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Manipulation of charged residues within the two-peptide lantibiotic lacticin 3147

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Summary

Lantibiotics are antimicrobial peptides which contain a high percentage of post-translationally modified residues. While most attention has been paid to the role of these critical structural features, evidence continues to emerge that charged amino acids also play a key role in these peptides. Here 16 'charge' mutants of the two-peptide lantibiotic lacticin 3147 [composed of Ltnα (2+, 2–) and Ltnβ (2+)] were constructed which, when supplemented with previously generated peptides, results in a total bank of 23 derivatives altered in one or more charged residues. When examined individually, in combination with a wild-type partner or, in some instances, in combination with one another, these mutants reveal the importance of charge at specific locations within Ltnα and Ltnβ, confirm the critical role of the negatively charged glutamate residue in Ltnα and facilitate an investigation of the contribution of positively charged residues to the cationic Ltnβ. From these investigations it is also apparent that the relative importance of the overall charge of lacticin 3147 varies depending on the target bacteria and is most evident when strains with more negatively charged cell envelopes are targeted. These studies also result in, for the first time, the creation of a derivative of a lacticin 3147 peptide (LtnβR27A) which displays enhanced specific activity.

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Introduction

Lantibiotics are antimicrobial peptides that have attracted increasing attention in recent years as a consequence of their activity at nanomolar concentrations, multiple combined mechanisms of action and activity against multidrug-resistant pathogens (Brotz *et al.*, 1998; Breukink *et al.*, 1999; Galvin *et al.*, 1999; Hasper *et al.*, 2006; Hyde *et al.*, 2006; Wiedemann *et al.*, 2006; Castiglione *et al.*, 2008; Parisot *et al.*, 2008; Smith *et al.*, 2008). These peptides are distinguished by the presence of the unusual lanthionine (Lan) and β-methyllanthionine residues which result from sulfide (or thioether) bridges formed between cysteines and neighbouring dehydrated residues during post-translational modification of ribosomally synthesized peptides (Pag and Sahl, 2002; Xie and van der Donk, 2004; Chatterjee *et al.*, 2005; Cotter *et al.*, 2005b; Willey and van der Donk, 2007). Other than the modified residues, charged amino acids have most frequently been associated with optimal lantibiotic activity (Rollema *et al.*, 1995; Kuipers *et al.*, 1996; Breukink *et al.*, 1997; Van Kraaij *et al.*, 1997; Szekat *et al.*, 2003; Yuan *et al.*, 2004; Chatterjee *et al.*, 2006; Cotter *et al.*, 2006; Field *et al.*, 2007; 2008). In fact, such is the significance of peptide charge that one of the original approaches to the classification of lantibiotics was based on this trait, i.e. linear, cationic peptides were classified as type A lantibiotics (originally thought to function exclusively through pore-formation) while type B lantibiotics consisted of globular uncharged or negatively charged peptides (functioning through the inhibition of enzymatic activity) (Jung, 1991). The role of positively charged residues is thought to be particularly important in pore-forming lantibiotics as the attraction between these residues and the anionic target membrane is considered to be the driving force for critical events such as insertion into the membrane and pore-formation. This is analogous to the mechanism of action of cationic antimicrobial peptides such as magainins and defensins (Sahl *et al.*, 2005) and could explain why an increase in the charge of the cell envelope of target cells, e.g. through D-alanylation of teichoic acid or lysinylation of phospholipids, can negatively impact on the effectiveness of cationic peptides (Peschel *et al.*, 1999; 2001; Abachin *et al.*, 2002; Poyart *et al.*, 2003; Kristian *et al.*, 2005; Kovacs *et al.*, 2006; Thedieck *et al.*, 2006; Herbert *et al.*, 2007).

Due to an enhanced appreciation of the mode of action of a few selected lantibiotics, it is now apparent that both uncharged globular binding domains and cationic linear pore-forming domains can coexist within a single peptide, e.g. the N- and C-termini, respectively, of the prototypical lantibiotic nisin (Breukink *et al.*, 1999; Wiedemann *et al.*, 2001). Studies with nisin have also provided evidence of the benefits of manipulating charged residues within lantibiotics. Conversion of the residues ISL within the N-terminal ring (ring A) of nisin to either KSI or KFI resulted in an increased IC_{50} against a number of target strains (Rink *et al.*, 2007) whereas the replacement of a lysine within the hinge region of the peptide, i.e. the region connecting the N- and C-terminal domains, with a threonine or serine, increased the activity against *Streptococcus agalactiae* and *Staphylococcus aureus* (Field *et al.*, 2008). In the case of the two-peptide lantibiotics such as lacticin 3147, globular and linear domains can be split across two peptides which each possess antimicrobial activities that are dramatically increased when combined (Morgan *et al.*, 2005; Wiedemann *et al.*, 2006; Lawton *et al.*, 2007b). In the case of lacticin 3147, the two peptides are Ltnα and β, encoded by *ltnA1* and *A2* respectively (Fig. 1). The Ltnα peptide is globular and has no net charge (2+; H23 and K30, and 2–; D10 and E24) whereas Ltnβ has a more elongated structure and is cationic (2+; K24 and R27) (Fig. 1). Converting each of the six charged residues to alanine as part of a complete alanine scanning mutagenesis study established that all of the newly generated producers, except *Lactococcus lactis* MGpMRαE24A, retained at least some bioactivity (i.e. a combined assessment that is impacted upon by both specific activity and production) (Cotter *et al.*, 2006). The lack of tolerance of E24 to change highlighted the importance of this negatively charged residue which is highly conserved among related peptides and is thought to have a key role in binding lipid II (Szekat *et al.*, 2003; Cotter *et al.*, 2006; Lawton *et al.*, 2007b). A seventh lacticin 3147 charge mutant, in which an additional lysine residue was introduced into Ltnα such that it more closely resembled the related C55α peptide of staphylococcin C55 (LtnαN15K), exhibited an only slightly reduced bioactivity. Unfortunately, however, a selection of seven 'charge' mutants can only provide us with a limited insight into the importance and tolerance to change of the charged residues within lacticin 3147. To address this, 16 additional mutants in which charged residues are introduced, replaced or exchanged were constructed to generate the largest bank of lantibiotic 'charge' mutants to date. This bank of 23 charge mutants (Fig. 2) allowed a detailed investigation of the importance of individual charged residues against wild-type target strains and strains with an altered cell envelope charge. This study provides further evidence of the varying extents to which these amino acids are tolerant of change, revealing that, in general, the consequences of the changes made depends more on location-specific impacts than on the overall effect on peptide charge and demonstrates that bioactivity can be retained even when the charge of these peptides can be altered by two, such as in the extreme example of a Ltnβ which is no longer cationic. This study is also noteworthy in that it describes the first example of a modified version of a lacticin 3147 peptide which displays enhanced antimicrobial activity.

