Investigation of Lantibiotic Regulation, Immunity and Synergy

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by

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I hereby declare that this thesis is my own work and has not been submitted for another degree, either at University College Cork or elsewhere.

Signed _____

Lorraine Draper

Abstract

Due to the increasing incidence of antibiotic resistant strains, the use of novel antimicrobials, such as bacteriocins, has become an ever more likely prospect. Lacticin 3147 (of which there are two components, Ltnα and Ltnβ) and nisin belong to the subgroup of bacteriocins called the lantibiotics, which has attracted much attention in recent years. The lantibiotics are antimicrobial peptides that contain unusual amino acids resulting from a series of enzyme-mediated post translational modifications. Given that there have been relatively few examples of lantibiotic-specific resistance; these antimicrobials appear to represent valid alternatives to classical antibiotics. However, the fact that lantibiotics are naturally only produced in small amounts often hinders their commercialisation. In order to overcome this bottleneck, several approaches can be employed. For example, we can create a situation that reduces the quantity of a lantibiotic required to inhibit a target by combining it with other antimicrobials. Here, following an initial screen involving lacticin 3147 and several classical antibiotics, it was observed between lacticin 3147 and the commercial antibiotics polymyxin B/E function synergistically. This reduced the amounts of the individual antimicrobials required for kill and broadened the spectrum of inhibition of both agents. Upon combination with polymyxins, lacticin 3147, which has been associated with Gram positive targets only, actively targeted Gram negative species such as *Escherichia coli* and *Cronobacter* sp. An alternative means of addressing problems associated with lantibiotic yield is to better understand how production is regulated, and ultimately use this information to enhance peptide levels. With this in mind the regulation of lacticin 3147 production from the promoter P_{bac} was investigated using a green fluorescent protein (GFP)

expression reporter system. This revealed that elements within both of the divergent operons of the lacticin 3147 gene cluster are involved in P_{bac} regulation. That is, LtnR, already established as a negative regulator of itself and the lacticin 3147 associated immunity genes, also acts as an activator of P_{bac} transcription. In contrast, an enhanced level of expression is observed in the absence of the lacticin 3147 structural genes, *ltnA*$_1$ and *ltnA*$_2$, indicating that these genes/gene products are involved in P_{bac} repression. In fact, through complementation of the *ltnA*$_2$ gene, it was revealed that this regulation is more likely to be dependent on the presence of the gene transcript rather that the corresponding prepropeptide or modified Ltnβ.

It may be that if lacticin 3147 production is successfully enhanced, the ability of the producing cell to protect itself may become an issue. To prepare for such a possibility a bioengineered derivative of the lacticin 3147 immunity protein LtnI (LtnI I81V) which provides enhanced protection was discovered through an in depth investigation involving the site and saturation mutagenesis of this protein. In addition, the creation of truncated forms of LtnI allowed the identification of important and essential regions of this immunity protein. Finally, as mentioned, self-immunity is essential to prevent self-killing. However the discovery of nisin U immunity and regulatory gene homologues (*spiFEGRR'K*) within the pathogenic strain *S. infantarius* subsp. *infantarius* is a cause for concern as it represents an example of immune mimicry, a form of lantibiotic-specific resistance. The ability of *spiFEG* to confer protection was apparent when they successfully provided protection to nisin A, F, Z, Q and U when expressed heterologously in the nisin sensitive *L. lactis* HP host.

As a consequence of the studies presented in this thesis, it is likely that strategies will emerge that will facilitate the production of greater levels of lacticin 3147 production

and lead to enhanced immunity in lactococcal backgrounds. Alternatively the need for enhanced production could be avoided through the use of antimicrobial combinations. In addition, providing awareness of the threats of the emergence of resistance through immune mimicry can allow researchers to develop strategies to prevent this phenomenon from leading to the dissemination of lantibiotic resistance.

Abstract

Due to the increasing incidence of antibiotic resistant strains, the use of novel antimicrobials, such as bacteriocins, has become an ever more likely prospect. Lacticin 3147 (of which there are two components, Ltnα and Ltnβ) and nisin belong to the subgroup of bacteriocins called the lantibiotics, which has attracted much attention in recent years. The lantibiotics are antimicrobial peptides that contain unusual amino acids resulting from a series of enzyme-mediated post translational modifications. Given that there have been relatively few examples of lantibiotic-specific resistance; these antimicrobials appear to represent valid alternatives to classical antibiotics. However, the fact that lantibiotics are naturally only produced in small amounts often hinders their commercialisation. In order to overcome this bottleneck, several approaches can be employed. For example, we can create a situation that reduces the quantity of a lantibiotic required to inhibit a target by combining it with other antimicrobials. Here, following an initial screen involving lacticin 3147 and several classical antibiotics, it was observed between lacticin 3147 and the commercial antibiotics polymyxin B/E function synergistically. This reduced the amounts of the individual antimicrobials required for kill and broadened the spectrum of inhibition of both agents. Upon combination with polymyxins, lacticin 3147, which has been associated with Gram positive targets only, actively targeted Gram negative species such as *Escherichia coli* and *Cronobacter* sp. An alternative means of addressing problems associated with

