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Effect of Bioengineering Lacticin 3147 Lanthionine Bridges on Specific Activity and Resistance to Heat and Proteases

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SUMMARY

Lacticin 3147 is a lantibiotic with seven lanthionine bridges across its two component peptides, Ltnα and Ltnβ. Although it has been proposed that the eponymous lanthionine and (β-methyl)lanthionine (Lan and meLan) bridges present in lantibiotics make an important contribution to protecting the peptides from thermal or proteolytic degradation, few studies have investigated this link. We have generated a bank of bioengineered derivatives of lacticin 3147, in which selected bridges were removed or converted between Lan and meLan, which were exposed to high temperature or proteolytic enzymes. Although switching Lan and meLan bridges has variable consequences, it was consistently observed that an intact N-terminal lanthionine bridge (Ring A) confers Ltnα with enhanced resistance to thermal and proteolytic degradation.

INTRODUCTION

Lantibiotics are a class of highly active antimicrobial peptides active against many Gram-positive bacteria (Brumfitt et al., 2002; Cotter et al., 2006; Galvin et al., 1999; Kruszewska et al., 2004; Piper et al., 2009a; Ryan et al., 1996, 1999a). These peptides undergo distinctive posttranslational modifications that result in the formation of lanthionine and/or (β-methyl)lanthionine bridges (Lan or meLan). Lanthionines are internal ring structures that result from the dehydration of serine and threonine to dehydroalanine (Dha) and dehydrobutyrine (Dhb), which then react with cysteine through a Michael-type addition, to form Lan and meLan bridges (Chatterjee et al., 2005; Cotter et al., 2005a; Pag and Sahl, 2002; Willey and van der Donk, 2007; Xie and van der Donk, 2004). There are extensive data demonstrating the importance of these bridges in providing lantibiotics with a biologically active conformation (Breukink and de Kruijff, 1999; Breukink et al., 1999; Brotz et al., 1998; Chatterjee et al., 2005). Numerous reviews focusing on these peptides also propose that these rings contribute to resistance to degradation by thermal stress or proteolytic enzymes. However, to date, there exists relatively little supporting evidence for this theory. Although a role for (me)Lan in thermal stability was first mooted by Hurst (1981) and has been reiterated on a number of occasions, there exists no example of a direct comparison between the thermotolerance of a peptide with and without a (me)Lan bridge. Evidence for a role in protecting against proteolytic degradation is much stronger in that a study by Bierbaum et al. (1996) elegantly demonstrated that the removal of a C-terminally located meLan or Lan structure from the lantibiotic Pep5 enhanced the sensitivity of the peptide to proteases, and, conversely, the introduction of a fourth bridge (MeLan) in the same peptide increased stability when exposed to chymotrypsin and Lys-C (Bierbaum et al., 1996). Further indirect evidence of the importance of (me)Lan bridges in lantibiotic stability has been provided by demonstrating the beneficial consequences of introducing such structures into non-lantibiotic peptides. Replacement of a cysteine bridge in Sandostatin with a lanthionine bridge improved the serum stability of the peptide (Osapay et al., 1997), whereas the introduction of lanthionine residues increased the bio-stability and bioavailability of enkephalin (Rew et al., 2002) and angiotensin (Kluskens et al., 2009) as well as the protease resistance of the peptide hormone LHRH (Rink et al., 2010).

For this study we selected lacticin 3147, a two-peptide lantibiotic active through the synergistic activity of Ltnα and Ltnβ (Figure 1). This lantibiotic was first isolated from *Lactococcus lactis* subsp. *lactis* DPC3147 in 1995 (Ryan et al., 1996) and exhibits broad-spectrum activity against a variety of Gram-positive targets (Field et al., 2008; Galvin et al., 1999; Piper et al., 2009b; Rea et al., 2007) as a consequence of a dual ability to inhibit peptidoglycan synthesis and form pores in target cell membranes (both of which are dependent on the interaction of the lantibiotic with the peptidoglycan precursor lipid II; Wiedemann et al. [2006]). Ltnα is a globular peptide consisting of 30 amino acids, of which 11 are posttranslationally modified (Martin et al., 2004). The structure of Ltnα is very similar to that of the α component of another two-peptide lantibiotic, haloduracin (Cooper et al., 2008), and the single peptide lantibiotic, mersacidin (Chatterjee et al., 1992). Indeed, it is believed that all members of the extended subgroup of mersacidin-like peptides have a similar conformation (Lawton et al., 2007). In the case of Ltnα, this means that three serines are ultimately modified to form two Lans (with two cysteines) and a D-alanine, whereas

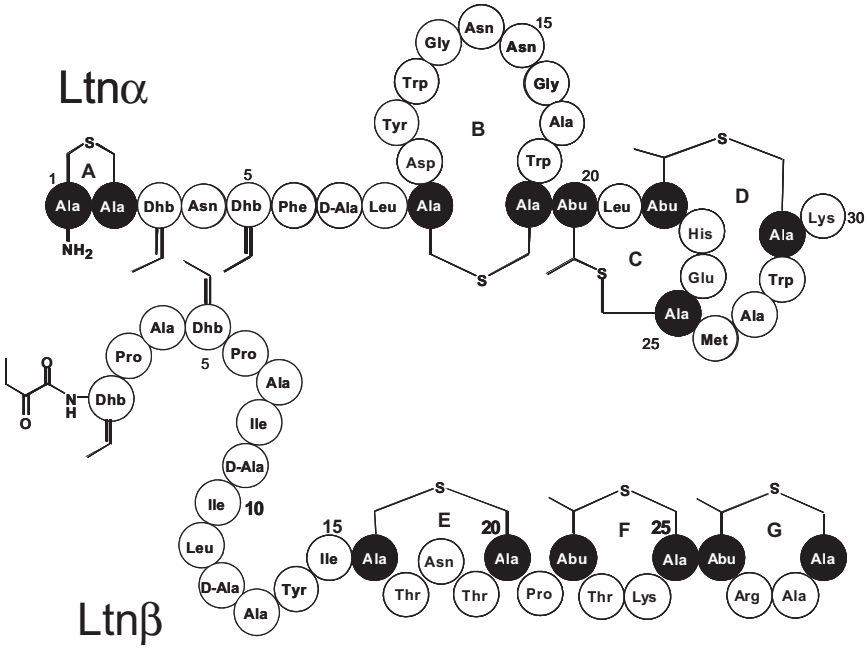


Figure 1. Structures of the Lacticin 3147 Peptides Ltnα and Ltnβ

Lan (Ala-S-Ala) and meLan (Abu-S-Ala) bridges are indicated. See also Figure S1.

four threonines are modified to become two meLans (with another two cysteines) and two Dhbs (Cotter et al., 2005b; Ryan et al., 1999b). Despite the generally conserved structure of these peptides, the presence of a Lan (or meLan) generated through the modification of two adjacent residues, as is the case with Ring A at the N terminus of the peptide, remains a rare feature across all lantibiotics and outside of Ltnα (and presumably the closely related C55α; Navaratna et al. [1998]; O'Connor et al. [2007]) has only been observed in mersacidin (meLan). It is interesting to note that investigations with mersacidin have revealed that the corresponding Ring A folds back into the lipid II binding pocket of this lantibiotic when in the presence of membrane-mimicking DPC micelles but that the addition of lipid II results in the reexposure of the lipid II binding site (Hsu et al., 2003). Ring A is also unusual by virtue of the fact that, in contrast to the vast majority of Lan/meLan bridges, the cysteine contributing to the bridge is located downstream of its serine partner (Martin et al., 2004). Ltnα is representative of a larger number of lantibiotic peptides (which includes the related C55α, Halα, mersacidin, and actagardine peptides) in which the N-terminal residue is involved in the formation of a bridge structure. Notably, studies with the haloduracin peptide, Halα, revealed that disrupting the disulphide bridge present at its N terminus resulted in additional N-terminal proteolysis (Cooper et al., 2008). In contrast to Ltnα, Ltnβ is a linear cationic peptide. It consists of 29 amino acids, of which 11 are posttranslationally modified. Five threonines are modified (whereas three remain unmodified) to form two meLan bridges (with two cysteines), two Dhbs, and a 2-oxobutyrl group (arising from the deamination of an N-terminal Dhb). All three serines are modified to generate one lanthionine bridge (with one cysteine) and two D-alanines (Martin et al., 2004).