Results

Design and construction of lacticin 3147 derivatives with altered charge residues

In addition to lacticin 3147, five related two-peptide lantibiotics, staphylococcin C55, haloduracin, plantaricin W, Smb and BHT-A, have been identified (Navaratna *et al.*, 1998; Holo *et al.*, 2001; Hyink *et al.*, 2005; Yonezawa and Kuramitsu, 2005; McClerren *et al.*, 2006; Lawton *et al.*, 2007a,b) (Fig. 1). An analysis of the amino acid sequence of the individual peptides in each case reveals that in all cases the β peptides are cationic with a positive charge ranging from +1 to +7 and all lack negatively charged residues (Fig. 1). These positively charged residues are C-terminally located, with all peptides containing a conserved Lys within the second last ring and/or an Arg within the last ring, while negatively charged residues are absent. Smbβ and BHT-Aβ are distinguished by the presence of an additional three residue stretch at the C-terminus, which includes a C-terminal Lys (Hyink *et al.*, 2005; Yonezawa and Kuramitsu, 2005) while the plantaricin W Plwβ peptide represents an extreme case possessing seven positively charged amino acids including the four most C-terminally located residues (Holo *et al.*, 2001). Curiously, Ltnα is the only example of an α component of a two-peptide lantibiotic which is not positively charged. Thus, although these peptides are structurally related to the classical, type B, one peptide, lantibiotic mersacidin (overall charge of –1; Altena *et al.*, 2000), the overall charge of this extended subgroup of lantibiotics varies. When Ltnα is compared with other α peptides, it is apparent that all contain a conserved, and essential, Glu (Cotter *et al.*, 2006; Cooper *et al.*, 2008). However, other charged residues are less highly conserved. Asp residues present at a similar location in Ltnα, Sacα and Plwα are the only other negatively charged residue in these peptides. With respect to positively charged residues, the His residue located adjacent to the conserved Glu in Ltnα is also located in Sacα and Plwα but is replaced by a Val in the other three peptides while residues corresponding to LtnαK30 are present in Sacα and Smbα. It is notable however that Ltnα is the only such peptide that does not possess a positively charged residue within its largest,

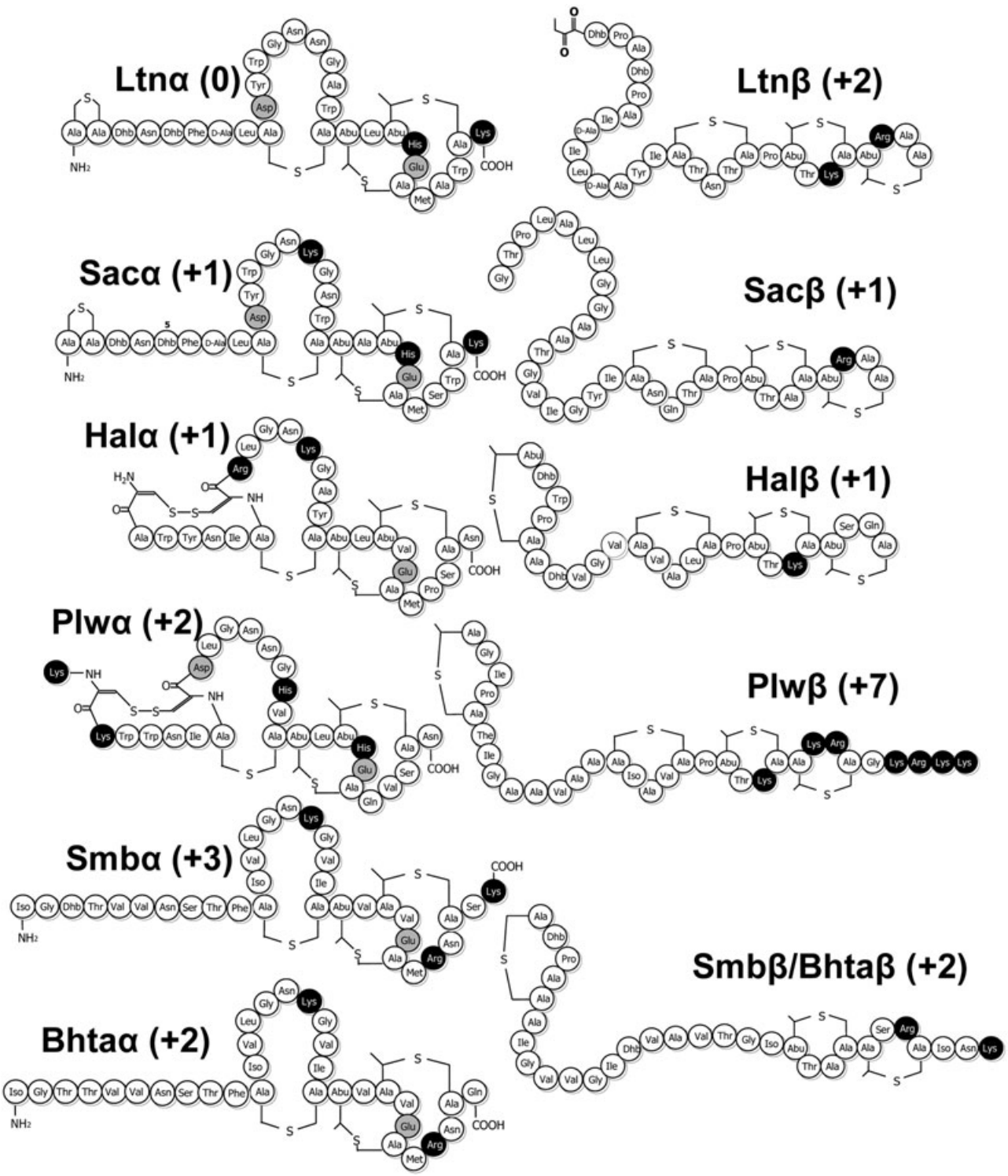

Fig. 1. Location of charged residues within the structures of two-peptide lantibiotics. Lacticin 3147 (Ltnα, Ltnβ) (Martin *et al*., 2004), staphylococcin C55 (Sacα and Sacβ; prediction based on lacticin 3147 structure), haloduracin (Halα, Halβ) (Cooper *et al*., 2008), plantaricin W (Plwα, Plwβ; prediction based on haloduracin structure), Smb (Smbα, Smbβ; predicted) and BHT-A (Bhtα, Bhtβ; predicted). In each case the charge of the individual peptides is indicated. Positively charged residues are represented by black circles while negatively charged residues are grey circles.