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CHAPTER I

Lantibiotic Resistance: A Review

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Summary

The dramatic rise in the incidence of antibiotic resistance demands that new or varied treatments will have to be developed. One potentially interesting class of antimicrobials are the modified bacteriocins termed lantibiotics, bacterially produced post-translationally modified, lanthionine/methyl-lanthionine containing peptides. This is in part due to the low levels of resistance reported for lantibiotics as compared with commercial antibiotics. Given that there are very few examples of naturally occurring lantibiotic resistance, the deliberate induction of such phenotypes and/or the creation of deletion mutations in resistance genes have been used to investigate this phenomenon. Mechanisms that hinder the action of lantibiotics are often innate systems that react to the presence of any cationic peptides/proteins, or which result from cell well damage, rather than being lantibiotic specific. Such resistance mechanisms often arise due to altered gene regulation following detection of antimicrobials/cell wall damage by sensory proteins at the membrane. This facilitates alterations to the cell wall or changes in the composition of the membrane. Other general forms of resistance include the formation of spores or biofilms, which are a common mechanistic response to many classes of antimicrobials. In rare cases bacteria have been shown to possess specific anti-lantibiotic mechanisms. These are often species specific and include the nisin lytic protein nisinase and the phenomenon of immune mimicry.

Introduction

Lantibiotics are ribosomally-synthesised antimicrobial peptides produced by Gram positive bacteria. They undergo post-translational modification which results in the presence of unusual amino acids such as dehydroalanine (Dha) and dehydrobutyrine (Dhb) as well as the eponymous lanthionine/methyllanthionine residues. Lantibiotics are subclassified on the basis of their biosynthetic machinery and the amino acid sequence of the structural peptide (Rea *et al.*, 2011). The most characterised lantibiotic is nisin (subclass 1), which has been used in the dairy and food industry for decades. It is a 3353 Da cationic, linear peptide of 34 amino acids produced by *Lactococcus lactis* subsp. *lactis* that contains five intramolecular ring structures. It has a dual mode of action, both preventing cell wall biosynthesis and also forming pores in the cell membrane of susceptible cells (Wiedemann *et al.*, 2001). Lantibiotics have been frequently suggested to have the potential to be utilised in a wide range of medical applications (see reviews (Cotter *et al.*, 2005a; Cotter *et al.*, 2013; Piper *et al.*, 2009). One of these reasons is that, in comparison to commercial antibiotics, resistance to lantibiotics is relatively rare. However, resistance has been induced in laboratory settings and this, in addition to the innate resistance characteristics of the bacterial cell, is the subject of this review.

Mode of action

In order to appreciate the various mechanisms by which bacteria are, or become resistant, to lantibiotics, it is first necessary to understand their mode of action. In the case of many cationic lantibiotics, or lantibiotics with cationic

domains, there is an initial attraction to the anionic cell membrane of target microbes (Asaduzzaman *et al.*, 2006; Deegan *et al.*, 2010; Moll *et al.*, 1996). Notably, changes in lantibiotic charge as a result of genetic engineering alters the efficacy of such lantibiotics, presumably due to reduced attraction/interaction with the cell membrane (Deegan *et al.*, 2010; Kraaij *et al.*, 1997). Once at the membrane, lantibiotics such as nisin, mersacidin, epidermin, plantaricin C and lacticin 3147 (Ltnα) bind and form a complex with a docking molecule, lipid II (Breukink *et al.*, 1999; Brotz *et al.*, 1998a; Brotz *et al.*, 1998b; Wiedemann *et al.*, 2006a; Wiedemann *et al.*, 2006b). Lipid II is a precursor of cell wall peptidoglycan and is found at the outer leaflet of the bacterial membrane, and so the lantibiotic: lipid II complex inhibits peptidoglycan synthesis. Nisin binds the pyrophosphate linker of lipid II (Hsu *et al.*, 2004), as does plantaricin C and lacticin 3147 (Ltnα) (Wiedemann *et al.*, 2006a; Wiedemann *et al.*, 2006b). Mersacidin and members of the mersacidin group of lantibiotics target the N-acetylglucosamine (GlcNAc) moiety and most probably the sugar and phosphate residues of lipid II (Bierbaum & Sahl, 2009). Lipid I has also been identified as a target of nisin and epidermin (Brotz *et al.*, 1998b); lipid I is essentially lipid II before the acquisition of the GlcNAc moiety. However, binding to lipid II has a more pronounced effect. It should be noted that lipid I or lipid II are not the target of all lantibiotics and the targets of some lantibiotics are yet to be found, such as those of Pep5 and epilancin K7 (Pag *et al.*, 1999). For some lantibiotics that bind lipid II, including nisin, this binding not only inhibits cell wall biosynthesis but also facilitates pore formation and leads to the release of ions and molecules from the target bacteria (Benz *et al.*, 1991; Bierbaum & Sahl, 2009). Two-component lantibiotics, such as lacticin 3147, are thought to function through the