Our knowledge to date of the importance of Lan and meLan structures in lacticin 3147 has been generated through in vivo bioengineering studies (Cotter et al., 2006; Field et al., 2007). These established that in six instances when a (me)Lan bridge is eliminated as a consequence of changing one of the associated Ser, Thr, or Cys residues (in most cases to an alanine residue), the antimicrobial activity was abolished. In contrast, manipulations of Cys1 or Ser2 of Ltnα, i.e., changes resulting in the removal of Ring A, had a more subtle impact on activity (Cotter et al., 2006; Field et al., 2007). Indeed, this represented only the fourth example of an in vivo bioengineered (me)Lan in which any antimicrobial activity was retained following bridge disruption; the other examples being mutacin II C27A (Chen et al., 1998) and Pep5 C27A and C33A (Bierbaum et al., 1996). More recently, an in vitro-based alanine mutagenesis strategy has also been employed to probe the consequences of mutating cysteines involved in (me)Lan formation in haloduracin (Halα, Halβ) (Cooper et al., 2008). In this instance, although the HalαC27A, C17A, and HalβC15A, C20A, and C24A peptides all displayed greatly reduced or no antimicrobial activity, HalαC23A and HalβC5A retained a significant degree of activity; in all instances these assays were carried out in the presence of unmutated sister peptide (Cooper et al., 2008).

In this study we use the lacticin 3147 peptides, Ltnα and Ltnβ, to carry out a systematic in vivo bioengineering-based investigation of the impact of the manipulation of Lan/meLan bridges on antimicrobial activity. As a consequence of its tolerance to change, Ring A of Ltnα was the subject of greatest focus, and the extent to which it can be manipulated was tested by adding five additional derivatives to the existing collection of two Ring A mutants. This included one peptide in which the existing Lan bridge was replaced by a meLan. To assess whether such Lan-meLan interconversion might be tolerated at other locations within the two peptides, a further six bioengineered strains/peptides, in which the remaining bridges were each manipulated, were created. In addition to assessing the nature of the peptides produced and quantifying the impact of these changes on specific activity, this collection of bioengineered peptides was also assessed to determine the consequences of these changes and, thus, the role of (me)Lan structures, with respect to the resistance of these peptides to proteases and thermal stress.

RESULTS

Design and Construction of Ltnα Ring A and Bridge Variants of Lacticin 3147

Alanine-scanning mutagenesis of lacticin 3147 revealed that both residues contributing to Ring A of Ltnα could be converted

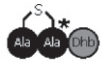
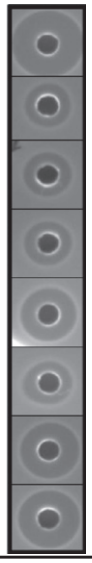
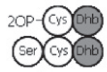
Ltnα mutant	Mass Da (+ cyan)[a]	Structure(s)	Activity vs. *L. lactis*	Ltnα (μM) vs. *L. lactis*	Plus β (nM) vs. *L. lactis*	Plus β (nM) vs. *M. luteus*
Wild type	3305 (+0)			1.25	9.8	156
C1A	3273.4			>2.5	312.5	1250
S2A	3307.7			>2.5	625	2500
C1S	3289.9			>2.5	312.5	2500
S2C	3337 (+50)			>2.5	2500	1250
C1S/S2C	3306.3 (+25), 3323.4			>2.5	1250, 1250	2500, 2500
S2T	3319.5 (+0)			>2.5	2500	2500
C1A/S2A	3275.1			>2.5	2500	1250

Figure 2. Ring A Mutants of Ltnα

Mass of the bioengineered peptides, predicted structures, and visual demonstration of antimicrobial activity of 50 μl bioengineered peptide when combined with wild-type sister peptide (both at 2.5 μM concentrations) against *L. lactis* HP and broth-based MIC determination of antimicrobial activity against *L. lactis* HP and *M. luteus*. [a], mass difference following cyanylation is in brackets; *, D-chiral center.

to alanine without eliminating the antimicrobial activity of the bioengineered strain (Cotter et al., 2006). As a consequence of the unusual size, location, and tolerance to change of Ring A, it became the focus of further investigation. More specifically, five bioengineered peptides were designed: LtnαC1S, S2C, S2T, and two double-mutants LtnαC1S-S2C and C1A-S2A. The creation of LtnαS2T allowed us to assess the consequences of replacing a Lan with a meLan bridge at this location, whereas LtnαS2C could result in several outcomes, including two unmodified cysteines at positions 1 and 2, the formation of a disulphide bridge between the same two cysteines, or the possible formation of a meLan between either cysteine and the Dhb located at position 3. There are also many possible consequences to the creation of the LtnαC1S prepeptide, including the introduction of an N-terminal serine or 2-oxopropionate (2-OP) following spontaneous deamination of an N-terminal Dha. The doubly altered peptides had the potential to invert the Lan bridge at the N terminus (LtnαC1S-S2C) or to create a peptide beginning with Ala-Ala (LtnαC1A-S2A), which would act as a mimic of the natural Lan (Ala-S-Ala), but without a thiol bridge and with an altered chirality of the second α carbon.

Following the successful creation of all of these strains, initial bioactivity based analyses revealed that in all cases antimicrobial activity against the lacticin 3147 sensitive indicator *Lactococcus lactis* HP was retained (data not shown). Colony mass spectrometry was performed to gain an insight into the nature of the modifications occurring at the N termini of the bioengineered peptides. In the case of LtnαC1S, a mass of 3289.9 daltons is consistent with the presence of an N-terminal 2-OP. LtnαS2C has a mass of 3337 daltons, and cyanylation of this peptide resulted in a 50 dalton increase in mass, consistent with the presence of two unmodified cysteines (Figure 2). The LtnαS2T peptide has a mass of 3319 daltons (Figure 2), which is consistent with either a Cys-Dhb N terminus or an N-terminal bridge between the residues (in this instance a meLan). Cyanylation was again employed and revealed that no free cysteines were present (Figure 2). The C1A-S2A mutation results in the replacement of the Lan bridge with two alanines, whereas the C1S-S2C change results in two peptides of mass 3306 and 3323 Da, consistent with the presence of an N-terminal 2-OP and serine, respectively, and, thus, inversion of the natural Lan bridge did not occur (Figure 2). The first of the two C1S-S2C peptides was subjected to cyanylation, which confirmed the presence of an unmodified cysteine at position 2 (Figure 2).

