17, 28). In general, these D-amino acids result as a consequence of the racemization of the corresponding L-forms. Three examples of D-alanine formation have been reported in microbially produced peptides, and all belong to the lantibiotic class of bacteriocins (antimicrobial peptides). These are lactocin S produced by *Lactobacillus sake* L45 which contains three D-alanines (29), and Ltn α and Ltn β , the individual components of the two-peptide lantibiotic lactacin 3147 originally produced by *Lactococcus lactis* DPC3147. Ltn α and Ltn β possess one and two D-alanines, respectively (6, 24) (Fig. 1). Although these peptides each exhibit antimicrobial activity, this activity is greatly enhanced when the peptides are combined. The mechanism by which D-alanines are generated in lantibiotics is unique and involves the conversion of a ribosomally introduced L-serine to a D-alanine, thus changing both the side group and chirality of the original residue. When this phenomenon was first noted in lactocin S, it was postulated that a conversion of L-serine to D-alanine occurred via a two-step process of α -carbon stereoinversion (29). The first step involves the dehydration of serine to dehydroalanine (Dha), a common occurrence during lantibiotic biosynthesis which, for the majority of lantibiotics, is catalyzed by a LanB or LanM enzyme. The second step predicted the stereospecific hydrogenation of the dehydrated residue by an as yet unidentified enzyme to form D-alanine. In the case of lactacin 3147 (Fig. 2), *in silico* analysis of *ltnJ* in the associated biosynthetic operon predicted a protein with significant similarity to zinc-dependent alcohol dehydrogenases and NAD(P)H-dependent quinone oxidoreductases of the zinc-containing alcohol dehydrogenase superfamily. Significantly, de-

letion of *ltnJ* resulted in the production of peptides of a mass consistent with the presence of Dha rather than D-alanine residues at the relevant locations. In *trans* complementation with *ltnJ* restored the ability to generate D-alanines (6).

The presence of D-amino acids in natural ribosomally synthesized peptides is significant. In the case of dermorphin, bombinin H, and ω -agatoxin IVb and IVc, these natural forms are more active than derivatives in which the D-amino acids have been replaced with their L-form counterparts (3, 12, 17). The greater activity of the D-amino acid-containing forms is likely due to differences with respect to peptide folding, enhanced resistance to proteases, and/or *in vivo* stability, while reduced cytotoxicity is also a common feature (11, 12, 16, 17, 21, 26, 30). In the case of the lactacin 3147 peptides, the consequences of replacing the D-alanines are variable and appear dependent on the chirality of the newly incorporated residue. More specifically, nonchiral amino acids such as glycine, dehydrobutyrine, and dehydroalanine are better tolerated than L-amino acids such as L-alanine and L-valine (6).

While lactacin 3147 and lactocin S are the only ribosomally synthesized prokaryotic peptides known to possess the D-amino acids, a number of *ltnJ* homologues have been identified in the genomes of *Staphylococcus aureus* C55 (SacJ), *Pediococcus pentosaceus* FBB61 (PenN), and *Nostoc punctiforme* PCC73102 (NpnJ), previously called NpunJ by Cotter et al. [6]). Here, we use an *trans* complementation strategy to determine which *ltnJ* homo-

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FIG 1 D-Alanine-containing lantibiotics, i.e., lactacin 3147 (Ltn α and Ltn β) and lactocin S. Ala-S-Ala (lanthionine) is shown in light gray, Abu-S-Ala (β -methyl lanthionine) is in dark gray. Other residues are as labeled.

logues can successfully complement a $\Delta ltnJ$ mutation in the lactacin 3147 gene cluster and to assess the impact of site-directed changes on LtnJ activity.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. All strains and plasmids used in this study are presented in Table S1 in the supplemental material. *L. lactis* strains were grown without aeration at 30°C in M17 broth supplemented with 0.5% glucose. The intermediate host Top10 *Escherichia coli* was grown with aeration at 37°C in Luria-Bertani (LB) broth. Antibiotics were used at the following concentrations: 10 μ g/ml of chloramphenicol (Cm) for *E. coli* and 5 μ g/ml of Cm for *L. lactis*. X-Gal (5-bromo-4-chloro-3-indole- β -D-galactopyranoside) at a concentration of 50 μ g/ml was used.