formation of a three member complex, with the α peptide targeting lipid II and then sequestering the β peptide for pore formation (Wiedemann *et al.*, 2006b). There are a few models which describe how the structural conformation of lantibiotics within the membrane leads to pore formation. This includes the barrel-stave mechanism described by Sahl (Sahl, 1991), the wedge model (Driessen *et al.*, 1995) and a model described by Chikindas (Chikindas *et al.*, 1995). However, not all lantibiotics form pores. Mersacidin, actagardine and cinnamycin (all members of the globular subclass II lantibiotics) only block cell wall/membrane synthesis by binding to receptors, i.e. lipid II or phosphatidylethanolamine (Brotz *et al.*, 1995; Machaidze & Seelig, 2003). Gallidermin (and related peptides) is unusual in that, while it possesses the same putative lipid II binding motif as nisin, it is considerably shorter (22 amino acids, compared to the 34 of nisin). As a result, the ability of gallidermin to form pores depends on the membrane thickness of the target cell and, thus, against some target cells it is the interaction with lipids I and II alone, and not pore formation, that is responsible for inhibiting these targets. The superior activity of gallidermin over nisin in a cell wall biosynthesis assay would seem to explain its high killing potency even in situations where it doesn't form pores (Bonelli *et al.*, 2006).

Another activity that has been attributed to a number of lantibiotics, including nisin, subtilin and sublancin, is the ability to prevent spore outgrowth by *Bacillus* and *Clostridium* species (Hurst, 1981; Paik *et al.*, 1998). An in depth study into the mechanism of action in *B. anthracis* identified that the initiation of germination is essential for the action of nisin. The action of nisin is governed by two events during the outgrowth of spores: the establishment of metabolism and

the shedding of the external spore structures (Gut *et al.*, 2008). In *L. lactis*, truncated nisin A mutants lacking rings D and E were unable to form pores in the membranes or cause a disruption of the membrane potential, but were still able to inhibit the outgrowth of *Bacillus subtilis* spores (Rink *et al.*, 2007). In contrast, mutants in the hinge region of nisin (N20P/M21P and M21P/K22P) which retained the ability to bind lipid II, but were unable to form pores, had antimicrobial activity against vegetative cells of *B. anthracis* but did not inhibit spore outgrowth. Therefore in *B. anthracis,* at least, pore formation is essential to limit spore outgrowth, which suggests that nisin utilizes lipid II as the germinated spore target during outgrowth inhibition and that nisin-mediated membrane disruption is essential to inhibit spore development into vegetative cells (Gut *et al.*, 2011).

It should be noted that there are a number of specific antimicrobial activities associated with individual lantibiotics (Asaduzzaman *et al.*, 2006). In addition, related peptides referred to as lanthipeptides have functions other than as antimicrobial agents (Arnison *et al.*, 2013). The morphogenetic peptides SapB and SapT from *Streptomycetes* act in such a manner (Kodani *et al.*, 2005). These peptides are believed to function as biosurfactants during the formation of aerial hyphae (Willey *et al.*, 2006).

The Role of Cell Wall Modifications in Resistance

Lipid II

In addition to many lantibiotics, a number of other antibiotics also interact with lipid II (e.g. vancomycin), albeit through different parts of the target molecule. Lipid II is the precursor of cell wall biosynthesis found at the outer leaflet of the

bacterial membrane. The main structural component of the bacterial cell wall consists of peptidoglycan which is a polymer of a repeating *N*-acetylmuramic acid (MurNAc) and GlcNAc motif, cross-linked via peptides attached to MurNAc. The final steps in the maturation are catalysed by a penicillin-binding protein (PBP), a bi-functional enzyme which catalyses polymerization of the sugar units (glycosyltransfer), as well as peptide cross-linking (transpeptidation), utilizing Lipid II as substrate (Schwartz *et al.*, 2001). Lipid II, as the corresponding monomer, consists of a single disaccharide pentapeptide bound to a polyisoprenoid anchor called undecaprenyl by a pyrophosphate linker. Binding of antimicrobial substances to lipid II interferes with peptidoglycan biosynthesis by physically sequestering the compound and preventing its utilization by transpeptidase and transglycosylase enzymes that install the cross-linked network of the bacterial cell wall (Lazar & Walker, 2002).

Recently, it was shown that nisin and gallidermin also bind to other intermediates of the wall teichoic acid (WTA) biosynthesis pathway, including lipid III (undecaprenol-pyrophosphate-*N*-acetylglucosamine) and lipid IV (undecaprenol-pyrophosphate-*N*-acetylglucosamine-*N*-acetyl-mannosamine), with the specific interaction with WTA precursors promoting pore formation in artificial lipid bilayers (Müller *et al.*, 2012).