Antimicrobial Activity of Ltnα Ring A Variants

Agar-based assays using purified Ring A variant peptides combined with native Ltnβ revealed differences in antimicrobial activity against *L. lactis* HP. Broth-based minimum inhibitory concentration (MIC) studies were carried out to test the activity of the peptides, both alone and in combination with equimolar concentrations of Ltnβ. All peptides were active, albeit at levels lower than that of the wild-type equivalent, confirming the tolerance of Ring A to change. The MIC of all individual mutant peptides was greater than 2.5 μM, but all peptides were active at or below this concentration when combined with Ltnβ (Figure 2). Of these, LtnαC1A and LtnαC1S were most active (MIC of 312.5 nM when combined with Ltnβ) (Figure 2), whereas LtnαS2A was 60-fold less active than the wild-type. Interestingly, those peptides that were created with the anticipation that their N terminus would most resemble that of the wild-type peptide were least active. The unsuccessful attempt to invert the Lan bridge (C1S-S2C) resulted in peptides (with N-terminal 2-OP-Cys and Ser-Cys sequences) that both had MICs of 1250 nM, and the replacement of the original Lan bridge with either MeLan, Cys-Cys, or Ala-Ala all resulted in the corresponding peptides having an MIC of 2.5 μM when combined with Ltnβ (Figure 2).

MIC investigations, with equimolar concentrations of purified Ltnα and β peptides, were also carried out using a second target strain, *Micrococcus luteus* DSM1790, to differentiate between generalized and strain-specific impacts. Although all combinations tested also exhibited antimicrobial activity against this target, the wild-type combination was again the most active (MIC 156 nM). The bioengineered peptides could be divided into two categories. When combined with Ltnβ, LtnαC1A, LtnαS2C, and LtnαC1A/S2A had a MIC of 1250 nM, whereas

Mutant	Mass Da (+ cyan)[a]	Activity vs. *L. lactis*	alone (μM) vs. *L. lactis*	Combined activity (nM)* vs. *L. lactis*	Combined activity (nM)* vs. *M. luteus*
Wild type	3305 (α) 2847(β)		1.25 2.5	9.8 9.8	156 156
LtnαS9T	3319.5		>2.5	312.5	2500
LtnαT20S	3291.3		>2.5	312.5	1250
LtnαT22S	3291		>2.5	78	625
LtnβS16T	2861.5		>2.5	156 (625 with αT22S)	625
LtnβT22S	2833.4		>2.5	625	1250
LtnβT26S	2833.4		>2.5	156 (625 with αT22S)	1250

Figure 3. Lan-meLan Interconversion of Rings B–F

Mass of the bioengineered peptides, predicted structures, and visual demonstration of antimicrobial activity of 50 μl bioengineered peptide when combined with wild-type sister peptide (both at 2.5 μM concentrations) against *L. lactis* HP and broth-based MIC determination of antimicrobial activity alone or with equimolar concentrations of complementary sister peptide against *L. lactis* HP and *M. luteus*. *, wild-type sister peptide used unless stated otherwise.

LtnαS2A, LtnαC1S, LtnαC1S/S2C, and LtnαS2T were 2-fold less active (MIC 2500 nM). Thus, whereas some impacts are consistent, e.g., LtnαC1A-Ltnβ is one of the most active combinations against both targets, there are target-specific differences. More specifically, LtnαS2A-Ltnβ is quite active against *L. lactis* HP but is one of the combinations with lesser activity against *M. luteus* DSM1790, whereas LtnαS2C-Ltnβ and LtnαC1A/S2A-Ltnβ are exceptional by virtue of being the only combinations that are more active against *M. flavus* than *L. lactis*.

Antimicrobial Activity of Lacticin 3147 Bridge Variants

Because alanine-scanning mutagenesis has already demonstrated that the mutagenesis of residues involved in the formation of the other lacticin 3147 bridges has very detrimental consequences on bioactivity, the benefits of manipulating these to the same extent as Ring A was debatable. Of the changes that could be made, it was anticipated that alterations resulting in the replacement of Lan bridges with meLans and vice versa might be best tolerated. The corresponding genetic manipulations were successfully completed, and following confirmation by colony mass spectrometry that peptides of correct mass were produced, the activity of the six resultant strains (Ltnα S9T, Ltnα T20S, Ltnα T22S, Ltnβ S16T, Ltnβ T22S, and Ltnβ T26S) was initially assessed by well diffusion assays using cell-free supernatant. This established that all retained some, albeit reduced, antimicrobial activity (data not shown). The peptides were purified, and their specific activities were determined as broth-based MICs. This revealed that in combination with Ltnβ, both LtnαS9T and LtnαT20S are 30-fold less active than wild-type Ltnα, whereas LtnαT22S is more active than other bridge variants, being only 8-fold less active than wild type (Figure 3). The tolerance at this location is also reflected in nature in that the related α component of the plantaricin W two-peptide lantibiotic has a serine at the location corresponding to LtnαT22. Of the Ltnβ peptides assessed in combination with Ltnα, both LtnβS16T and LtnβT26S are 15-fold less active than wild-type Ltnβ, and LtnβT22S is a further 4-fold less active (Figure 3). The greater consequences of manipulating LtnβT22S were not anticipated because natural Lan-meLan variation occurs across the natural β peptides, i.e., Ring D corresponds to a meLan in Smbβ/Bhtaβ, Bliβ, and the putative Pnmβ, Ring E corresponds to a Lan in SmbAβ/bhtAβ, and Ring F corresponds to a Lan in Plwβ (see Figure S1 available online). The peptides with Lan and meLan interconversions that are most active against *L. lactis* HP are LtnαT22S, LtnβS16T, and LtnβT26S. Both combinations (i.e., LtnαT22S-LtnβS16T and LtnαT22S-LtnβT26S) resulted in a 64-fold reduction in activity against *L. lactis* HP relative to the wild-type combination (MIC 625 nM), which was 2-fold less than the anticipated impact (8-fold reduction × 16-fold reduction = 128-fold reduction) (Figure 3). MIC assays with the bioengineered peptides, in combination with an equimolar concentration of the relevant sister peptide, established that antimicrobial activity was also retained, to at least some extent, against *M. luteus* DSM1790. It was noted that LtnαT22A-Ltnβ and Ltnα-LtnβS16T were the most active combinations (MIC 625 nM) and that in contrast to peptides in which Ring A was bioengineered, the consequences of interconverting Lan-MeLan residues had less dramatic strain-variable consequences (Figure 3).

Thermotolerance of Lacticin 3147 and Bridge Variants

Although thermotolerance is one of the characteristics frequently associated with lantibiotics, it has not been definitively demonstrated that the loss of an individual (me)Lan bridge leads to increased thermal sensitivity. We assessed the impact of high temperature (80°C for 30 min) on the antimicrobial activity of Ltnα, Ltnβ, and a selection of peptides in which bridges were manipulated or removed, including LtnαC1A, LtnαC1S (the most active of the Ring A mutants), LtnαC1A/S2A, LtnαT20S, LtnαT22S, LtnβS16T, and LtnβT26S (representing other bridge mutants of differing activity), was assessed against *L. lactis* HP

Table 1. Impact of Heat (80°C for 30 min) on the Antimicrobial Activity of Lacticin 3147 Peptides and Bioengineered Derivatives as Determined by MIC Assay Against *L. lactis* HP

Lacticin 3147 peptides	MIC Value (nm) without Heating	MIC Value (nm) After Heating
WT Ltnα + Ltnβ	9.8	19.5
αC1A + Ltnβ	312.5	1250
αC1S + Ltnβ	312.5	1250
αC1A/S2A + Ltnβ	2500	>2500
αT20S + Ltnβ	312.5	625
αT22S + Ltnβ	78	156
Ltnα + βS16T	156	312.5
Ltnα + βT26S	156	312.5

Table 2. Impact of Heat (80°C for 30 min) on the Lacticin 3147 Peptides and Bioengineered Derivatives as Determined by Mass Spectrometry

Peptide	T_0	T_{30}
WT Ltnα	3305	3305.4, 3323.5
LtnαC1A	3273.5	2533.4, 2632.4
LtnαC1S	3290	2549.4
LtnαC1A/S2A	3275.1	3275.6, 3292.6
LtnαT20S	3291	3291.4, 3308.5
LtnαT22S	3291	3291.8, 3307.9
WT Ltnβ	2848	2847.6
LtnβS16T	2861.5	2864.57
LtnβT26S	2834	2838.58
Blank without peptide	No peak	No peak

Masses of resultant fragments are presented. See also Figure S2.