Heterologous expression of genes encoding LtnJ homologues. The genes encoding SacJ, PenN and NpnJ were amplified from *S. aureus* C55, *P. pentosaceus* FBB61, and *N. punctiforme* PCC73102, respectively, with the relevant cloneFor/cloneRev primers (see Table S2 in the supplemental material), ligated with pNZ44 (18) digested with the relevant restriction endonucleases (Table S1), and introduced into *E. coli* Top10. Following DNA sequencing to ensure the absence of errors, the pNZ44 derivatives were isolated and introduced into MG1363/pMRC01 $\Delta ltnJ$ (19).

SDM of *ltnJ*. Specific changes to the pNZ44-*ltnJ* construct in *E. coli* were generated through use of a QuickChange site-directed mutagenesis (SDM) strategy (Stratagene) as employed previously (5, 6) with the exception that the Top10 *E. coli* was used as the intermediate host. *E. coli* bacteria putatively containing the mutated pNZ44-*ltnJ* derivatives were selected as colonies on LB-Cm plates and further assessed by PCR analysis, which required the use of one check PCR primer designed specifically to anneal to the newly incorporated codon in each case. These and other oligonucleotides employed in this study are listed in Table S1 in the supplemental material. DNA sequencing was finally employed to ensure that the correct changes had been incorporated and that no other untargeted mutations had occurred. The engineered plasmids were then isolated and introduced into MG1363/pMRC01 $\Delta ltnJ$.

Peptide production, purification, and mass analysis. An overnight culture of the lactacin 3147 producer (or derivative thereof) was inoculated into 1 liter of modified TY (tryptone, yeast extract) broth (1% inoculum) and incubated overnight at 30°C, and the cells were harvested by centrifugation (7,000 $\times g$ for 20 min) and resuspended in 250 ml of 70% propan-2-ol, pH 2.0 (adjusted to pH 2.0 by addition of HCl). The preparation was stirred for 4 h at 4°C, the cell debris was removed by centrifugation, and the bacteriocin-containing supernatant was reduced to approximately 60 ml by removing propan-2-ol via rotary evaporation. The

FIG 2 Depiction of the lacticin 3147 biosynthetic genes and the contribution of the associated products to conversion of L-serine to D-alanine.

resultant preparation was applied to a 10-g (60-ml volume) Varian C₁₈ Bond Elute Column (Varian, Harbor City, CA) preequilibrated with methanol and water. The column was subsequently washed with 120 ml of 30% ethanol followed by elution with 100 ml of 70% propan-2-ol, pH 2. Ten- and 20-ml volumes of the 100-ml elute were reduced to 2 ml by rotary evaporation, and aliquots of 1,750 μ l were then applied to a Phenomenex (Phenomenex, Cheshire, United Kingdom) C₁₂ reverse-phase high-performance liquid chromatography (RP-HPLC) column (Jupiter 4u Proteo; 90 Å, 250 by 10.0 mm; 4- μ m particle size) previously equilibrated with 25% propan-2-ol–0.1% trifluoroacetic acid (TFA), and this step was repeated until all of the 100-ml elute was concentrated. The column was subsequently developed in a gradient of 30% propan-2-ol containing 0.1% TFA to 60% propan-2-ol containing 0.1% TFA from 4 to 40 min at a flow rate of 1.2 ml/min. HPLC fractions were collected at peaks corresponding to Ltn α and Ltn β from each run; they were pooled together after all the HPLC runs, rotary evaporated to reduce the propan-2-ol content in the HPLC fractions, and then treated with N₂ gas and kept frozen at –80°C. These frozen samples were freeze-dried to get a powder form of the peptides to be used for quantified antimicrobial assays and mass spectrometry (MS) analysis of the peptides. The purified peptides were subjected to matrix assisted laser desorption ionization–time of flight (MALDI-TOF) MS analysis (Shimadzu) to check the purity of the peptides.

Chiral amino acid analysis. For chiral amino acid analysis, purified peptides were first hydrolyzed in 6 N DCl/D₂O at 110°C for 21 h prior to derivatization and analysis. Alanine was measured as TFA-alanine *i*-propyl ester (15) in selected ion monitoring (SIM) mode for ion 140 and ion 168. The enantiomeric purity of amino acids was determined by gas chromatography (GC) coupled with mass spectrometry (MS) on a stationary-phase chiral-sil-Val column by a previously described method (9, 24).