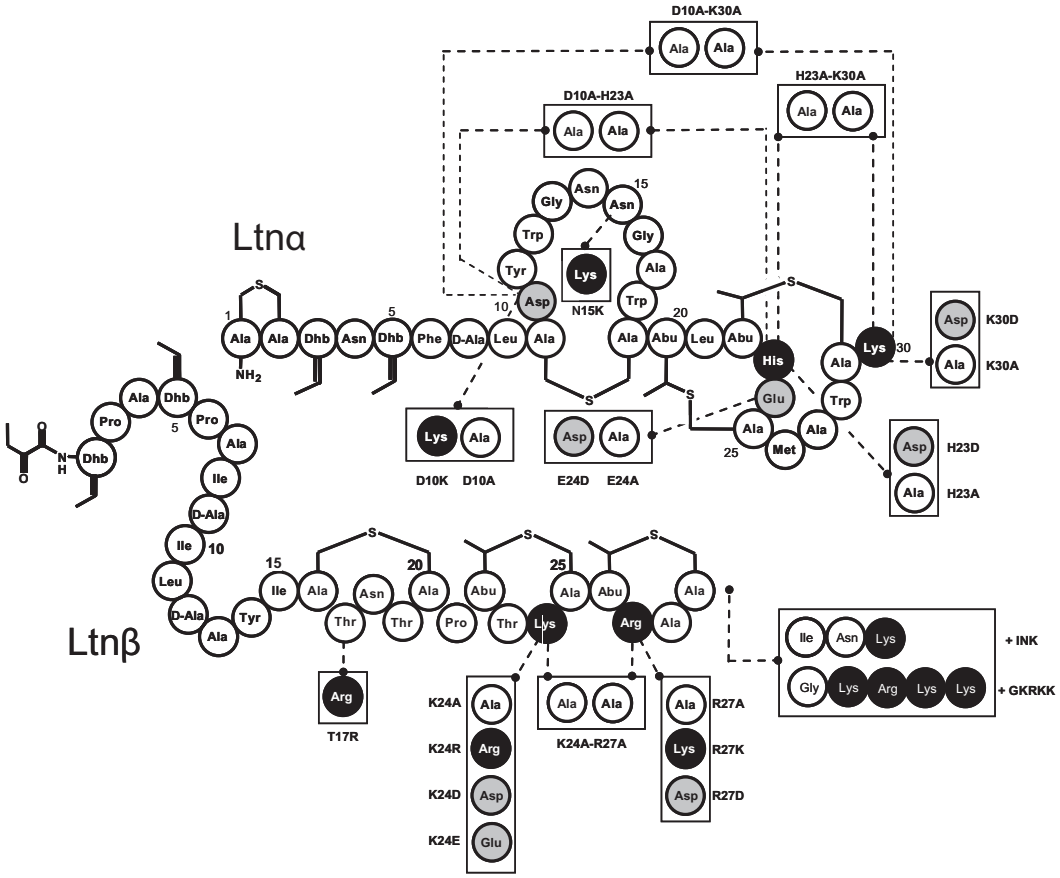

Fig. 2. Structure of the lacticin 3147 peptides, Ltnα and Ltnβ, and the location of bioengineered 'charge' changes.

centrally located, ring. Given the somewhat conserved nature of charged residues in these two-peptide lantibiotics, the consequences of manipulation of these amino acids in lacticin 3147 has the potential to provide data that are pertinent not only to lacticin 3147 but indeed all two-peptide lantibiotics as well as more distantly related mersacidin-like and lacticin 481-like peptides. Here, in addition to a more thorough investigation of the seven 'charge' mutants generated to date, 16 mutants were created to yield an overall total of 23 'charge' derivatives (Table 1, Fig. 2). Although the original investigations revealed that five of the original six charged residues were tolerant to the introduction of an alanine residue, i.e. LtnαD10A, H23A and K30A and LtnβK24A and R27A, they, or producers thereof, retained at least some antimicrobial activity. Here, these five residues were replaced by an amino acid of opposite charge (resulting in a charge alteration of ±2) to test in a more extreme way the consequences of manipulating charge at these locations i.e. LtnαD10K, H23D, K30D, LtnβK24E, K24D and R27D. A number of additional mutants were created with a view to altering the overall charge of the peptides produced to the same extent, but through changes at two locations; i.e. LtnαH23A-K30A and LtnβK24A-R27A. Additional producers of bioengineered peptides, altered at each of two locations (LtnαD10A-H23A and D10A-K30A), were targeted to allow an assessment of altering charged residues without impacting on overall peptide charge. In three instances charged residues were replaced with a similarly charged equivalent (LtnαE24D, LtnβK24R and R27K), while finally, a number of mutants were generated with a view to the production of peptides in which additional positively charged residues were incorporated at a location known to be tolerant of change (i.e. LtnβT17R) or at the C-terminus of Ltnβ (i.e. the residues INK and GKRKK are added at the C-terminus of two derivatives such that they would more closely resemble Smbβ/BHT-Aβ or Plwβ respectively). These changes were brought about through a combination of PCR-based alterations and, in the majority of cases, the mutated genes were introduced in place of *ltnA1* or *ltnA2* by double-crossover recombination. Mass spectrometry established that 18 of the mutants produced peptides of expected mass in all except two cases, bioengineering resulted in the production of a peptide of expected mass. The exceptions were LtnαD10K (Ltnβ+INK and Ltnβ+GKRKK), which were not

Table 1. Mass spectrometric and bioactivity analysis of strains producing lacticin 3147 'charge' derivatives.