(Table 1). It was established that this heat treatment brought about a 2-fold decrease in the specific activity of the wild-type combination and of LtnαT20S, LtnαT22S, LtnβS16T, and LtnβT26S (when combined with the corresponding sister peptide), whereas αC1A and αC1S exhibited greater sensitivity in the form of a 4-fold reduction in activity. Heat treatment of LtnαC1A/S2A, which had limited antimicrobial activity prior to treatment, reduced activity below the detectable threshold. It would seem that, whereas replacement of one (me)Lan bridge with another does not impact on heat resistance, the removal of a bridge has more severe consequences, thereby providing the first experimental evidence that Lan bridges contribute to thermotolerance. Mass spectrometric analysis was also carried out after heat treatment of the peptides (Figure S2; summarized in Table 2). This revealed that in many cases, i.e., Ltnα, LtnαC1A/S2A, LtnαT20S, and LtnαT22S, the peptides may be oxidized. However in the case of the Ltnα peptides, which on the basis of MIC results, are most thermosensitive, i.e., LtnαC1A and LtnαC1S, the peptides appear to be cleaved. This is indicated by a 741 dalton reduction in the mass of both peptides, which is consistent with the loss of the six most C terminally located residues, raising the intriguing possibility that the N-terminal Ring A structure protects the C terminus of the peptide from high temperature-induced cleavage. Cleavage of the LtnαC1A/S2A peptide is not apparent, which perhaps reflects the fact that its N terminus more closely resembles that present in the wild-type peptide. With respect to the Ltnβ peptides, the wild-type form of the peptide was not affected by thermal treatment, whereas thermal treatment of LtnβS16T and LtnβT26S resulted in a 3–4 dalton increase in mass, the cause of which is not known.

Impact of Proteases on Lacticin 3147 and Bridge Variants

Lacticin 3147 is sensitive to a number of proteases, such as α-chymotrypsin (Gardiner et al., 2007). In this study wild-type peptides and a selection of bioengineered peptides (LtnαC1A, LtnαC1S, LtnαC1A/S2A, LtnαT20S, LtnαT22S, LtnβS16T, LtnβT26S) were exposed over a period of 3 hr to a variety of different proteases, including pepsin, α-chymotrypsin, pronase, and proteinase K (100 μg/ml). There was a particular interest in determining the extent to which the peptides were digested after 1 and 3 hr as assessed by mass spectrometric analysis (Figures S3–S7; summarized in Table 3). First, the stability of the mutant peptides in buffer over this period was assessed. Some oxidation of Ltnβ (wild-type peptide, LtnβT26S, and, to a lesser extent, LtnβS16T) was apparent after 3 hr, but no degradation was evident. Exposure to pepsin revealed that both wild-type Ltnα and Ltnβ were quite resistant to the enzyme. and digestion is not evident after 3 hr. However, it is important to note that digested fragments with poor ionization efficiency can go undetected when MALDI-ToF is employed. At least some of the LtnβS16T, LtnβT26S, and LtnT22S peptides remain undigested after this time, whereas undigested LtnαC1A, LtnαC1S, and LtnαT20S could not be detected. As expected from previous lacticin 3147 studies (Gardiner et al., 2007), α-chymotrypsin had a dramatic impact on peptide integrity, with all peptides having been digested after 3 hr. However, it was notable that wild-type Ltnα, Ltnβ, and LtnβS16T appear to be less sensitive to the activity of this protease because the undigested form of the peptides could still be detected after 1 hr exposure. A similar pattern was apparent on exposure to pronase, a combined protease from *Streptomyces griseus* (contains at least three proteolytic activities, including an extracellular serine protease), in that the undigested forms of these peptides remained, to at least some extent, after 3 hr. Of the remaining peptides LtnαC1A and C1S were most sensitive, providing the only examples where undigested peptide could not be detected after 1 hr. Finally, all peptides were highly sensitive to proteinase K with the wild-type Ltnα peptide and the three Ltnβ peptides being detected in their undigested form after 1 hr exposure. When examined in combination, these studies indicate that Ltnβ, despite its more linear structure, is generally less sensitive to the proteases employed than Ltnα. Of the bioengineered forms of Ltnβ, LtnβS16T exhibited a level of sensitivity that was quite similar to that of the wild-type form, whereas LtnβT26S was more sensitive. Of the Ltnα peptides the wild-type peptide was consistently the most resistant to proteases, followed by LtnαT22S. LtnαT20S and, to an even greater extent, LtnαC1A/S2A, LtnαC1A, and C1S were more sensitive to these enzymes. Thus, it would seem that exchanging Lan and meLan bridges can, in some instances, lead to increased protease sensitivity,

Table 3. Summary of Consequences of Digestion of Ltnα, Ltnβ, and Derivatives Thereof (10 μM/ml) with Proteases (100 μg/ml) over 1 and 3 hr at 37°C as Assessed by Mass Spectrometry

		No protease		Pepsin		α-Chymotrypsin	
	0 hr	1 hr	3 hr	1 hr	3 hr	1 hr	3 hr
WT Ltnα	**3305**	**3305.1**, 3321.2°	3306.2, **3322.3°**	**3305.2**	**3305.4**	3084.2, **3305.3**	**2486.1**
Ltnα C1A	**3273.5**	**3274.8**	3274.8, **3291.4°**	2866.2	1998.4	**2489.2**	**1579**
Ltnα C1S	**3290**	**3290.3**	3289.9, **3305.3°**	3253.1	2640.2	**1625.7,** 3025	**1578.9**
Ltnα C1A/S2A	**3275.1**	**3275.1, 3292.1**	3276.08, 3292.1	2266.34, 2786.7, 3275.01	1562.2	**1657.82, 2111.5**	**1994.29, 2493.41**
Ltnα T20S	**3291**	**3291.4**	3291.4, **3307.4°**	**2549.4**	2124.4, **2549.6**	**2109.11**	**1855**
Ltnα T22S	**3291**	**3291.5**	**3291.7**, 3309	**2549.5**, 3307.6°	2552.4, **3307.4°**	**2124.4**	**1855.2,** 2453.7
WT Ltnβ	**2848**	**2847.8**	2847.2, **2863.2°**	**2847.5**	**2847.4**	1546, **2847.8**	**1545.7**
Ltnβ S16T	**2861.5**	**2861.3**	**2861.6**, 2877.6°	**2861.2**	**2861.5**	**1559.9,** 2861.4	**1560**
Ltnβ T26S	**2834**	**2833.8**	2835.4, **2851.4°**	2172.9, **2763.8,** 2848.6°	**2848.6°**	**1545.9,** 2511.4 2752.6	1531.9, **1545.9.**