Despite the importance of lipid II to the activity of many lantibiotics, quantification of the levels of lipid II in nisin-sensitive *Micrococcus flavus*, nisin-sensitive *Listeria monocytogenes* and their nisin-resistant variants indicated that nisin resistance was independent of lipid II levels (Kramer *et al.*, 2004).

DltA

Bacterial membranes usually have an overall negative net charge. Bacteriocins, including the lantibiotics, resemble a considerable proportion of the antimicrobial molecules associated with human, vertebrate, invertebrate and plant host defence systems in that they have cationic properties that confer a high affinity for the anionic bacterial cell envelope (Weidenmaier *et al.*, 2003). The Gram positive bacterial cell wall comprises a thick peptidoglycan fabric as well as polymers of alternating phosphate and alditol groups called teichoic acids. Teichoic acids are classified in two groups: WTA (as mentioned previously), which are phosphodiester-linked via a linkage unit to muramic acid residues of peptidoglycan (Hancock & Baddiley, 1985; Ward, 1981), and lipoteichoic acids (LTA), which are macroamphiphiles anchored hydrophobically through the fatty acid residues of their glycolipid component in the outer layer of the cytoplasmic membrane (Fischer, 1988). The *dlt* (D-alanyl-lipoteichoic acid) operon is responsible for D-alanine esterification of both LTA and WTA (Fig. 1(a)) *(Perego et al., 1995).* The *dlt* operon has been characterized across many species and contains four genes required for functionality: *dltABCD* (Neuhaus & Baddiley, 2003). The role of DltA is to act as a D-alanine-D-alanyl carrier protein ligase (Dcl), which activates D-alanine by hydrolysis of ATP and transfers it to the phosphopantetheine co-factor of a specified-alanine carrier protein (Dcp), encoded by *dltC (Heaton & Neuhaus, 1992).* The hydrophobic DltB, which may have a transmembrane location, is required for D-alanine incorporation into teichoic acids, possibly through the transfer of activated D-alanine across the cytoplasmic membrane (Perego *et al.*, 1995). It is thought that DltD transfers D-alanine from the membrane carrier to

teichoic acids, as indicated by the apparent presence of an N-terminal signal peptide. The *dltABCD* gene sequence and organization is highly conserved across a range of species (Peschel *et al.*, 1999). In *Streptococcus agalactiae*, however, in addition to these genes, two regulatory genes of the *dlt* operon, designated *dltR* and *dltS* are located upstream of *dltA* (Poyart et al., 2001). In *C. difficile*, a putative regulatory protein (CD2850) is thought to negatively regulate the *dlt* operon (McBride & Sonenshein, 2011a).

As a result of the D-alanylation of teichoic acids, positive charges are incorporated into the cell wall. Hence, cationic antimicrobial peptides (CAMPs) such as nisin and gallidermin are repelled from the cell envelope of target microorganisms such as *L. monocytogenes, B. cereus, Clostridium difficile, S. pneumoniae* and *S. aureus* (Abachin *et al.*, 2002; Khattar *et al.*, 2009; Kovacs *et al.*, 2006; McBride & Sonenshein, 2011a; Peschel *et al.*, 1999). This is a form of innate lantibiotic resistance and becomes particularly evident in the presence of antimicrobial peptides that trigger a signalling pathway that up-regulates the process (Peschel *et al.*, 1999). This phenomenon was first reported by Peschel *et al*., who determined that a *dltA* mutant of *S. aureus* and *dltA, dltB*, and *dltD* mutants of *Staphylococcus xylosus* did not produce D-alanine esters in their teichoic acids, and that these conditions increased their sensitivity to CAMPs, including the lantibiotic gallidermin. Additionally this study showed that wild-type strains of *Staphylococcus* bearing additional copies of the *dlt* operon contained greater amounts of D-alanylated teichoic acids, and hence repelled cationic proteins more effectively and were less sensitive to gallidermin (Peschel *et al.*, 1999). It was also notable that spontaneously nisin-resistant mutants of *L. lactis* IL1403 expressed the *dlt* and *gal*

operons at a higher level (Kramer *et al.*, 2006). The relevance of the *gal* operon, associated with the Leloir pathway (Grossiord *et al.*, 2003), is that *galE* encodes a UDP-glucose 4-epimerase that is responsible for the synthesis of α-galactose, which is transported across the membrane to become a substituent of LTA. This observation led to further studies in *L. lactis* MG1363, which established that *L. lactis* MG1363Δ*galAMK* was twice as sensitive to nisin as the wild-type, suggesting that α-galactose incorporation has an effect on LTA structure and thus is important in nisin resistance. In addition the LTA of the resistant strain contains twice as much α-galactose as the wild-type, which could indicate a more densely packed LTA, and ultimately making the cell-wall barrier less negatively charged due to the action of DltA (Kramer *et al.*, 2006). Nisin-resistant cells also appeared to have more lipoteichoic acid than nisin-sensitive cells. In addition de-esterified lipoteichoic acids from nisin-resistant cells migrated more slowly through a polyacrylamide gel than those from nisin-sensitive cells. These results indicated that lipoteichoic acids could be modified to increase the resistance of *Streptococcus bovis* to nisin (Mantovani & Russell, 2001). LTA alanylation also seems to affect the susceptibility of *Streptococcus pneumoniae* to nisin (Kovacs *et al.*, 2006)