Strain	Peptide charge	Predicted mass (Da)	Actual mass (Da)	Bioactivity (mm)[a]
MG1363 pMRC01	Ltnα 0; Ltnβ +2	Ltnα 3305; Ltnβ 2847	3305.29, 2847.32	15.3 (±0.6)
L. lactis MGpMRα				
D10A	Ltnα +1	3261	3261.58[b]	8.3 (±0.6)
D10K	Ltnα +2	3318	No data	No inhibition
H23A	Ltnα −1	3239	3239.9[b]	14.3 (±0.6)
H23D	Ltnα −2	3283	3283.23	11
E24A	Ltnα +1	3247	3247.7[b]	No inhibition
E24D	Ltnα 0	3291	3291.6	7.6 (±0.6)
K30A	Ltnα −1	3248	3248.47[b]	14.3 (±0.6)
K30D	Ltnα −2	3292	3291.69	10.3 (±0.6)
D10A-K30A	Ltnα 0	3204	3206.7	No inhibition
D10A-H23A	Ltnα 0	3195	3195.58	9.3 (±0.6)
H23A-K30A	Ltnα −2	3182	3181.29	11.3 (±0.6)
N15K	Ltnα +1	3319	3319.46[c]	14.3 (±0.6)
L. lactis MGpMRβ				
K24A	Ltnβ +1	2790	2790.49[b]	10.3 (±0.6)
K24D	Ltnβ 0	2834	2833.97	No inhibition
K24E	Ltnβ 0	2848	2848.68	7.3 (±0.6)
K24R	Ltnβ +2	2875	2875.33	13.3 (±0.6)
R27A	Ltnβ +1	2762	2762.35[b]	14.3 (±0.6)
R27D	Ltnβ 0	2806	2806.77	No inhibition
R27K	Ltnβ +2	2819	2819.69	13
K24A-R27A	Ltnβ 0	2705	2728.13 (+23 Da)	10.7 (±0.6)
T17R	Ltnβ +3	2903	2902.72	9
+INK	Ltnβ +3	3256	Not detected	No inhibition
+GKRKK	Ltnβ +6	3535	Not detected	No inhibition

a. Diameter of well = 6 mm.
b. Cotter and colleagues (2006).
c. O'Connor and colleagues (2007).

detected, LtnαD10A-K30A (mass of 3206.7 Da rather than the expected 3204) and LtnβK24A-R27A (mass of 2728 Da rather than the expected 2705 Da; Table 1). The latter two represent additional examples of bioengineered Ltnβ peptides of unexpected mass, the other being LtnβP21A, the mass of which was 3 Da less than the expected value of 2821 (Cotter *et al*., 2006). The basis for these unexpected masses has not been determined, but in the case of LtnβK24A-R27A may be as a result of the formation of an adduct.

(Bio)Activity of Ltnα charge mutants against L. lactis *HP*

The bioactivity of the cell free supernatant from the 12 Ltnα charge variant-producing strains was initially assessed using the well diffusion assay technique against the sensitive indicator *L. lactis* HP. All of these mutants produce both the mutated Ltnα and an unaltered Ltnβ peptide (Table 1). The bioactivity of these strains was assessed in order to provide an insight into the consequences of these changes and to facilitate the selection of peptides that merit closer inspection. The reduced bioactivity of strains in which changes were made with a view to the generation of Ltnα peptides with a net positive charge, indicated that such alterations appear to negatively impact on peptide production and/or specific activity (Table 1).

Three mutant strains were constructed such that there would be no change to the overall charge of Ltnα (*L. lactis* MGpMRαD10A-K30A, D10A-H23A and E24D). In the case of *L. lactis* MGpMRαD10A-K30A, the previously observed negative impacts of changing D10 and K30 to alanines individually were cumulative in that no bioactivity was apparent (Table 1). In contrast, the bioactivity of the LtnαD10A-H23A-producing mutant was greater than that of its Ltnα-D10A-producing counterpart, establishing that the additional H23A change partially compensates for the negative consequences of the D10A change. For this reason the LtnαD10A and D10-H23A were among those selected for further investigations involving the purified peptides (see below). Finally, given the lack of bioactivity of *L. lactis* MGpMRαE24A, it was postulated that bioactivity might only be retained if another negatively charged residue occupied this location. While the mutant strain created (*L. lactis* MGpMRαE24D), which produced a α peptide which more closely resembles a number of lacticin 481-like peptides, did retain some slight bioactivity, indicating that the charge of this residue is important, the preference for the native glutamate was evident (Table 1). In contrast to the detrimental effect of manipulating the negatively charged residues of Ltnα, the consequences of altering the two positively charged residues, alone and in combination, on the bioactivity of the associated strains

Table 2. Specific activity of selected charge mutants against *Lactococcus lactis* HP.

	Combined activity (nM)[a]	Peptide alone (μM)
Peptide(s)		
Ltnα	9.8*	1.25
αN15K	40*	> 2.5
αH23A-K30A	156*	> 2.5
αH23D	78*	ND
αK30A	312.5*	> 2.5
αD10A	312.5*	> 2.5
αD10A-H23A	625*	> 2.5
Ltnβ	9.8*	2.5
βK24R	156*	> 2.5
βR27K	625*	> 2.5
βR27A	78*	1.25
βR27D	2500*	> 2.5
βK24A-R27A	312.5*	> 2.5
Bioengineered peptides in combination		
αK30A + βK24R	1250	NA
αK30A + βR27A	2500	NA
αN15K + βK24R	625	NA
αN15K + βR27A	1250	NA

a. Specific activity of bioengineered peptide with natural (*) or bioengineered sister peptide at a 1:1 ratio.
ND, not determined; NA, not applicable.

were considerably more subtle. Other mutants producing Ltnα peptides with the same –2 overall charge, but with more dramatic localized impacts on charge (LtnαH23D and K30D), retained similar levels of bioactivity, highlighting the extreme tolerance to change at these locations.