Antimicrobial activity assays. A variety of antimicrobial assays were employed. Agar well diffusion assays were carried out as described previously (25). Briefly, molten GM17 agar was cooled to 48°C and seeded with the indicator strain *L. lactis* subsp. *cremoris* HP (~2 $\times$ 10⁷ fresh overnight-grown cells). The inoculated medium was dispensed immediately into sterile petri plates, and plates are allowed to solidify and were dried for 30 min under a laminar flow hood. Wells (6-mm diameter) were bored in the seeded agar plates. Aliquots (50 μ l) of cell-free supernatant from the producing strains were dispensed into wells, and the plates were incubated overnight at 30°C before being assessed. Deferred antagonism assays were carried out by spotting 2 μ l of an overnight culture (2 $\times$ 10⁸ CFU/ml) of the producing strains onto GM17 agar plates. Spotted plates were incubated at 30°C overnight; after incubation these plates were subjected to UV irradiation (30 min) to prevent further growth/antimicrobial production, and molten soft agar containing 1 $\times$ 10⁶ CFU/ml of the *L. lactis*

subsp. *cremoris* HP indicator was used to overlay the UV-irradiated plates. Results were ascertained after overnight incubation at 30°C; the sensitivity of the indicator strain against bacteriocin producer is measured by diameter of the inhibition zone. MIC determinations were carried out in 96-well microtiter plates. In each case the respective lacticin 3147 peptides were combined in equimolar concentrations (1:1 ratio with its sister peptide) as described previously (4). *L. lactis* subsp. *cremoris* HP was grown overnight and then inoculated into fresh GM17 broth (1% inoculum) and grown to logarithmic phase (optical density at 600 nm [OD₆₀₀] of 0.5). Serial 2-fold dilutions of an equimolar mixture of the respective Ltn α and Ltn β peptides (or derivatives thereof) were made in GM17, i.e., the growth medium of the indicator strain, and added to the individual wells of the microtiter plate in a 0.1-ml volume. The diluted HP indicator was added to give a final inoculum of 10⁵ CFU/ml in a combined volume of 0.2 ml. After incubation for 16 h at 30°C, the MIC value was read as the lowest peptide concentration causing inhibition of visible growth. Results given are mean values of three independent determinations.

RESULTS

Heterologous expression of *ltnJ* homologues. *In silico* analysis has previously identified a number of *ltnJ*-like genes in other genera (6). These are *sacJ*, present within the gene cluster associated with synthesis of the lacticin 3147-like lantibiotic staphylococcin C55; *penN*, required for production of the bacteriocin pediocin A; and *nprJ*, identified during genome sequencing of *Nostoc punctiforme* (ATCC 29133) (Fig. 3). However, it is not apparent if the encoded proteins are also capable of catalyzing Dha to D-alanine formation. LtnJ and SacJ are very similar, both in sequence and in context, since the SacA1 propeptide (the unmodified form of Sac α) differs from LtnA1 by only 4 residues and also has a serine at position 7, which is likely to be converted to a D-alanine (20). However, SacA2 does not have serine residues corresponding to those converted to D-alanine in Ltn β . In the case of the plasmid associated pediocin A, neither the antimicrobial peptide nor the corresponding gene has been identified, and this, coupled with the absence of an associated *lanM* and *lanB*, means that the role of PenN in this system is unclear. It is interesting, however, that subclones of the putative pediocin A gene cluster lacking *penN* do not lead to a bacteriocin-positive phenotype (10). The role of *nprJ* is not clear although it is notable that *lanM* homologues have been identified in the genome of *N. punctiforme* PCC 73102 (2).

FIG 3 Sequence alignment of LtnJ, SacJ, PenN, NpnJ, Pfam00107 (Zn-dependent alcohol dehydrogenase superfamily), and COG0604 (NADPH-quinone oxidoreductases). Residues in light blue are 100% conserved, residues in dark blue are at least 50% conserved, dashed lines are indicative of domains conserved in all cases, solid lines represent domains conserved in LtnJ-like proteins, residues selected for SDM are highlighted in red in yellow boxes, and conserved cysteine ligands are highlighted in open boxes.

To investigate if *sacJ*, *penN*, or *npnJ* can catalyze the formation of D-alanine, we took advantage of a system previously used to complement a $\Delta ltnJ$ mutant through the introduction of *ltnJ* on a plasmid (pNZ44) under the control of a constitutive promoter. In the same manner *sacJ*, *penN*, or *npnJ* was similarly cloned and introduced into the $\Delta ltnJ$ mutant.

