Manipulation of charged residues within the two-peptide lantibiotic lactacin 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 lactacin 3147 [composed of Ltnα (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 lactacin 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 lactacin 3147 peptide (LtnβR27A) which displays enhanced specific activity.

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-methylanthionine 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, 2005). From these investigations it is also apparent that the relative importance of the overall charge of lactacin 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 lactacin 3147 peptide (LtnβR27A) which displays enhanced specific activity.
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 Saccharomyces cerevisiae strains (Rink et al., 1999; Wiedemann 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, has 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 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 mesacidin (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, Saccα 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 Saccα and Plwα but is replaced by a Val in the other three peptides while residues corresponding to LtnαK30 are present in Saccα and Smbα. It is notable however that Ltnα is the only such peptide that does not possess a positively charged residue within its largest.

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 mesacidin (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, Saccα 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 Saccα and Plwα but is replaced by a Val in the other three peptides while residues corresponding to LtnαK30 are present in Saccα 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.
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 mesasacidin-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 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-Αβ or P1β 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
detected, Ltn<sub>a</sub>D10A-K30A (mass of 3206.7 Da rather than the expected 3204) and Ltn<sub>β</sub>K24A-R27A (mass of 2728 Da rather than the expected 2705 Da; Table 1). The latter two represent additional examples of bioengineered Ltn<sub>β</sub> peptides of unexpected mass, the other being Ltn<sub>β</sub>P21A, 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 Ltn<sub>β</sub>K24A-R27A may be as a result of the formation of an adduct.

(Bio)Activity of Ltn<sub>a</sub> charge mutants against L. lactis HP

The bioactivity of the cell free supernatant from the 12 Ltn<sub>a</sub> 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 Ltn<sub>a</sub> and an unaltered Ltn<sub>β</sub> 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 Ltn<sub>a</sub> 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 Ltn<sub>a</sub> (L. lactis MGpMR<sub>a</sub>D10A-K30A, D10A-H23A and E24D). In the case of L. lactis MGpMR<sub>a</sub>D10A-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 Ltn<sub>a</sub>D10A-H23A-producing mutant was greater than that of its Ltn<sub>a</sub>D10A-producing counterpart, establishing that the additional H23A change partially compensates for the negative consequences of the D10A change. For this reason the Ltn<sub>a</sub>D10A 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 MGpMR<sub>a</sub>E24D, it was postulated that bioactivity might only be retained if another negatively charged residue occupied this location. While the mutant strain created (L. lactis MGpMR<sub>a</sub>E24D), 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 Ltn<sub>a</sub>, the consequences of altering the two positively charged residues, alone and in combination, on the bioactivity of the associated strains

![Table 1. Mass spectrometric and bioactivity analysis of strains producing lacticin 3147 'charge' derivatives.](image-url)
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).

| Peptide(s) | Combined activity (nM)* | Peptide alone (μM) |
|------------|------------------------|------------------|
| Ltnα       | 9.8* 1.25              |                  |
| αN15K      | 40* > 2.5              |                  |
| αH23A-K30A | 156* > 2.5             |                  |
| αH23D      | 79* 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 |

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

**Note:** Specific activity of bioengineered peptide with natural (*) or bioengineered sister peptide at a 1:1 ratio.

ND, not determined; NA, not applicable.

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 Ltn, 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 Ltn : Ltn[R27K] was lower than expected (MIC of 625 nM; Table 2). Of the two uncharged Ltn peptides, K24A-R27A was most active when combined with Ltn (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 Ltn is also active in the absence of Ltn (albeit at μM concentrations; specific activity of 2.5 μM – Table 2), the specific activity of the five modified Ltn 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’ Ltn activity was at least partially responsible for the reductions in combined activity (Ltn and Ltn[R27K]). In the remaining case, it was established that Ltn[R27A] exhibited a level of activity that was greater than that of wild-type Ltn (Table 2) and thus represents the first example of a derivative of either Ltn or Ltn[R27K] that is enhanced in any manner. The fact that the enhanced specific activity of Ltn[R27A] did not result in an enhanced ‘combined’ activity in the presence of Ltn establishes that while this alteration enhances the potency of the β peptide in isolation, it has a negative impact on the ability of Ltn and Ltn[R27A] to function synergistically.

**Combined activity of Ltn 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. LtnN15K, LtnK30A, Ltn[K24R] and Ltn[R27A] 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. Ltn \( \Delta \text{charge} +1 \), \( \Delta \text{charge} -1 \) and Ltn \( \Delta \text{charge} 0 \), \( \Delta \text{charge} -1 \) 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 negatively charged Ltn (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 Ltn[R27A] by combining it with another bioengineered Ltn 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 Ltn[R27A] in isolation. In contrast, Ltn[K24R], despite being less active than Ltn[R27A] when assessed alone and in combination with wild-type Ltn, was the more active of the two Ltn peptides when combined with LtnN15K 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 Ltn 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 lysisin of membrane phospholipid (Peschel et al., 1999, 2001; Abachin et al., 2002; Poyart et al., 2003; Kristian et al., 2005; Kovacs et al., 2006; Thiedeck 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 Ltn-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 Sa113 \( \Delta \text{dltA} \) and \( \Delta \text{mprF} \) 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 Sa113 \( \Delta \text{dltA} \) and Sa113 \( \Delta \text{mprF} \) (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 \( \Delta \text{dltA} \) or \( \Delta \text{mprF} \) mutants (Table 4).

However, it was noted that, although bioactivity was not

| Peptide | Sa113 | \( \Delta \text{mprF} \) | \( \Delta \text{dltA} \) |
|---------|-------|----------------|----------------|
| Wild type | 937.5 nM | 156.25 nM | 156.25 nM |
| LtnN15K : Ltn | 1.25 μM | 625 nM | 625 nM |
| Ltn : Ltn[R27K] | > 2.5 μM | 1.25 μM | 625 nM |
| Ltn : Ltn[K24A-R27A] | > 2.5 μM | > 2.5 μM | > 2.5 μM |
| Ltn : Ltn[R27D] | > 2.5 μM | 2.5 μM | 2.5 μM |

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enhanced, four of the strains that were active against Sa113ΔltIA and ΔmprF produced peptides into which a positively charged residue had been introduced (L. lactis MGpMRα 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 ΔltIA and ΔmprF 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 ΔltIA and ΔmprF. 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 ΔdltA and ΔmprF. 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-ΔdltA and anti-ΔmprF bioactivity of L. lactis MGpMRjK24A 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 (lip)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 (lip)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 ΔdltA and ΔmprF 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-ββ, 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 ΔdltA and ΔmprF 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 LtnxN15K and K30A peptides. It has previously been postulated that the C-terminal region of Ltnβ may be involved in the interaction with Ltnx 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 Ltnx : 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 lysinilation and, for the first time, identify a derivative of one of the lactacin 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 lactacin 3147 (McAuliffe et al., 2000) and bioengineered derivatives thereof were cultured at 30°C in M17 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, Sa113ΔmprF (Peschel et al., 1999) and Sa113ΔdltA (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 lactacin 3147 producing plasmid) through use of the Quikchange site-directed mutagenesis strategy (Stratagene) and pORi280lnnA1A2 (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 pORi280lnnA1A2 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 × 10⁷ 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 × 10⁷ 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 × 10⁸ 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 lactacin 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 milliliter volumes of the 100 ml elute were reduced to 2 ml by rotary evaporation and aliquots of 1650 μl 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 10⁶ 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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