In the absence of *dltA*, Abachin *et al*. discovered that *L. monocytogenes* was severely impaired in a mouse infection model (4 log increase in the LD_{50}) and, *in vitro*, the adherence of the mutant to various cell lines was impaired, although the amounts of surface proteins associated with virulence (ActA, InlA and InlB) remained unaffected. These results show that the D-alanylation of the LTAs contributes to the virulence of the intracellular pathogen *L. monocytogenes* (Abachin *et al.*, 2002). However, to date, the contrary has not been identified where

increased levels of DltA may contribute to virulence. So whether such a mechanism could enhance virulence due to sensing increased levels of DltA as well as enhancing the lantibiotic resistance of a strain has yet to be determined.

Penicillin-Binding Proteins

Multi-modular penicillin-binding proteins (PBPs) are responsible for peptidoglycan assembly. They are subcategorized as class A or B, depending on the structure and the catalytic activity of their N-terminal module. The C-terminal of the penicillin-binding protein modules of both classes catalyse peptide cross-linking between two adjacent glycan chains through their transpeptidase activity (Goffin & Ghuysen, 1998). The N-terminal module of PBPs belonging to class A are responsible for the glycosyltransferase activity leading to glycan chain elongation (Terrak *et al.*, 1999), whereas in class B PBPs this domain is responsible for interactions with other proteins during septation and also regulates the shape of bacterial cells (Marrec-Fairley *et al.*, 2000).

There have been a number of instances in which a relationship between lantibiotic resistance and PBPs has been noted. First, the expression level of a putative PBP was found to be significantly increased in a spontaneous nisin-resistant strain of *L. monocytogenes* compared to the wild-type non-resistant strain (Gravesen *et al.*, 2001). This strain was also slightly more resistant to mersacidin but was sensitive to a variety of different beta-lactam antibiotics. Similarly, expression of the gene encoding PBP2A (class B PBP) was higher in a nisin-resistant mutant of *L. lactis* IL1403 than in the corresponding wild-type strain, leading to speculation

that resistance was provided by a thicker and more densely packed cell wall (Kramer *et al.*, 2006).

The class A penicillin-binding protein PBP4 (encoded by *lmo2229*) of *L. monocytogenes* has been implicated in nisin resistance as disruption of the encoding gene enhanced nisin sensitivity, with a 1.66-fold decrease in MIC (Guinane *et al.*, 2006). It was also observed that increased expression of LiaS, a histidine kinase element which regulates PBP2229 (PBP4), resulting in increased levels of PBP and nisin resistance in *Listeria* (Gravesen *et al.*, 2004). Gravesen *et al*. suggest that PBP2229 mediates enhanced nisin resistance by shielding lipid II and in addition reduces the extracellular lipid II concentration (Gravesen *et al.*, 2004). This hypothesis is in accordance with previous studies that suggest nisin sensitivity is affected by the accessibility of lipid II (Breukink *et al.*, 1999; Gravesen *et al.*, 2001). Curiously, mutation of another histidine kinase element, LisK, results in enhanced nisin resistance despite reducing expression of both *pbp2229* and *liaS* (Cotter *et al.*, 2002).

IrpT/RmlD

Another novel gene involved in nisin resistance is *irpT*, disruption of which leads to an increased level of resistance. While IrpT is not directly involved in nisin resistance, it has a role in gene regulation. In the absence of IrpT, the gene *rmlD* and four others are up-regulated in *Lactococcus lactis* subsp. *lactis* N8 (Xuanyuan *et al.*, 2010). RmlD is involved in the synthesis of dTDP-l-rhamnose, which is a precursor involved in cell wall polysaccharides backbone production (Tsukioka *et al.*, 1997). The importance of RmlD was confirmed when it was established that

overexpression of the corresponding gene in *L. lactis* MG1363 enhanced nisin resistance (Xuanyuan *et al.*, 2010).

Resistance of Spores to Lantibiotics

Bacterial spores are extremely resistant to biocides, thanks in particular to their thickly layered proteinaceous spore coat (for review see (Leggett *et al.*, 2012)). Although they do not kill spores, lantibiotics such as nisin can be sporostatic, preventing spore outgrowth. An investigation of nisin resistant mutants of *Clostridium botulinum* created through continuous exposure to the lantibiotic resulted in resistant mutants (both spores and vegetative cells) which had the ability to germinate in levels of nisin that reduced the parental strain by 7-8 log cycles (Mazzotta *et al.*, 1997). Although the mechanism by which the action of nisin is circumvented in these resistant mutants is unknown, it appears that it is not a nisin specific phenomenon as resistance was observed to a variety of bacteriocins, across a range of classes.