The antimicrobial activity of the resultant strains was assessed, and it was established that heterologous expression of *penN*, but not *sacJ* or *npnJ*, resulted in the restoration of activity (Table 1 and Fig. 4). Mass spectrometric analysis of these strains established that the expression of *penN* resulted in the production of peptides of the correct masses, consistent with the introduction of

TABLE 1 Bioactivity and colony mass spectrometry of heterologously expressed LtnJ homologues

Strain description	Peptide mass (Da)		Bioactivity (mm) ^a
	Ltn α	Ltn β	
MG1363/pMRC01 $\Delta ltnJ$	3,303	2,843	9.2 ($\pm$ 0.3)
pNZ44 <i>ltnJ</i> pMRC01 $\Delta ltnJ$	3,305.37	2,847.47	16.8 ($\pm$ 0.6)
pNZ44 <i>sacJ</i> pMRC01 $\Delta ltnJ$	3,305	2,843	No activity
pNZ44 <i>penN</i> pMRC01 $\Delta ltnJ$	3,305	2,847	10.6 ($\pm$ 0.6)
pNZ44 <i>npnJ</i> pMRC01 $\Delta ltnJ$	3,303	2,843	No activity

^a Bioactivity is determined using a deferred antagonism agar-based assay with *L. lactis* HP as the indicator strain, and results are presented as the diameter of the corresponding zone of inhibition (in mm). Diameter of colony, 6 mm; no activity, no inhibitory zone.

FIG 4 Antimicrobial activity of the $\Delta ltnJ$ mutant following the introduction of genes encoding LtnJ homologues. Activity of the associated cell-free supernatant against *L. lactis* subsp. *cremoris* HP was determined alone (control) or when it was combined with cell-free supernatant containing Ltn α (MG1363/pOM31) or Ltn β (MG1363/pOM39).

D-alanines (3,305 Da for Ltn α and 2,847 Da for Ltn β). Mass spectrometry also revealed that the production of SacJ resulted in wild-type Ltn α , with the correct mass of 3,305 Da. However, an Ltn β peptide with a mass of 2,843 Da is consistent with residues 9 and 12 remaining as Dhas rather than being converted to D-alanines. Peptide complementation studies demonstrated that exogenous Ltn β , but not Ltn α , enhanced the antimicrobial activity of the SacJ-producing strain (Fig. 4). It would thus seem that while SacJ can modify Sac α -like peptides, it is incapable of modifying related peptides such as Ltn β . This is consistent with the lack of suitable substrates in Sac β (Fig. 4). Mass spectrometry and peptide complementation studies confirmed that NpnJ was unable to modify either peptide. This may reflect the relatively low percent identity between LtnJ and NpnJ. It is also notable that NpnJ lacks a Cys residue which is conserved in LtnJ, SacJ, and PenN and which is thought to act as a zinc ligand (Fig. 3). The absence of this ligand in NpnJ may also explain its inability to functionally replace LtnJ. It should be noted, however, that the extent to which NpnJ is pro-

duced in the *L. lactis* background was not quantified and, thus, cannot be ruled out as a mitigating factor.

Site-directed mutagenesis of LtnJ. As LtnJ, SacJ, and PenN are all capable of incorporating D-alanine residues into Ltn α , they are likely to share a number of residues essential for enzymatic activity. A number of fully conserved residues are apparent from alignment of the predicted amino acid sequences (Fig. 3). Given the lack of information available with respect to this group of enzymes, we endeavored to gain insight into the identity of important residues within these enzymes. Thus, 15 conserved residues were targeted for site-directed mutagenesis to determine their importance with respect to the activity of LtnJ, i.e., L54, N63, S89, G99, G127, A157, T166A, L185, A189, T213, G272, G308, L317, D335, and K359. In each case the codon for the targeted residue was replaced with a codon for L-alanine, except for A157 and A189, where a codon for glycine was introduced. As LtnJ activity has not been reconstituted *in vitro*, the impact of these mutations was investigated *in vivo* through site-directed mutagenesis of the pNZ44-*ltnJ* construct, prior to expression of the mutated gene in *trans* in the $\Delta ltnJ$ mutant. The consequences of these changes were assessed through bioactivity-based investigations and mass spectrometry (Table 2). Each mutant fell into one of four subgroups. In subgroup 1 were those that did not reconstitute bioactivity or mass (L54A, G99A, T166A, A189G, and L317A). We conclude that each of these residues is required for the functionality of LtnJ. Subgroup 2 consists of mutations which produce peptides with wild-type bioactivity and mass (N63A, S89A, G127A, A157G, L185A, T213A, and G308A). We conclude that while the amino acids at these positions are conserved, they are tolerant of change. Subgroup 3 has only one member, the K359A mutation. In this instance the peptides generated had a mass corresponding to masses of the wild-type peptides (suggesting the hydrogenation of Dha to D-alanine), but no activity was apparent from either agar well diffusion or deferred antagonism assays. Finally, subgroup 4