		Pronase		Proteinase K	
	0 hr	1 hr	3 hr	1 hr	3 hr
WT Ltnα	**3305**	2724.1, 2795.2, **3305.3**	2538.3, **2724.4,** 3306.8	2724.1, 3209.4, **3306.4**	2314.3, **2626.4,**
Ltnα C1A	**3273.5**	**2596,**	2289.8, **2474.9**	**1993.1,** 2438.3, 2907.4	**1992.6,** 2438.8
Ltnα C1S	**3290**	-	-	**1993.4,** 2438.6, 2907.8	**1993.3,** 2438.5, 2908.6, 2947.5
Ltnα C1A/S2A	**3275.1**	2146.1, 2273.9, 2308.8, 2436.9	2146.1, 2274.2, 2688.5	**1856.9, 1993.3, 2171.9, 2907.3**	**1993.3, 2174.2, 2657.6, 2907.3**
Ltnα T20S	**3291**	**2496.9,** 3291.2	1990.8, 2248.8, **2376.9**	**1993.2,** 2438.4	**1993.1,** 2439
Ltnα T22S	**3291**	**2381.1,** 2527.1, 3308.7	**2540.1**	**2249.9,**	**2156.5,**
WT Ltnβ	**2848**	**2847.8**	**2292.9,** 2847.5	1709.1, **1851.2,** 2847.9	1709, 1851
Ltnβ S16T	**2861.5**	2275.2, **2861.3**	2275.2, **2861.5**	1723.2, **1865.3,** 2861.5	1722.9, **1865.1**
Ltnβ T26S	**2834**	2074.3, **2377.5,** 2564.5, 2833.7, 2850.5°	**2071.8,** 2485.3	**1708.6,** 1851.2, 2438.7	**1708.6,** 1850.7, 2439, 2834

Masses of resultant fragments are presented. Dominant peak is in bold, and mass corresponding to undigested peptide is underlined (peaks corresponding to those observed in control reactions, i.e., enzyme without peptide, have been excluded). See also Figures S3–S7.

whereas the peptides in which Ring A of Ltnα was removed were consistently among the most protease sensitive.

DISCUSSION

Lanthionine bridges are the key distinctive features that differentiate lantibiotics from other antimicrobials. The importance of these bridges with respect to imposing structure on these peptides is very evident. However, only one study involving Pep5 has investigated the role of specific bridges in providing resistance to proteases (Bierbaum et al., 1996), and no corresponding thermotolerance studies have taken place. In this study an extensive mutagenesis of Ring A of the lacticin 3147 Ltnα peptide was carried out to determine the impact of the changes made on the antimicrobial activity of these peptides. This strategy was prompted by an alanine-scanning mutagenesis study that previously established that the LtnαC1A and LtnαS2A mutants both retained significant levels of antimicrobial activity. The tolerance of Ltnα Ring A to change, together with its atypical orientation and the fact that it bridges two adjacent residues, makes it particularly fascinating. In this study the Ring A mutants LtnαS2T, S2C, C1S, and C1S-S2C and C1A-S2A were generated. Although analysis of the extended set of "Ring A" mutants established that all peptides retained activity (reaffirming the nonessential nature of the natural Lan structure), the potency of the peptides relative to one another differed from one target to another, indicating that Ring A, and perhaps the extended N-terminal domain, plays a role in the target specificity of the peptide. Other notable features included the failure to introduce new structures at the N terminus (other than a simple Lan to meLan exchange) and the relatively poor activity of LtnαS2T. As a result of the negative consequences that were apparent when other residues involved in (me)Lan formation were converted to alanine (Cotter et al., 2006), the more subtle Lan/meLan interconversion strategy was employed at other locations. There have also been examples of the successful interconversion of Lan and MeLan bridges, in that in lacticin 481 the engineered peptides T33S (MeLan to Lan, Ring A), S35T, and S42T (Lan to MeLan; Ring B and C) all retained antimicrobial activity (Chatterjee et al., 2006). The production of bioengineered derivatives of the closely related nukacin ISK-1 peptide in which Lan-MeLan interconversions (or other bridge manipulations) have taken place has also been demonstrated. However, the impact of these changes on antimicrobial activity is not known (Shioya et al., 2009). The (me)Lan bridge variants of lacticin 3147, i.e., LtnαS2T (as noted above), LtnαS9T, LtnαT20S and LtnαT22S, LtnβS16T, LtnβT22S, and LtnβT26S, all retained at least some antimicrobial activity. When one focuses on the bridges that are conserved among other lacticin 3147-like two-peptide lantibiotics (all except Ltnα Ring A), natural variation is, in general, a strong indicator of how well Lan-meLan interconversion will be tolerated. For example both Ser and Thr residues are found at locations corresponding to LtnαT22, LtnβS16, and LtnβT26, whereas those corresponding to LtnαS9 and LtnαT20 are invariable. LtnβT22S is exceptional in this regard in that, despite more closely resembling the SmbA and bhtA-β peptides, this peptide retains unexpectedly low activity against *L. lactis* HP. The corresponding bridge is located within a domain of Ltnβ that is proposed to be involved in Ltnα-Ltnβ synergy (Cotter et al., 2006) and, on the basis of this result, may be more important than other adjacent bridges in this regard.

The availability of these various bioengineered peptides facilitated an investigation of their relative sensitivities to high temperature and proteolytic enzymes. The impact of the heat treatment employed had a similar effect on both wild-type lacticin 3147 peptide and (β-methyl) lanthionine bridge variant peptides with the exception of the representative Ring A mutants, αC1A and αC1S, the antimicrobial activity of which was particularly affected by high temperature treatment. Although many lantibiotics exhibit a high innate thermotolerance, which has been assumed to be due at least in part to the (me)Lan bridges, this is the first instance in which it has been demonstrated that removal of a Lan bridge results in thermal sensitivity. Mass spectrometry indicates that this reduced activity is due to the cleavage of the peptide between E24 and the meLan (Ring C) derived from a C25 in the LtnA1 propeptide. Proteolytic enzyme studies combined with mass spectrometry revealed that the natural Ltnα and Ltnβ peptides were consistently the most resistant to digestion. Of the bioengineered peptides the Ltnβ derivatives, and LtnβS16T in particular, were least susceptible to the activities of the various enzymes, whereas LtnαT20S and, to a greater extent, LtnαC1A/S2A, LtnαC1A, and C1S were particularly sensitive. This sensitivity was also apparent in the form of reduced antimicrobial activity after pepsin treatment, which did not alter the activity of the other peptides. This study provides further evidence of the benefits of possessing (me)Lan or related cyclic structures (Cooper et al., 2008) at the N terminus of lantibiotics, as is the case for the Ltnα-like peptides and, indeed, many of the extended mersacidin-like peptide group. It also reveals the target-specific consequences of manipulating these residues. More significantly, it has confirmed the importance of such structures in providing resistance to proteolytic enzymes and, for the first time, high temperature and will presumably attract even greater interest in the use of (me)Lan structures to enhance the stability of non-lantibiotic compounds.