To determine to what extent alterations in bioactivity were as a consequence of impacts on the specific activity of the altered peptide and not due to differences in their production or the rate at which they diffuse in agar, broth-based minimum inhibitory concentration (MIC) determinations were carried out with five representative bioengineered Ltnα peptides (alone and/or in combination with an equimolar concentration of Ltnβ against the *L. lactis* HP indicator). In all cases MIC determinations involved the comparison of equimolar concentrations of 100% pure RP-HPLC-purified peptides in isolation or combination. It was established that in many cases the impact on the specific activities of these peptides (when combined with Ltnβ) did indeed reflect the impact on bioactivity. The LtnαN15K peptide possessed the greatest specific activity of the three, with a MIC that was only four times greater than that of the wild-type combination (40 nM). LtnαH23A-K30A and H23D were more dramatically affected, with MICs increasing 16- and 8-fold respectively (Table 2). The negative impacts resulting from these changes were at least partially due to reduced Ltnα activity (i.e. were not solely as a consequence of a reduced ability to function synergistically with Ltnβ), as, where tested, the specific activity of the peptide in isolation was also reduced relative to that of the native Ltnα (Table 2). The MIC investigations were also carried out to establish if the enhanced bioactivity of LtnαD10A-H23A over LtnαD10 was due to differences in specific activity. However, it was established that this was not the case as the specific activity of the D10A peptide (312.5 nM) was twice that of D10A-H23A (625 nM), when combined with Ltnβ. It was postulated that this phenomenon may be due to the LtnαD10A-H23A peptide diffusing to a greater extent in agar (relevant for bioactivity studies) or that this peptide may be produced in greater quantities than its LtnαD10A counterpart. Although agar-based studies with purified peptides eliminated the former possibility (data not shown), we cannot exclude the latter in that while the amount of LtnαD10A-H23A isolated from the surface of producing cells is not relatively enhanced, we were unable to quantify the relative amounts of the peptides that were released into the supernatant (i.e. the basis for supernatant-based bioactivity studies).

(Bio)Activity of Ltnβ charge mutants against L. lactis *HP*

The bioactivity of the 11 strains (two previously generated and nine newly created) producing Ltnβ derivatives in which charged residues were altered was also assessed. It was established that the replacement of one positively charged residue with another, i.e. LtnβK24R and LtnβR27K, or with alanine, i.e. LtnβK24A, LtnβR27A, LtnβK24A-R27A, did not severely impact on bioactivity of the associated strain (Table 1), despite the fact that in the last case the form of Ltnβ produced was no longer cationic. In contrast, attempts to eliminate the net cationic charge of Ltnβ by replacing a positive with a negative residue resulted in a dramatic loss of bioactivity (i.e. *L. lactis* MGpMRβK24E, βK24D and βR27D). Indeed, of the three strains in question, only the LtnβK24E producer inhibited the indicator strain to any degree (Table 1). As with Ltnα, the creation of a strain that produces a Ltnβ derivative, LtnβT17R, that contains an additional positively charged residue at a location known to be tolerant of change (Cotter *et al*., 2006), was targeted. However, in this instance the additional positive charge had a significantly negative impact on the bioactivity of the culture supernatant (Table 1). Finally, other efforts to make versions of Ltnβ more cationic through the introduction of additional C-terminally located residues, such that the peptide would more closely resemble Plwβ and Smbβ/BHT-Aβ, were unsuccessful in that although the modified genes were generated, mass spectrometry revealed that these peptides were not produced.

In five instances, LtnβK24R, R27K, R27A, R27D and K24A-R27A, bioengineered Ltnβ peptides were purified and their specific activities in broth were determined, alone or in combination with equimolar concentrations of Ltnα. In agreement with the bioactivity studies, it was apparent that the LtnβR27A and K24R peptides retained

considerable potency when combined with Ltnα, albeit at levels that are reduced relative to the wild-type combination (8- and 16-fold reduced activity respectively; Table 2) while the activity of Ltnα : LtnβR27K was lower than expected (MIC of 625 nM; Table 2). Of the two uncharged Ltnβ peptides, K24A-R27A was most active when combined with Ltnα (312.5 nM MIC), while the introduction of a negatively charged residue had a particularly detrimental impact on combined specific activity (R27D; Table 2). As Ltnβ is also active in the absence of Ltnα (albeit at μM concentrations; specific activity of 2.5 μM – Table 2), the specific activity of the five modified Ltnβ peptides in isolation was also determined. In four instances the bioengineered changes had a detrimental impact on specific activity in that they no longer inhibited the target strain at 2.5 μM (Table 2). It is logical to assume that in these cases the reduced 'solo' Ltnβ activity was at least partially responsible for the reductions in combined activity (Ltnα and Ltnβ). In the remaining case, it was established that LtnβR27A exhibited a level of activity that was greater than that of wild-type Ltnβ (Table 2) and thus represents the first example of a derivative of either Ltnα or Ltnβ that is enhanced in any manner. The fact that the enhanced specific activity of LtnβR27A did not result in an enhanced 'combined' activity in the presence of Ltnα establishes that while this alteration enhances the potency of the β peptide in isolation, it has a negative impact on the ability of Ltnα and Ltnβ to function synergistically.

Combined activity of Ltnα and Ltnβ charge mutants against L. lactis *HP*

Four purified bioengineered peptides were the subject of further investigation to determine the consequences of their use in various combinations against *L. lactis* HP. LtnαN15K, LtnαK30A, LtnβK24R and LtnβR27A were selected due to being among the more active of the bioengineered 'charge' peptides and because they represented peptides whose overall charges had been altered in different ways i.e. Ltnα Δcharge +1, −1 and Ltnβ Δcharge 0, −1 respectively. The results revealed that none of these peptides combined in a manner that compensated for individual reductions in specific activity. Yet again it was apparent that localized impacts are more significant than the overall role of peptide charge in that combining a more positively charged Ltnα (N15K) with a more negatively charged Ltnβ (R27A) did not enhance synergism (1250 nM; Table 2). Other attempts to harness the enhanced solo activity of LtnβR27A by combining it with another bioengineered Ltnα peptide (K30A) were similarly unsuccessful and, indeed, in this latter case there was a negative impact on activity as the combined MIC (MIC 2500 nM; Table 2) was lower than that of LtnβR27A in isolation. In contrast, LtnβK24R, despite being less active than LtnβR27A when assessed alone and in combination with wild-type Ltnα, was the more active of the two Ltnβ peptides when combined with LtnαN15K and K30A. Overall, it was apparent that although some bioengineered 'charge' peptides can function synergistically, further developments are required in order to generate combinations that are more active than the wild-type pair of Ltnα and β.