TABLE 2 Bioactivity and colony MS analysis of strains expressing site-directed *ltnJ* mutants

Strain or mutant description	Ltn α mass (Da)	Ltn β mass (Da)	Diam (mm) of inhibition zone by: ^a	
			Well diffusion assay	Deferred antagonism assay
MG1363/pMRC01	3,305.45	2,847.5	15.3 $\pm$ 0.6	24.2 $\pm$ 0.6
pMRC01 $\Delta ltnJ$	3,303.6	2,843.79	No activity	9.2 $\pm$ 0.3
pNZ44 <i>ltnJ</i> pMRC01 $\Delta ltnJ$	3,305.37	2,847.47	10.7 $\pm$ 0.6	16.8 $\pm$ 0.6
pNZ44 <i>ltnJ</i> (L54A)	3,303.72	2,843.86	8.3 $\pm$ 0.3	12.5 $\pm$ 0.3
pNZ44 <i>ltnJ</i> (N63A)	3,305	2,847.3	10.9 $\pm$ 0.6	17.3 $\pm$ 0.6
pNZ44 <i>ltnJ</i> (S89A)	3,305.57	2,847.58	7.8 $\pm$ 0.3	13.5 $\pm$ 0.3
pNZ44 <i>ltnJ</i> (G99A)	3,303.64	2,843.58	7.5 $\pm$ 0.3	11.2 $\pm$ 0.3
pNZ44 <i>ltnJ</i> (G127A)	3,305.48	2,847.44	7.8 $\pm$ 0.3	13.8 $\pm$ 0.6
pNZ44 <i>ltnJ</i> (A157G)	3,305.3	2,847.33	8.2 $\pm$ 0.3	13.5 $\pm$ 0.6
pNZ44 <i>ltnJ</i> (T166A)	3,303.49	2,843.49	7.3 $\pm$ 0.3	9.6 $\pm$ 0.3
pNZ44 <i>ltnJ</i> (L185A)	3,305.4	2,847.41	7.5 $\pm$ 0.3	10.8 $\pm$ 0.3
pNZ44 <i>ltnJ</i> (A189G)	3,303.66	2,843.6	No activity	10.5 $\pm$ 0.3
pNZ44 <i>ltnJ</i> (T213A)	3,305.73	2,846.98	10.4 $\pm$ 0.6	14.8 $\pm$ 0.6
pNZ44 <i>ltnJ</i> (G272A) ^b	3,305.92	2,845.73, 2,847.81	8.2 $\pm$ 0.3	11.3 $\pm$ 0.3
pNZ44 <i>ltnJ</i> (G308A)	3,305.51	2,847.51	10.9 $\pm$ 0.6	17.2 $\pm$ 0.6
pNZ44 <i>ltnJ</i> (L317A)	3,303.7	2,843.44	7.5 $\pm$ 0.3	12.4 $\pm$ 0.6
pNZ44 <i>ltnJ</i> (D335A) ^b	3,303.9	2,843.55, 2,847.56	8.2 $\pm$ 0.3	12.0 $\pm$ 0.3
pNZ44 <i>ltnJ</i> (K359A)	3,305.36	2,847.82	No activity	No activity

^a Bioactivity is determined using *L. lactis* subsp. *cremoris* HP as an indicator strain, and results are presented as the diameter of the corresponding zone of inhibition (measured in mm). Values are means $\pm$ standard deviations. Diameter of colony, 6 mm; diameter of well, 5.8 mm; no activity, no inhibitory zone.

^b The mutation generated two forms of Ltn β with two masses.