Cell Membrane Modifications

Lipopolysaccharide (LPS)

Lantibiotics are generally produced by Gram positive bacteria and are most active against other Gram positive strains. Gram negative target microorganisms are usually very resistant to lantibiotics as the architecture of their outer membrane prevents the penetration of the lantibiotics (with sizes of approximately 1800–4600 Da) to the cytoplasmic membrane (Fig. 1(f)) (Brotz & Sahl, 2000; Schved *et al.*, 1994;

Stevens *et al.*, 1991). The outer layer of the outer membrane serves as the site of attachment for lipid A, an anionic dimer of glucosamine linked to fatty acid chains and flanked by polar phosphate groups. Lipid A is covalently bonded to a core polysaccharide and a specific O-chain consisting of repeating oligosaccharide units. This complex is referred to as lipopolysaccharide (LPS), unless it lacks the O-chain, in which case it is named lipooligosaccharide (LOS) (Rietschel *et al.*, 1994). Chelating agents such as EDTA as well as the application of sublethal stresses such as heating or freezing can disrupt the LPS barrier leading to an increased sensitivity among enterobacteria to lantibiotics (Boziaris & Adams, 2000; Kalchayanand *et al.*, 1992; Stevens *et al.*, 1991). Even without such treatments some lantibiotics possess limited activity against Gram negative targets. Microbisporicin, which selectively blocks peptidoglycan biosynthesis causing cytoplasmic UDP-linked precursor accumulation, has some activity against Gram negative species (such as *E. coli*, *Moraxella catarrhalis*, *Neisseria* spp., and *Haemophilus influenza*), which is unseen in other lantibiotics (Castiglione *et al.*, 2008). However, the mechanistic basis for this enhanced activity has yet to be elucidated. Some bioengineered nisin mutants, altered at serine residue 29, are also exceptional by virtue of their enhanced activity against Gram negative strains such as *Cronobacter sakazakii, E. coli* and *Salmonella* Typhimurium (Field *et al.*, 2012).

Lipid Composition

The lipid composition of the bacterial cell membrane also impacts on the levels of resistance. Membranes contain phospholipids whose composition and relative abundance vary profoundly, both between species and under various

environmental conditions and growth phases (DiRusso & Nystrom, 1998; Ratledge & Wilkinson, 1988). The most common bacterial phospholipids are phosphatidylglycerol (PG) and diphosphatidylglycerol (cardiolipin), whose head groups are negatively charged (Huijbregts *et al.*, 2000). Others, such as phosphatidylethanolamine (PE) (a zwitterion), are found in enterobacteriaceae (Matsumoto, 2001) and bacilli (De Mendoza *et al.*, 1993) but not in *Staphylococcus* (Nahaie *et al.*, 1984) or *Listeria* species *(Fischer & Leopold, 1999)*. In a nisin-resistant variant of *L. monocytogenes* Scott A increased levels of PG over cardiolipin was identified as the mechanism of resistance (Verheul *et al.*, 1997). There are corroborating reports that nisin has a greater propensity to penetrate membranes composed of cardiolipin over PG, PE, phostaphatidylcholine (PC), monogalactisyldiacylglycerol (MGDG) and digalactisyldiacylglycerol (DGDG) (Demel *et al.*, 1996). Decreased levels of the anionic phospholipids PG and cardiolipin and increased levels of the neutrally charged PE were also associated with nisin resistance in *L. monocytogenes* ATCC 700302 (Crandall & Montville, 1998). Such a phospholipid composition results in a decrease in membrane net negative charge and hence diminishes the ability of nisin to interact with the membrane (Fig. 1(b)). Nisin resistant cells have been reported to have decreased levels of phospholipids and to have a greater ration of straight-chained:branch-chained fatty acids than parental cells (Fig. 1(c)) (Ming & Daeschel, 1995). Reduced membrane fluidity has also been associated with nisin resistance, with increased long-chain fatty acids and reduced ratios of C15/C17 fatty acids contributing to a more rigid membrane conformation (Martinez & Rodriguez, 2005; Mazzotta & Montville, 1997; Ming & Daeschel, 1993).

MprF

Lysine esterification of one of the two hydroxyl groups of PG results in the free amino groups imparting a net positive charge on PG, creating lysyl-phosphatidylglycerol (L-PG) (Peschel *et al.*, 2001; Ratledge & Wilkinson, 1988). The presence of L-PG and the process of lysinylation has been described in several bacterial pathogens, including *S. aureus (Nahaie et al., 1984; Peschel et al., 2001), L. monocytogenes* (Fischer & Leopold, 1999; Thedieck *et al.*, 2006) and *Mycobacterium tuberculosis* (Maloney *et al.*, 2009), as well as some soil organisms such as *Bacillus species* (Samant *et al.*, 2009; Staubitz & Peschel, 2002). This lysinylation process is performed by MprF (multiple peptide resistance factor) which catalyses the transfer of lysine residues from lysyl-tRNAs to PG (Gould & Lennarz, 1967; Lennarz *et al.*, 1966; Staubitz *et al.*, 2004) and which appears to have a major role in resistance to antimicrobial peptides (Fig. 1(e)). Identification of the protein responsible has led to the corresponding gene being detected in a considerable number of Gram positive and Gram negative genomes (Weidenmaier *et al.*, 2003). In fact, sequence comparisons with known PG synthase sequences allowed the identification of *mprF* homologues in 347 microorganisms, including 31 genera of Gram positive bacteria (mostly Firmicutes and Actinobacteria), 59 genera of Gram negative bacteria (mostly Proteobacteria), and in three species of Archaea within the genus *Methanosarcina* (Roy, 2009).