Impact of altered cell envelope charge on the sensitivity of S. aureus *to lacticin 3147*

The innate resistance of *S. aureus* to cationic antimicrobial peptides has been attributed to a decrease in the negative charge of its cell envelope due to D-alanylation of cell wall teichoic acid and the lysinylation of membrane phospholipid (Peschel *et al*., 1999; 2001; Abachin *et al*., 2002; Poyart *et al*., 2003; Kristian *et al*., 2005; Kovacs *et al*., 2006; Thedieck *et al*., 2006; Herbert *et al*., 2007). Thus mutation of genes responsible for these cell envelope decorations, such as *dltA* and *mprF*, has been shown to result in an enhanced sensitivity to the lantibiotics nisin and gallidermin as well as other non-lantibiotic cationic antimicrobial peptides (Peschel *et al*., 1999; 2001). This enhanced sensitivity is not evident upon exposure to non-polar lantibiotics such as the Ltnα-like mersacidin (Kramer *et al*., 2006). Here we establish that cell envelope modifications do protect *S. aureus* (strain Sa113) against lacticin 3147 as evident from bioactivity studies which demonstrate that Sa113Δ*dltA* and Δ*mprF* are more sensitive to the lantibiotic than the parent Sa113 (Table 3). Here it is established that lacticin 3147 is more active against Sa113 (937.5 nM) than nisin (14 μM) (Peschel *et al*., 2001) but that its activity is increased a further sixfold against both Sa113Δ*dltA* and Δ*mprF* (Table 3). Bioactivity studies, using the deferred antagonism approach, were also carried out to investigate the consequences of exposing cell envelope of these strains with altered membrane charge to the bioengineered lacticin 3147 'charge' mutants. In all cases the bioactivity of the bioengineered strains was reduced to the extent that wild-type Sa113 was not inhibited and only six exhibited any bioactivity against either the *dltA* or *mprF* mutants (Table 4). However, it was noted that, although bioactivity was not

Table 3. Specific activity of selected charge mutants against Sa113 wild-type and mutant strains.

Peptide	Sa113	Δ*mprF*	Δ*dltA*
Wild type	937.5 nM	156.25 nM	156.25 nM
LtnαN15K : Ltnβ	1.25 μM	625 nM	625 nM
Ltnα : LtnβR27K	> 2.5 μM	1.25 μM	625 nM
Ltnα : Ltnβ K24A-R27A	> 2.5 μM	> 2.5 μM	> 2.5 μM
Ltnα : Ltnβ R27D	> 2.5 μM	2.5 μM	2.5 μM

Table 4. Bioactivity[a] of lacticin 3147 charge mutants against *S. aureus* Sa113, Δ*dltA* and Δ*mprF*.

Lacticin 3147 producer	Overall Charge of peptide	Sa113	Δ*dltA*	Δ*mprF*
Wild type	+2	+	++++	+++++
L. lactis MGpMRα				
D10A	+3	–	–	–
D10K	+4	np	np	np
N15K	+3	–	+++	++++
H23A	+1	–	+/–	+
H23D	0	–	–	–
E24A	+3	–	–	–
E24D	+2	–	–	–
K30A	+1	–	–	–
K30D	0	–	–	–
D10A-H23A	+2	–	–	–
D10A-K30A	+2	–	–	–
H23A-K30A	0	–	–	–
L. lactis MGpMRβ				
T17R	+3	–	++	++
K24A	+1	–	–	–
K24R	+2	–	+++	+++
K24E	0	–	–	–
K24D	0	–	–	–
R27A	+1	–	+	+
R27K	+2	–	+	+
R27D	0	–	–	–
K24A-R27A	0	–	–	–
+INK	+3	np	np	np
+GKRKK	+6	np	np	np

a. Bioactivity: –, no activity (diameter of bacterial growth 6 mm); +/–, faint activity 6–8 mm diameter; +, 9–12 mm diameter; ++, 13–15 mm diameter; +++, 16–18 mm diameter; ++++, 19–20 mm diameter; +++++, > 20 mm diameter. np – corresponding peptides were not produced leading to a lack of bioactivity.

enhanced, four of the strains that were active against Sa113Δ*dltA* and Δ*mprF* produced peptides into which a positively charged residue had been introduced (*L. lactis* MGpMRαN15K and βT17R) or had been replaced with a similarly charged amino acid (*L. lactis* MGpMRβK24R and βR27K) (Table 4). It would appear from these investigations that the presence of positively charged residues in the Ltnβ peptide is of particular importance when strains with more negatively charged cell envelopes are targeted. To confirm this conclusion a number of purified bioengineered peptides (LtnαN15K, LtnβR27K, LtnβK24A-R27A and LtnβR27D) were employed to again determine whether bioactivity results accurately reflected the specific activity of the mutant peptides in broth. Of the bioengineered peptides only the consistently active LtnαN15K peptide, when combined with its companion peptide, inhibited Sa113 at the concentrations employed (MIC 1.25 μM; Table 3). The relative resistance of the *S. aureus* strains also precluded an assessment of sensitivity to the individual Ltnα and Ltnβ peptides. However, it was apparent that the LtnαN15K : Ltnβ, Ltnα : LtnβR27K and Ltnα : LtnβR27D combinations all inhibited the Δ*dltA* and Δ*mprF* Sa113 strains. The activity of Ltnα : LtnβR27D, despite the producing strain lacking detectable bioactivity, can be attributed to the higher concentrations of the peptides employed for specific activity studies. As with *L. lactis* and *S. aureus* MIC and bioactivity studies, Ltnα : LtnβR27K again retained a greater level of activity than Ltnα : LtnβR27D against Δ*dltA* and Δ*mprF*. However, the inability of Ltnα : LtnβK24A-R27A to inhibit the Sa113 mutants, even at a concentration of 2.5 μM, was notable. Thus, although the net charge of Ltnα : LtnβR27D and Ltnα : LtnβK24A-R27A is the same (i.e. neutral), the retention of one positively charged residue provides the former combination with an advantage with respect to the targeting of strains with more negatively charged cell envelopes.

Discussion

The gene-encoded nature of lantibiotics makes them ideal for bioengineering (Kuipers *et al*., 1992; 1996; Wiedemann *et al*., 2001; Szekat *et al*., 2003; Xie *et al*., 2004; Yuan *et al*., 2004; Chatterjee *et al*., 2006; Rink *et al*., 2007; Cooper *et al*., 2008; Field *et al*., 2008; Patton *et al*., 2008). While a number of studies have reported the importance of specific charged residues in a selection of lantibiotics (Cotter *et al*., 2005a) or a tolerance of the introduction of additional charged residues in others (Chatterjee *et al*., 2006), this study represents the most comprehensive investigation of the consequences of manipulating charged residues of any lantibiotic. As a consequence of our investigation of 23 charge mutants we can make a number of general observations. It is evident that of all of the mutants generated, those in which LtnαE24 was altered suffered the most negative consequences. These negative consequences are more likely due to the importance of the specific residues rather than a general requirement that Ltnα should have a net neutral or negative charge as is apparent from the (bio)activity of LtnαN15K (+1) and specific activity of LtnαD10A. With respect to Glu24, it was apparent that not even an aspartate residue, which occupies the corresponding location in other lantibiotics such as salivaricin A (Ross *et al*., 1993) and nukacin ISK-1 (Sashihara *et al*., 2000) although not in the α components of two-peptide lantibiotics, could efficiently replace this glutamate residue. Differences with respect to the importance of this glutamate residue in α components/mersacidin-like peptides relative to lacticin 481-like peptides, where for example conversion of the corresponding glutamate to alanine is tolerated (Patton *et al*., 2008), suggests that the manner in which peptides belonging to these respective subgroups target receptors varies. With respect to the other negatively charged residue in Ltnα, Asp10, it is apparent that while it plays an important role in lacticin 3147 activity (combined specific activity of LtnαD10A drops 32-fold relative to control), this