SIGNIFICANCE

This study is focused on the consequences of manipulating the Lan and meLan bridges of the lacticin 3147 peptides (Ltnα and Ltnβ) with respect to antimicrobial activity and resistance to thermal stress and proteolytic digestion. These investigations are particularly significant in that, although often claimed to be important, the actual contribution of Lan and meLan structures to the stability of lantibiotics has not been the subject of such a detailed study previously. It is revealed that, whereas the interconversion of Lan and meLan bridges has varying consequences with respect to activity and stability, Ltnα bridge mutants are consistently more sensitive to thermal treatment and proteolytic digestion than natural Ltnα. In contrast, their Ltnβ counterparts display a resistance to thermal and proteolytic cleavage that is comparable with that of natural Ltnβ. Most notably, it is established that, although Ring A of Ltnα is not essential for bioactivity, it does provide protection against thermal stress and proteolytic digestion,

thereby suggesting that the introduction of such structures could also enhance the stability of non-lantibiotic peptides.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, Enzymes, Antibiotics, and Growth Conditions

Wild-type Lacticin 3147 producer *Lactococcus lactis* MG1363 pMRC01 (Cotter et al., 2005b; McAuliffe et al., 2000)) and all the bridge variants of Lacticin 3147 and indicator strain *L. lactis* HP were grown without aeration at 30°C in M17 broth supplementing with 0.5% glucose. *Micrococcus luteus* was grown in Muller Hinton Broth (MHB) at 30°C, *Escherichia coli* EC101 was grown with aeration at 37°C in Luria–Bertani (LB) broth. Antibiotics were used at the following concentrations: 150 μg/ml erythromycin for *E. coli* and 5 μg/ml erythromycin; 5 μg/ml chloramphenicol for *L. lactis*; and tetracycline 10 μg/ml for *L. lactis*. X-gal (5-bromo-4-chloro-3-indole-β-D-galactopyranoside) at a concentration of 50 μg/ml was used. 100mM Tris-HCl-10mM $CaCl_2$ solution was used to dissolve the enzymes Pepsin, α-Chymotrypsin, Protease, and Proteinase K (chemicals and enzymes from Sigma-Aldrich, Ireland). All bacterial strains and plasmids used in this study are presented in Table S1, and oligonucleotides used in study are presented in Table S2.

Site-Directed Mutagenesis

The QuickChange site-directed mutagenesis system (Stratagene) was employed to engineer the *ltnA1* and *A2* genes within the pORI280/*ltnA1A2* (RepA−, LacZ+) vector as described previously by Cotter et al. (2003, 2005b). The QuickChange protocol was followed in all steps, except in one step where *E. coli* host, EC101 (RepA+), was used. Transformants were screened with a colony check PCR, using LtnA1SOED and a mutation-specific primer, which was designed to amplify mutated region. Candidates were confirmed by DNA sequencing to ensure only mutated change was present and no other alterations in the gene. The mutated plasmid was then electroporated into competent *L. lactis* MG1363 pMRC01 pVE6007 (Cotter et al., 2003).

The pVE6007 plasmid is temperature-sensitive $RepA^+$ helper plasmid and needed because the MG1363, pMRC01, and pORI280 lack the RepA gene. The mutated pORI280 plasmids were integrated by single crossover with the LtnA1A2 genes on pMRC01 by growing the cultures initially overnight at 30°C (GM17 + Ery), followed by two passages (0.1% inoculum) at 37°C. Because pVE6007 is temperature sensitive, growing at 37°C will lead to the loss of this plasmid and, consequently, pORI280. Only the cells in which a single crossover happened are Ery resistant and will survive during growth. The second transfer was streaked out (GM17agar + Xgal + Ery), and colonies were checked for chloramphenicol sensitivity, which confirms the loss of pVE6007. Individual colonies were incubated in GM17 at 37°C and subcultured continuously (0.1% inoculum). At regular intervals the cultures were diluted and plated out on pre-warmed GM17-Xgal plates and incubated at 37°C to find $LacZ^-$ colonies. White colonies ($LacZ^-$) indicated the loss of pORI280 and the occurrence of a second homologous recombination event. These colonies were again checked with check PCR to differentiate between mutant and wild-type revertants and sequenced to confirm PCR results. MGpMRαT22S and βS16T were generated through bioengineering of pDF01, i.e., pCi372-PbacA1A2 (Field et al., 2007). Mutagenesis was performed via PCR with Phusion DNA polymerase using 5′ phosphorylated forward primer and nonphosphorylated reverse primers (Table 2). Amplified PCR product was then subjected to DpnI treatment for 1 hr at 37°C and introduced into *E. coli* TOP10. The respective plasmid pCI372-Pbac A1A2 was isolated from overnight culture, and the mutated PbacA1A2 insert was amplified using pPTPLA1A2For(BglII) and pPTPLA1A2Rev(XbaI), digested with BglII and XbaI enzymes (Roche) ligated into similarly digested pPTPL and then cloned into *E. coli* MC1000. The recombinant plasmids were isolated and, following DNA sequence-based confirmation that mutagenesis has been successful, were introduced in to *L. lactis* MG1363 pOM44.

Antimicrobial Activity Assays

The bioactivity of the mutated peptides was tested by doing well-diffusion assays with the lacticin 3147 sensitive *L. lactis* HP as an indicator strain, as described previously (Ryan et al., 1996). Fifty microliters overnight culture of *L. lactis* HP was added to 20 ml molten GM17 agar, which was cooled to 50°C (~10^6 overnight-grown cells per milliliter), poured into a sterile Petri dish, and allowed to solidify and dried. Wells, 5.8 mm in diameter, were made in the solidified agar plates and filled with 50 μl of cell-free supernatant from overnight cultures of the peptide-producing strains. The plates were then incubated overnight at 30°C, and the zones of inhibition were measured. Differed antagonism assays were carried out by spotting 2 μl of overnight culture (~2×10^8 CFU/ml) of the producing strain onto GM17 agar plates. Spotted GM17 plates were incubated at 30°C overnight and then plates were treated with UV irradiation for 30 min, then molten GM17 agar with 1×10^6 CFU/ml of *L. lactis* HP was overlayed and allowed to solidify and then incubated at 30°C overnight. MIC determinations were performed as described previously (Cotter et al., 2006) in micro-titer plates. *L. lactis* HP was grown in M17 broth plus 0.5% glucose (Oxoid), and *M. luteus* DSM1790 was grown at 30°C in MHB. Serial 2-fold dilutions of the peptides were made in the growth medium of the indicator strain (GM17 or MHB). Bacteria were then added to give a final inoculum of 10^5 CFU/ml in a volume of 0.2 ml. After incubating at 30°C for 16 hr (optimum growth conditions for *L. lactis* HP and *M. luteus*), MIC values were taken as the lowest peptide concentration causing inhibition of visible growth. MIC results given are mean values of three independent determinations.