MprF proteins are integral membrane proteins with two separable functional domains; a well-conserved hydrophilic cytoplasmic domain at the C terminus that synthesizes L-PG and a large hydrophobic domain at the N terminus that facilitates the flipping of L-PG (Ernst *et al.*, 2009). Both domains of MprF are

necessary for the resistance phenotype. The C-terminal domain and six of the fourteen proposed transmembrane segments of MprF are sufficient for the full synthesis of L-PG, but they do not lead to an efficient resistance phenotype, since most of the L-PG remains in the inner layer of the cytoplasmic membrane. However, full resistance is achieved when the N-terminal domain is co-expressed with the L-PG synthase domain and the L-PG is translocated to the outer layer of the membrane (Ernst *et al.*, 2009). L-PG-deficient mutants exhibit increased susceptibilities to many cationic peptides. This was first noted when a mutant of *S. aureus* devoid of L-PG was found to be more susceptible to gallidermin and nisin (Peschel *et al.*, 2001). Comparable effects due to loss of L-PG synthesis were observed for *L. monocytogenes*, with the corresponding deletion mutant being significantly more susceptible to gallidermin and the α-defensins HNP-1 and HNP-2 (Thedieck *et al.*, 2006).

Some Gram positive bacteria, such as *Clostridium perfringens* SM101, possess two *mprF* homologous genes (*mprF1* and *mprF2*). While *mprF2* encodes a lysyl-phosphatidylglycerol synthase (L-PGS), *mprF1* encodes an alanyl phosphatidylglycerol synthase (A-PGS). The formation of alanyl phosphatidylglycerol (A-PG) and L-PG was shown to be tRNA-dependent using Ala-tRNAs and Lys-tRNAs as substrates, respectively (Roy & Ibba, 2008; Staubitz *et al.*, 2004). In *E. faecalis, mprF1* and *mprF2* homologues have been identified, although only MprF2 is involved in aminoacylation and formation of L-PG, A-PG, and R-PG (Bao *et al.*, 2012), a task also carried out by a single MprF protein found in *E. faecium* (Roy & Ibba, 2009). Aminoacylation of PG with glycine and ornithine has also been described (Gould & Lennarz, 1967; Houtsmuller & van, 1963). By

producing the MprF A-PGS protein of *C. perfringens* in an *S. aureus mprF* deletion mutant, it was established that the production of A-PG rather than L-PG production did not affect susceptibility to antimicrobials such as nisin and gallidermin or to the antibiotic daptomycin (Slavetinsky *et al.*, 2012). This indicates that the zwitterion A-PG is just as effective as the cationic L-PG in protecting the cell and maintaining resistance. A-PG contributes up to 6% to the overall lipid content of *Pseudomonas aeruginosa.* A-PG deficiency (due to deletion of an *mprf* homologue) leads to increased susceptibility to β-lactam antibiotics (ampicillin, oxacillin, and cefsulodin), the lipopeptide antibiotic daptomycin and other antimicrobials (protamine sulfate, poly-l-lysine, and polymyxin E) as well as chromium ions (Arendt *et al.*, 2012; Klein *et al.*, 2009), indicating that MprF contributes to reducing the antimicrobial susceptibility in both Gram positive and Gram negative microorganisms.

A similar protective mechanism has been identified in *M. tuberculosis*. In these bacteria, the *lysX* gene, encoding the two-domain lysyl-transferase (MprF)-lysyl-tRNA synthetase (LysU) protein, is responsible for L-PG production. A *lysX* deletion mutant shows sensitivity to cationic antibiotics and antimicrobial peptides. This mutant also shows altered membrane potential compared to the wild-type strain and an increased association with lysosome-associated membrane protein–positive vesicles. This indicates that the *lysX* mutant strain is not as adept at preventing fusion of phagosomes with lysosomes, a process which is partly required to allow intramacrophage replication (Maloney *et al.*, 2009). It is interesting to note that the expression of the *mprF* fragment of *lysX* alone does not lead to the production of L-PG. This is notable since two lysU genes are encoded in *M. tuberculosis*, one is a cytosolic essential gene, the other as a domain of lysX. It

therefore appears that unlike other bacteria, the cytosolic LysU and the membrane-bound MprF do not cooperate to produce L-PG (Maloney *et al.*, 2009).