TABLE 3 MIC of selected peptides from *ltnJ* mutated strains against *L. lactis* subsp. *cremoris* HP indicator

Description of LtnJ derivative	MIC of Ltn α plus Ltn β from the LtnJ derivative strain (nM) ^a
pMRC01 Δ <i>ltnJ</i>	2,500
pNZ44 <i>ltnJ</i> pMRC01 Δ <i>ltnJ</i>	156
pNZ44 <i>ltnJ</i> (L54A) pMRC01 Δ <i>ltnJ</i>	2,500
pNZ44 <i>ltnJ</i> (N63A) pMRC01 Δ <i>ltnJ</i>	234
pNZ44 <i>ltnJ</i> (S89A) pMRC01 Δ <i>ltnJ</i>	625
pNZ44 <i>ltnJ</i> (A189G) pMRC01 Δ <i>ltnJ</i>	1,875
pNZ44 <i>ltnJ</i> (K359A) pMRC01 Δ <i>ltnJ</i>	3,750

^a Peptides were combined in equimolar concentrations.

contains mutants that do not clearly fall into any of the previous subgroups (G272A and D335A). LtnJ(G272A) yielded Ltn α peptides with wild-type mass but generated two forms of Ltn β with masses of 2,845.73 and 2,847.81 Da. Altering this residue seems to specifically impact the ability of LtnJ to modify Ltn β but not Ltn α . LtnJ(D335A) also impacts D-alanine formation in Ltn β , with both unmodified (2,843.55 Da) and modified forms of the peptide being produced, but differs in that Ltn α remains unmodified. Both the G272A and D335A changes improved bioactivity compared to that of the Δ *ltnJ* background but not to wild-type levels.

As bioactivity-based investigations do not distinguish between impacts arising as a consequence of reduced production or reduced activity, a number of peptides produced by LtnJ mutants (L54A, N63A, S89A, A189G, and K359A) were selected and purified with a view of carrying out MIC studies using equimolar concentrations of each peptide (Table 3). MIC analysis with the peptides from the subgroup 1 mutants highlighted the importance of the L54 residue in LtnJ in that this derivative resulted in the production of peptides with activity identical to the activity of the background Δ *ltnJ* strain. The S89A and A189G changes resulted in peptides with slightly better activity than that of the Δ *ltnJ* background, but these mutants were still not as active as wild-type peptides. As expected the N63A change (subgroup 2) did not impact negatively on antimicrobial activity. Peptides from the K359A subgroup 3 mutant were less active than the peptides produced by the Δ *ltnJ* mutant, thus establishing that LtnJ(K359A) is acting on the peptides in a manner which negatively impacts their activity.

Chiral amino acid analysis. The data establish that LtnJ (K359A) modifies Ltn α and Ltn β peptides in a manner which reduces their antimicrobial activity while not altering their mass. Given that LtnJ catalyzes Dha to D-alanine formation, we postulated that this form of the enzymes might produce a similar reaction, but one that results in the formation of either L-alanine or a mixture of L-alanine and D-alanine residues rather than exclusively producing D-alanine. This is consistent with the previous observation that activity is impaired when L-alanine is introduced in place of D-alanine in Ltn α and Ltn β (6). To test this theory, chiral-phase gas chromatography was performed to estimate the proportions of L- to D-alanines in Ltn α and Ltn β generated by LtnJ(K359A) and compare the results to the corresponding wild-type peptides (Table 4). Wild-type Ltn α contains one D-alanine and two L-alanine residues, and Ltn β contains two D-alanine and four L-alanine residues (ratio of 1:2 in both peptides). Chiral amino acid analysis reveals that the ratio of D- to L-alanines in the K359A-generated Ltn α is 1:4.4 (Table 4). For Ltn β we determined

TABLE 4 Chiral amino acid composition of selected *ltnJ* SDM derivatives

Strain description	Area D-Ala ^a	Area L-Ala ^a	D-Ala (%) ^b	L-Ala (%) ^b	Ratio of D-Ala to L-Ala
pMRC01 Ltn α	1,430,420	2,934,517	32.77	67.23	1:2
pMRC01 Δ <i>ltnJ</i>	91,882	412,877	18.2	81.8	1:4.4
pNZ44 <i>ltnJ</i> (K359A) α					
pMRC01 Ltn β	13,197,118	27,823,729	32.17	67.83	1:2
pMRC01 Δ <i>ltnJ</i>	3,213	21,300	13.11	86.89	1:6.6
pNZ44 <i>ltnJ</i> (K359A) β					

^a Arbitrary units.^b Percentage of total area.

a ratio of 1:6.6 for the peptide generated by K359A (Table 4). We conclude that the diminished activity of the strain producing LtnJ(K359A) is as a consequence of conversion of Dha to Ala formation in a nonstereospecific manner by these enzymes, leading to peptides with a mix of L- and D-alanines instead of complete conversion to D-alanine.