Purification and Production of Peptides

Strains from which peptide was purified were grown in modified TY broth. An overnight culture of the producing strain (in GM17) was inoculated into 1 liter of modified TY broth (1% inoculum) and grown overnight at 30°C. The cells were harvested by centrifugation at 7000 rpm for 20 min, and the cell pellet was resuspended in 250 ml of 70% propan-2-ol (pH 2.0 adjusted with concentrated HCl) and stirred for 4 hr at 4°C. The mixture was then centrifuged at 7000 rpm for 20 min; the supernatant was reduced to 60 ml by removing propan-2-ol by rotary evaporation. A Varian C18 Bond Column 10 g (60 ml volume) was washed consecutively with 60 ml 100% methanol and 60 ml water. Then the rotary evaporated sample was loaded on this column, washed with 120 ml 30% ethanol, and finally peptide eluted in 100 ml 70% 2-propanol (pH 2.0) and stored at 4°C. Twenty milliliter volumes of the 100 ml elute were reduced to 2 ml by rotary evaporation, and aliquots of 1650 μl were then applied to a Phenomenex (Phenomenex, Cheshire, UK) C12 reverse-phase RP-HPLC column (Jupiter 4u Proteo 90 Å, 250 × 10.0 mm, 4 μm) previously equilibrated with 25% propan-2-ol, 0.1% trifluoroacetic acid (TFA), which was repeated until all 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. The HPLC fractions collected at peaks of the peptide A1 or A2, after all the HPLC runs the fractions were pooled and rotary evaporated to reduce the amount of propan-2-ol and then treated with N2 gas and kept frozen at −80°C, these frozen samples were freeze dried to get powder form of peptide, which will be used for quantified antimicrobial assay and mass spectrometric analysis of peptide. The purified proteins were subjected to MALDI-ToF MS analysis (Shimadzu) to check the purity of the peptides.

Cyanylation

Purified freeze-dried lacticin 3147 peptides were dissolved in a reducing buffer 22 μl of 10 mM TCEP (aqueous solution) (Wu, 2008; Wu and Watson, 1998) for 15 min at room temperature and then combined by adding 6 μl of 100 mM CDAP (made with citrate buffer [pH3.0]) (Wu, 2008; Wu and Watson, 1998) incubating another 15 min at room temperature.

After cyanylation the reaction mixture was then separated and purified using RP-HPLC, and HPLC fractions were collected and further analyzed by combining 1 μl of HPLC fraction with 1 μl of α-CHCA (Wu, 2008) using MALDI-ToF MS analysis (Shimadzu) to measure the mass of cyanylated peptides.

Impact of Proteases on (β-methyl) Lanthionine Bridges/Protease Assay

Stability of wild-type and mutant peptides against proteases was carried out with RP-HPLC purified peptides as described previously (Gardiner et al., 2007). Enzymes were dissolved in 100 mM Tris HCl-10 mM $CaCl_2$ at 30°C. Peptides at 10 μg/ml concentrations were treated with enzymes at 100 μg/ml

concentrations. Mass spectrometric analysis of the peptides was carried out at 1 and 3 hr time points as described previously (Cotter et al., 2006). Pepsin, α-chymotrypsin, pronase, and proteinase K (Sigma-Aldrich, Dublin, Ireland) dissolved in buffer (100 mM Tris HCl-10 mM $CaCl_2$) at their optimum pH (8, 2, 7.8, 7.0, and 7.5, respectively). Well assay with supernatant after treating with enzymes and MIC determination with peptides after treating with enzymes at a 1 hr time point were also carried out.

Assessment of the Impact of High Temperature on Lacticin 3147 Peptides

To assess the impact of high temperature on the lacticin 3147 peptides, and bioengineered derivatives, the peptides are dissolved at a concentration of 10 μg/ml in 1% 100 mM Tris HCl-10 mM $CaCl_2$ buffer; peptides used for treatment are heated to 80°C for 30 min, after which time antimicrobial activity and mass spectrometric analyses were carried out.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and two tables and can be found with this article online at doi:10.1016/j.chembiol.2010.08.011.

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REFERENCES

Bierbaum, G., Szekat, C., Josten, M., Heidrich, C., Kempter, C., Jung, G., and Sahl, H.G. (1996). Engineering of a novel thioether bridge and role of modified residues in the lantibiotic Pep5. Appl. Environ. Microbiol. *62*, 385–392.

Breukink, E., and de Kruijff, B. (1999). The lantibiotic nisin, a special case or not? Biochim. Biophys. Acta *1462*, 223–234.

Breukink, E., Wiedemann, I., van Kraaij, C., Kuipers, O.P., Sahl, H., and de Kruijff, B. (1999). Use of the cell wall precursor lipid II by a pore-forming peptide antibiotic. Science *286*, 2361–2364.

Brotz, H., Josten, M., Wiedemann, I., Schneider, U., Gotz, F., Bierbaum, G., and Sahl, H.G. (1998). Role of lipid-bound peptidoglycan precursors in the formation of pores by nisin, epidermin and other lantibiotics. Mol. Microbiol. *30*, 317–327.

Brumfitt, W., Salton, M.R., and Hamilton-Miller, J.M. (2002). Nisin, alone and combined with peptidoglycan-modulating antibiotics: activity against methicillin-resistant Staphylococcus aureus and vancomycin-resistant enterococci. J. Antimicrob. Chemother. *50*, 731–734.

Chatterjee, S., Lad, S.J., Phansalkar, M.S., Rupp, R.H., Ganguli, B.N., Fehlhaber, H.W., and Kogler, H. (1992). Mersacidin, a new antibiotic from Bacillus. Fermentation, isolation, purification and chemical characterization. J. Antibiot. (Tokyo) *45*, 832–838.

Chatterjee, C., Paul, M., Xie, L., and van der Donk, W.A. (2005). Biosynthesis and mode of action of lantibiotics. Chem. Rev. *105*, 633–684.

Chatterjee, C., Patton, G.C., Cooper, L., Paul, M., and van der Donk, W.A. (2006). Engineering dehydro amino acids and thioethers into peptides using lacticin 481 synthetase. Chem. Biol. *13*, 1109–1117.

Chen, P., Novak, J., Kirk, M., Barnes, S., Qi, F., and Caufield, P.W. (1998). Structure-activity study of the lantibiotic mutacin II from Streptococcus mutans T8 by a gene replacement strategy. Appl. Environ. Microbiol. *64*, 2335–2340.

Cooper, L.E., McClerren, A.L., Chary, A., and van der Donk, W.A. (2008). Structure-activity relationship studies of the two-component lantibiotic haloduracin. Chem. Biol. *15*, 1035–1045.

Cotter, P.D., Hill, C., and Ross, R.P. (2003). A food-grade approach for functional analysis and modification of native plasmids in Lactococcus lactis. Appl. Environ. Microbiol. *69*, 702–706.

Cotter, P.D., Hill, C., and Ross, R.P. (2005a). Bacterial lantibiotics: strategies to improve therapeutic potential. Curr. Protein Pept. Sci. *6*, 61–75.

Cotter, P.D., O'Connor, P.M., Draper, L.A., Lawton, E.M., Deegan, L.H., Hill, C., and Ross, R.P. (2005b). Posttranslational conversion of L-serines to D-alanines is vital for optimal production and activity of the lantibiotic lacticin 3147. Proc. Natl. Acad. Sci. USA *102*, 18584–18589.

Cotter, P.D., Deegan, L.H., Lawton, E.M., Draper, L.A., O'Connor, P.M., Hill, C., and Ross, R.P. (2006). Complete alanine scanning of the two-component lantibiotic lacticin 3147: generating a blueprint for rational drug design. Mol. Microbiol. *62*, 735–747.

Field, D., Collins, B., Cotter, P.D., Hill, C., and Ross, R.P. (2007). A system for the random mutagenesis of the two-peptide lantibiotic lacticin 3147: analysis of mutants producing reduced antibacterial activities. J. Mol. Microbiol. Biotechnol. *13*, 226–234.

Field, D., Connor, P.M., Cotter, P.D., Hill, C., and Ross, R.P. (2008). The generation of nisin variants with enhanced activity against specific gram-positive pathogens. Mol. Microbiol. *69*, 218–230.