is not true of all α peptides as is evident by its absence from a number of these other peptides, including Halα which possesses a positively charged arginine at the corresponding location. Bioactivity studies also revealed a previously unobserved phenomenon in that the negative consequences of a D10A manipulation of Ltnα (8.3 mm zone) were partially compensated for by an additional H23A change to the peptide (9.3 mm zone). However, this enhanced bioactivity is not as a consequence of either enhanced specific activity or diffusion rate but may be as a consequence of differences in the relative amounts of each peptide that the associated producers release.

While the tolerance to change of the positively charged residues in lacticin 3147 had been indicated previously (Cotter *et al*., 2006), the full extent to which they could be manipulated without eliminating (bio)activity against HP became apparent when it was established that strains producing LtnαH23D, K30D, H23A-K30A, LtnβK24A-R27A or K24E and as well as the Ltnα : LtnβR27D combination of purified peptides all exhibited (bio)activity in at least some circumstances. As with Asp 10, the positively charged residues in Ltnα are only partially conserved in α peptides. The retention of at least some antimicrobial activity by LtnαH23A-K30A is consistent with the fact that Halα is also active despite naturally lacking positively charged residues within the C-terminal half of the peptide. That said, it was noteworthy that the LtnαH23A-K30A, H23D and K30D peptides and/or the corresponding producers all retained at least some (bio)activity despite the overall charge of the peptide being significantly altered. The consequences of manipulating positively charged residues in Ltnβ are variable. Ltnβ is a cationic peptide, but the addition of an additional arginine residue at a location known to be tolerant of change had a negative rather than a positive impact. In fact, the importance of the charged residues in this peptide seems to be dependent on the strain targeted. The strain producing LtnβK24A-R27A and the purified K24A-R27A peptide (when combined with Ltnα) both retain a significant level of (bio)activity against HP despite the β peptide having no positively charged residues. It may be that, as a consequence of a mode of action which involves interacting with Ltnα or a Ltnα-lipid II complex, Ltnβ is not as dependent as other cationic peptides on the electrostatic interaction between positively charged residues and the negatively charged cell membrane to trigger pore-formation. Notably, while eliminating the cationic nature of Ltnβ by double alanine substitution has a less detrimental impact on anti-HP activity than positive to negative substitution, this trend is reversed with respect to the targeting of Sa113 Δ*dltA* and Δ*mprF*. In this situation the retention of at least one positively charged residue in Ltnβ (Ltnα : LtnβR27D, Table 3) results in this combination exhibiting greater activity than Ltnα : LtnβK24A-R27A, despite both having an overall neutral charge. The further benefit of possessing two positively charged residues is evident from the poor anti-Δ*dltA* and anti-Δ*mprF* bioactivity of *L. lactis* MGpMRβK24A and R27A which contrasts with these strains being among the more potent against HP.

This is the first occasion upon which the combined consequences of altering target cell envelope charge and of bioengineering charge residues in a targeting lantibiotic (or indeed any cationic antimicrobial peptide) has been investigated. This is of significance as *S. aureus*, and a number of other Gram positive pathogens, are partially protected against cationic peptides through the alanylation of (lipo)teichoic acid and the lysinylation of the membrane phospholipids. These cell envelope modifications counter the natural tendency of cationic peptides to accumulate at the negatively charged cell envelope and thus, theoretically, reducing the charge of a cationic peptide should result in it being less adversely affected by the degree of alanylation of (lipo)teichoic acid or lysinylation of the membrane. Although such a trend was not apparent with the derivatives assessed here, this does not discount the possibility that this goal could be achieved through the creation of another generation of 'charge' variants. The corollary was also investigated i.e. does an increase in charge enhance activity against the relatively more negatively charged cell envelopes of the Δ*dltA* and Δ*mprF* mutants. While the associated results highlight the importance of positively charged residues in these circumstances, it was evident that an increase in overall positive charge (i.e., LtnαN15K and especially LtnβT17R) does not necessarily enhance antimicrobial activity. In fact, efforts to incorporate additional positively charged residues at the C-terminus of Ltnβ, as is naturally the case with Plwβ and Smbβ/BHT-Aβ, resulted in the peptide not being synthesized. Nonetheless, given that increasing the charge of other cationic peptides can have beneficial consequences (Dathe *et al*., 2001), it may be that the introduction of positively charged residues into the peptides at other locations could result in enhanced activity targets with cell envelopes that are less positively charged than staphylococci or against the Δ*dltA* and Δ*mprF* strains employed in this study.

This study also highlights the challenges involved in creating lantibiotic derivatives with enhanced antimicrobial activity. This challenge is even greater when two-peptide lantibiotics are the template as changes that may increase the activity of one peptide may have a detrimental impact on its ability to function synergistically with its partner. Here, for the first time, a lacticin 3147 peptide with enhanced antimicrobial activity is identified. LtnβR27A exhibits twofold greater activity against *L. lactis* HP than its wild-type counterpart. However, although a synergistic impact is apparent when this peptide is combined with Ltnα, the combined activity is eightfold lower than that of

the wild-type pair and is particularly reduced when combined with the LtnαN15K and K30A peptides. It has previously been postulated that the C-terminal region of Ltnβ may be involved in the interaction with Ltnα when it was observed that a LtnβP21A peptide, despite retaining high levels of solo activity, exhibited greater reduced combined activity (Cotter *et al.*, 2006). Such a role would also explain the reduced combined activity of Ltnα : LtnβR27A. Despite the lack of enhanced combined activity, the identification of a Ltnβ peptide with enhanced activity is a notable event given the extreme rarity to date with which lantibiotics with enhanced features are identified and could be the first step on a path that could lead to the creation of a Ltnβ derivative that is sufficiently active to be of commercial interest in its own right.