DISCUSSION

Lacticin 3147 is one of only two lantibiotics that contains D-alanines and is the only example whereby the enzyme(s) responsible for this modification has been identified (6). Although this specific modification is unusual, posttranslational modification is an intrinsic feature of all lantibiotics, most notably with respect to the modifications which result in the formation of dehydroalanine from serine, dehydrobutyrine from threonine, and (β -methyl) lanthionine bridges formed by bridging Dha and Dhb residues with cysteines. The associated modification enzymes are quite flexible and can modify a variety of peptides, even if they differ quite substantially from the natural substrate (1, 4, 7, 23, 32). In contrast, there have been relatively few studies which have focused on the manipulation of the modification enzymes in order to identify key residues and functional domains. A notable exception is LctM, the enzyme which is responsible for dehydration and (β -methyl) lanthionine formation in lacticin 481. Several LctM mutants have been created, which has resulted in the identification of residues of key importance in terms of the functionality of the enzyme (31, 33). In this study the *ltnJ* dehydrogenase-encoding gene was subjected to site-directed mutagenesis to identify amino acid residues that are of key importance in the activity of LtnJ. It should be noted that further analysis will be required to discriminate between changes that have an impact due to the importance of a specific residue being changed and those which impact the tertiary structure of the enzyme in a more general manner.

We previously established the existence of a number of LtnJ homologues. These include SacJ, present within the operon responsible for synthesis of the lacticin 3147-like lantibiotic staphylococcin C55; PenN, required for production of the bacteriocin pediocin A; and NpnJ, identified during genome sequencing of *N. punctiforme* (NCTC29133). Of these, only PenN successfully modified both lacticin 3147 peptides. In the case of PenN, the associated antimicrobial peptide pediocin A has not been identified. However, as a consequence of the activity of PenN, there is a strong possibility that it, too, contains D-amino acids. SacJ catalyzed D-alanine formation only in Ltn α . Further investigation is

required to determine if the inability of SacJ to modify Ltn β is due to SacJ's inability to detect a specific motif within the Ltn β peptide, the generally greater difference between LtnA2 and SacA2 (45% identity) relative to that between LtnA1 and SacA1 (86%), or the composition of the leader regions (4% identity between A1 leaders but only 10% identity between A2 leaders). Nonetheless, the activity of SacJ and the similarities between Ltn α and Sac α peptides strongly suggest that Sac α is another example of a D-alanine-containing ribosomally synthesized peptide. The availability of predicted amino acid sequences for these homologues allowed us to identify conserved amino acids and use site-directed mutagenesis (SDM) to attempt to link individual residues with functionality. The consequences of altering L54, G99, T166, A189, and L317 indicate that each of these is of importance with respect to optimal formation of D-alanine from Dha in lactacin 3147. G272 and D335 seem to be of intermediate importance, in that at least one peptide of correct mass resulted in both cases. Mutating N63, T213, and G308 had no detrimental impact, and thus these residues do not appear to have a critical role; mutation of S89, G127, A157, and L185 resulted in the production of peptides of correct masses but slightly reduced activity levels. Given the subsequent findings, it may well be that these alterations affect the stereospecific formation of D-alanine in a subtle fashion. However, the most unexpected finding was the identification of a change which resulted in the associated peptides having less antimicrobial activity than those produced in the Δ ltnJ background, despite having masses corresponding to those of the wild-type proteins. The basis for this phenomenon only became clear upon chiral amino acid analysis to differentiate the enantiomers of alanine, which established that LtnJ(K359A) no longer completely converts Dha to D-alanine but also incorporates some L-alanines, thereby explaining the negative impact on antimicrobial activity despite the production of peptides of wild-type mass.

In conclusion, this study has established that at least two homologues of LtnJ can also catalyze D-alanine formation, it has revealed the relative importance of specific conserved residues in LtnJ, and it shows for the first time that LtnJ can be mutated in a manner that eliminates its ability to catalyze D-alanine formation in a stereospecific manner.

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