Galvin, M., Hill, C., and Ross, R.P. (1999). Lacticin 3147 displays activity in buffer against gram-positive bacterial pathogens which appear insensitive in standard plate assays. Lett. Appl. Microbiol. *28*, 355–358.

Gardiner, G.E., Rea, M.C., O'Riordan, B., O'Connor, P., Morgan, S.M., Lawlor, P.G., Lynch, P.B., Cronin, M., Ross, R.P., and Hill, C. (2007). Fate of the two-component lantibiotic lacticin 3147 in the gastrointestinal tract. Appl. Environ. Microbiol. *73*, 7103–7109.

Hsu, S.T., Breukink, E., Bierbaum, G., Sahl, H.G., de Kruijff, B., Kaptein, R., van Nuland, N.A., and Bonvin, A.M. (2003). NMR study of mersacidin and lipid II interaction in dodecylphosphocholine micelles. Conformational changes are a key to antimicrobial activity. J. Biol. Chem. *278*, 13110–13117.

Hurst, A. (1981). Nisin. Advances in Applied Microbiology, Volume 27, D. Perlman and A.I. Laskin, eds. (New York: Academic Press), pp. 85-123.

Kluskens, L.D., Nelemans, S.A., Rink, R., de Vries, L., Meter-Arkema, A., Wang, Y., Walther, T., Kuipers, A., Moll, G.N., and Haas, M. (2009). Angiotensin-(1-7) with thioether bridge: an angiotensin-converting enzyme-resistant, potent angiotensin-(1-7) analog. J. Pharmacol. Exp. Ther. *328*, 849–854.

Kruszewska, D., Sahl, H.G., Bierbaum, G., Pag, U., Hynes, S.O., and Ljungh, A. (2004). Mersacidin eradicates methicillin-resistant Staphylococcus aureus (MRSA) in a mouse rhinitis model. J. Antimicrob. Chemother. *54*, 648–653.

Lawton, E.M., Ross, R.P., Hill, C., and Cotter, P.D. (2007). Two-peptide lantibiotics: a medical perspective. Mini Rev. Med. Chem. *7*, 1236–1247.

Martin, N.I., Sprules, T., Carpenter, M.R., Cotter, P.D., Hill, C., Ross, R.P., and Vederas, J.C. (2004). Structural characterization of lacticin 3147, a two-peptide lantibiotic with synergistic activity. Biochemistry *43*, 3049–3056.

McAuliffe, O., Hill, C., and Ross, R.P. (2000). Each peptide of the two-component lantibiotic lacticin 3147 requires a separate modification enzyme for activity. Microbiology *146*, 2147–2154.

Navaratna, M.A., Sahl, H.G., and Tagg, J.R. (1998). Two-component anti-Staphylococcus aureus lantibiotic activity produced by Staphylococcus aureus C55. Appl. Environ. Microbiol. *64*, 4803–4808.

O'Connor, E.B., Cotter, P.D., O'Connor, P., O'Sullivan, O., Tagg, J.R., Ross, R.P., and Hill, C. (2007). Relatedness between the two-component lantibiotics lacticin 3147 and staphylococcin C55 based on structure, genetics and biological activity. BMC Microbiol. *7*, 24.

Osapay, G., Prokai, L., Kim, H.S., Medzihradszky, K.F., Coy, D.H., Liapakis, G., Reisine, T., Melacini, G., Zhu, Q., Wang, S.H., et al. (1997). Lanthionine-somatostatin analogs: synthesis, characterization, biological activity, and enzymatic stability studies. J. Med. Chem. *40*, 2241–2251.

Pag, U., and Sahl, H.G. (2002). Multiple activities in lantibiotics–models for the design of novel antibiotics? Curr. Pharm. Des. *8*, 815–833.

Piper, C., Cotter, P.D., Ross, R.P., and Hill, C. (2009a). Discovery of medically significant lantibiotics. Curr. Drug Discov. Technol. *6*, 1–18.

Piper, C., Draper, L.A., Cotter, P.D., Ross, R.P., and Hill, C. (2009b). A comparison of the activities of lacticin 3147 and nisin against drug-resistant Staphylococcus aureus and Enterococcus species. J. Antimicrob. Chemother. *64*, 546–551.

Rea, M.C., Clayton, E., O'Connor, P.M., Shanahan, F., Kiely, B., Ross, R.P., and Hill, C. (2007). Antimicrobial activity of lacticin 3,147 against clinical Clostridium difficile strains. J. Med. Microbiol. *56*, 940–946.

Rew, Y., Malkmus, S., Svensson, C., Yaksh, T.L., Chung, N.N., Schiller, P.W., Cassel, J.A., DeHaven, R.N., Taulane, J.P., and Goodman, M. (2002). Synthesis and biological activities of cyclic lanthionine enkephalin analogues: delta-opioid receptor selective ligands. J. Med. Chem. *45*, 3746–3754.

Rink, R., Arkema-Meter, A., Baudoin, I., Post, E., Kuipers, A., Nelemans, S.A., Akanbi, M.H., and Moll, G.N. (2010). To protect peptide pharmaceuticals against peptidases. J. Pharmacol. Toxicol. Methods *61*, 210–218.

Ryan, M.P., Rea, M.C., Hill, C., and Ross, R.P. (1996). An application in cheddar cheese manufacture for a strain of Lactococcus lactis producing a novel broad-spectrum bacteriocin, lacticin 3147. Appl. Environ. Microbiol. *62*, 612–619.

Ryan, M.P., Flynn, J., Hill, C., Ross, R.P., and Meaney, W.J. (1999a). The natural food grade inhibitor, lacticin 3147, reduced the incidence of mastitis after experimental challenge with Streptococcus dysgalactiae in nonlactating dairy cows. J. Dairy Sci. *82*, 2625–2631.

Ryan, M.P., Jack, R.W., Josten, M., Sahl, H.G., Jung, G., Ross, R.P., and Hill, C. (1999b). Extensive post-translational modification, including serine to D-alanine conversion, in the two-component lantibiotic, lacticin 3147. J. Biol. Chem. *274*, 37544–37550.

Shioya, K., Harada, Y., Nagao, J.I., Nakayama, J., and Sonomoto, K. (2009). Characterization of modification enzyme NukM and engineering of a novel thioether bridge in lantibiotic nukacin ISK-1. Appl. Microbiol. Biotechnol. *86*, 891–899.

Wiedemann, I., Bottiger, T., Bonelli, R.R., Wiese, A., Hagge, S.O., Gutsmann, T., Seydel, U., Deegan, L., Hill, C., Ross, P., et al. (2006). The mode of action of the lantibiotic lacticin 3147–a complex mechanism involving specific interaction of two peptides and the cell wall precursor lipid II. Mol. Microbiol. *61*, 285–296.

Willey, J.M., and van der Donk, W.A. (2007). Lantibiotics: peptides of diverse structure and function. Annu. Rev. Microbiol. *61*, 477–501.

Wu, J. (2008). Disulfide bond mapping by cyanylation-induced cleavage and mass spectrometry. Methods Mol. Biol. *446*, 1–20.

Wu, J., and Watson, J.T. (1998). Optimization of the cleavage reaction for cyanylated cysteinyl proteins for efficient and simplified mass mapping. Anal. Biochem. *258*, 268–276.

Xie, L., and van der Donk, W.A. (2004). Post-translational modifications during lantibiotic biosynthesis. Curr. Opin. Chem. Biol. *8*, 498–507.