In conclusion, as a consequence of creating the largest collection of 'charge' lantibiotic derivatives (and the largest collection of targeted derivatives of lantibiotics in general), we have been able to confirm the importance of LtnαE24, the requirement for positively charged residues in Ltnβ when targeting cells with reduced levels of cell envelope-associated D-alanylation or lysinylation and, for the first time, identify a derivative of one of the lacticin 3147 peptides with enhanced antimicrobial activity.

Experimental procedures

Strains and growth conditions

The *L. lactis* strains HP (indicator), *L. lactis* MG1363 pMRC01 (producer of wild-type lacticin 3147 (McAuliffe *et al.*, 2000) and bioengineered derivatives thereof were cultured at 30°C in MI7 broth supplemented with 0.5% glucose (GM17) without aeration. *Escherichia coli* EC101 (Law *et al.*, 1995) was grown in Luria–Bertani (LB) broth at 37°C with aeration. *S. aureus* Sa113, Sa113Δ*dltA* (Peschel *et al.*, 1999) and Sa113Δ*mprF* (Peschel *et al.*, 2001) were grown in BHI at 37°C with aeration. Antibiotics were used at the following concentrations: erythromycin 150 μg ml^{-1} for *E. coli* and 5 μg ml^{-1} for *L. lactis*; chloramphenicol 5 μg ml^{-1} for *L. lactis* and Xgal was used at 50 μg ml^{-1}.

Site-directed mutagenesis

Individual amino acids were changed on pMRC01 (the native lacticin 3147 producing plasmid) through use of the Quikchange site-directed mutagenesis strategy (Stratagene) and pORI280*ltnA1A2* (RepA–, LacZ+) as described previously by Cotter and colleagues (2003; 2005c). The Quikchange protocol was followed according to the manufacturer's instructions except that EC101 (RepA+) was used as the *E. coli* host. EC101 putative containing mutated derivatives of pORI280*ltnA1A2* were identified as blue colonies on LB-Ery-Xgal plates and successful mutation was screened for using a check primer designed specifically to anneal to the newly incorporated codon (used in conjunction with either of two standard oligonucleotide – ltnA1A or ltnA1D – that anneal to unmutated regions of the *ltnA1A2* insert elsewhere). These and other oligonucleotides employed in this study are listed in Table S1. Candidates were further checked by DNA sequencing to ensure that the correct change had been incorporated and that no other mutations had been incorporated elsewhere. The plasmids were then isolated and electroporated into *L. lactis* MG1363.pMRC01.pVE6007 and transformants were selected on GM17-Ery-Xgal plates. Loss of pVE6007 and integration of pORI280 occurred following growth at 37°C in GM17Ery broth and streaking onto GM17Ery-Xgal. Candidates were also streaked onto GM17Cm to ensure the loss of pVE6007 and subsequently subcultured in GM17 at 37°C to induce excision and curing of pOri280. At regular interval *lacZ*– colonies were identified by plating onto GM17-Xgal and the resultant potential mutants where checked as before by PCR and sequencing.

To manipulate ltnA2 with a view to the production of Ltnβ+INK and Ltnβ+GKRKK, the plasmid pDF01 (i.e. pCi372-PbacA1A2; Field *et al.*, 2007) was used as a template to introduce the additional amino acids via PCR using 5′ phosphorylated forward primers and non-phosphorylated reverse primer (Table S1). PCR amplification was performed with Phusion DNA polymerase (Finnzymes). Amplified products were treated with DpnI restriction endonuclease for 1 h at 37°C and introduced into *E. coli* TOP10 cells. The respective pCi372-PbacA1A2 derivatives were isolated and the PbacA1A2 inserts were amplified with pPTPLA1A2For(BglII) and pPTPLA1A2Rev(XbaI), digested with BglII and XbaI (Roche) and ligated with similarly digested pPTPL before being introduced into *E. coli* MC1000. The recombinant plasmids were isolated, inserts were sequenced and the plasmid was introduced into *L. lactis* MG1363 pOM44.

Bioactivity assays

Well diffusion assays were carried as described previously (Ryan *et al.*, 1996). Briefly, molten agar was cooled to 48°C and seeded with the indicator strain *L. lactis* ssp. *cremoris* HP (~2×10^7 fresh overnight-grown cells). The inoculated medium was dispensed into sterile Petri plates, allowed to solidify and dried. Wells (6 mm diameter) were made in the seeded agar plates. Aliquots of culture supernatant from the producing strains were dispensed into wells, and the plates were incubated overnight at 30°C. Deferred antagonism assays were carried out by spotting 2 μl of an overnight culture (2×10^8 cfu ml^{-1}) of the producing strains onto GM17 agar plates. Spotted plates were incubated at 30°C overnight, after which subjected to UV irradiation and molten agar containing 1×10^6 cfu ml^{-1} of the relevant *S. aureus* indicator was used to overlay the plate with the irradiated spotted cultures and incubated at 30°C overnight.

Peptide purification

An overnight culture of the strain producing lacticin 3147 (or derivative thereof) was inoculated into 1 l modified TY broth (1% inoculum), incubated overnight at 30°C and the cells were harvested by centrifugation (7000 *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 concentrated HCl). The preparation was stirred for 4 h at 4°C, and the cell debris was removed by

centrifugation and the bacteriocin-containing supernatant reduced to approximately 60 ml by removing propan-2-ol via rotary evaporation. The resultant preparation was applied to a 10 g (60 ml volume) Varian C18 Bond Elute Column (Varian, Harbor City, CA) pre-equilibrated with methanol and water. The column was subsequently washed with 120 ml of 30% ethanol and elution with 100 ml 70% propan-2-ol, pH 2. Ten and twenty millilitre 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^{-1}.

Minimum inhibition concentration determination

The MIC determinations were carried out in microtitre plates as described previously (Cotter *et al.*, 2006). *Lactococcus lactis* HP was grown in M17 broth plus 0.5% glucose (Oxoid) and SA113 and mutants thereof were grown in LB broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl). Serial twofold dilutions of the peptides were made in the growth medium of the respective indicator strain. Bacteria were added to give a final inoculum of 10^5 cfu ml^{-1} in a volume of 0.2 ml. After incubation for 16 h at 30°C for *L. lactis* or at 37°C for *S. aureus* the MIC was read as the lowest peptide concentration causing inhibition of visible growth. Results given are mean values of three independent determinations.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1. Oligonucleotides used in this study.

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