In conclusion, the presence of these aminoacylated PGs provides a way for bacteria to shield themselves from the action of lantibiotics, and CAMPs in general. The extent of this protection can be considerable ranging from a 1.5 fold increase in the amount of gallidermin required to inhibit *L. monocytogenes* (Thedieck *et al.*, 2006), to a 7- and 28- fold increase in resistance to gallidermin and nisin, respectively, in *S. aureus* (Peschel *et al.*, 2001).

fab Operon

In a nisin resistant *L. lactis* strain, the operon *fabDG1G2Z1Z2* is expressed to a lower extent than in the sensitive wild-type (Kramer *et al.*, 2006). This operon is involved in membrane synthesis via saturation and elongation of phospholipids (Heath & Rock, 1995). The decreased expression of the *fab* operon might lead to a reduced amount of saturated fatty acids and less elongated fatty acids in the membrane, making it less densely packed. Such alterations in cytoplasmic membrane composition might influence the ability for nisin to interact with the membrane and thus increase resistance. However, further investigations are required to definitively establish the contribution of this operon in providing protection against lantibiotics.

Two Component Systems Associated With Lantibiotic Resistance

Two-component systems (TCS) are thought to function as a monitor that allows the cell to adapt to specific environmental conditions. It contains a sensor that encodes a sensory histidine-kinase (HK) and a regulator that encodes a cognate response regulator. In addition, an intramembrane-sensing HK (IM-HK) lack an extracytoplasmic domain, indicative for a sensing process at or from within the membrane interface. Two major groups are found in Firmicutes linked to maintaining cell envelope integrity, mediating antibiotic resistance, or detoxification processes and can be differentiated based on sequence similarity and genomic context: (1) BceS-like IM-HK that are functionally and genetically linked to ABC transporters, and (2) LiaS-like IM-HK, as part of three-component systems (Fig. 2).

1. BceRS-like Two Component Systems

BceRS of B. subtilis

BceS-like IM-HKs possess two transmembrane helices but lack any extracellular sensory domains. They are functionally and genetically linked to ABC transporters named BceAB (Dintner *et al.*, 2011; Mascher, 2006). They are almost exclusively found in Firmicutes, with 80% of the transporters associated with a BceRS-like TCS (Dintner *et al.*, 2011). It would appear that the presence of antimicrobial peptides cannot be detected by these histidine kinases alone, but rather that this resistance module relies on the transporters for stimulus recognition i.e. in the presence of an antimicrobial peptide, the transporter somehow communicates with the sensor kinase. It is believed that this involves

direct contact between the transporter and histidine kinase (Dintner *et al.*, 2011; Falord *et al.*, 2012). However, the mechanism by which signalling occurs is as yet unknown. This communication leads to the activation of the cognate response regulator and, subsequently, an induction of transporter gene expression. Importantly, ATP hydrolysis by the transporter, and therefore active transport, are required for the signalling process (Fig. 2) (Hiron *et al.*, 2011; Rietkotter *et al.*, 2008).

Experimental evidence from a number of homologous systems from *B. subtilis, Staphylococcus aureus, Streptococcus mutans,* and *Lactobacillus casei* confirms that this signalling pathway is a general characteristic of the Bce-type modules and, on a number of occasions has been associated with lantibiotic resistance (Falord *et al.*, 2012; Hiron *et al.*, 2011; Ouyang *et al.*, 2010; Revilla-Guarinos *et al.*, 2013; Rietkotter *et al.*, 2008; Staron *et al.*, 2011). For example in *L. lactis*, a homologue named LlrG/KinG, is found encoded either side of a VanZ-like protein. VanZ is found in *E. faecium* and confers resistance to teicoplanin (Arthur *et al.*, 1995). Downstream from *llrG/kinG,* the ABC transporter *ysaBC* is located. The expression of this transporter is induced by nisin and consequently confers resistance to this lantibiotic (Kramer *et al.*, 2006).

The BceAB-type transporters are well characterised and have been classified within the peptide-7 exporter (Pep7E) family in the Transport Classification Database (TCDB) (Saier *et al.*, 2009). These transporters are composed of an ATPase (BceA) and a permease (BceB) component with 10 transmembrane helices and a characteristic, large, extracellular domain of approximately 200 amino acids between helices VII and VIII. These transporters contribute to *B. subtilis* bacitracin

and lantibiotic resistance. With the obseved MIC of bacitracin for the *bceAB* mutant reduced ~30 fold and resistance to actagardine and mersacidin reduced ~2- to 4-fold (Mascher *et al.*, 2003; Ohki *et al.*, 2003; Rietkotter *et al.*, 2008; Staron *et al.*, 2011). Through random mutagenesis, the specific functions of the transporter were found to be primarily associated with the C-terminal region of the permease, BceB, and particularly in the eighth transmembrane helix. Furthermore, although signalling and resistance are functionally interconnected, several mutations identified had stronger effects over one than the other (Kallenberg *et al.*, 2013). The co-occurrence and coevolution of Pep7E transporters and the BceRS-like TCS has led to the hypothesis that a functional link always exists between these systems, in which they cooperate in both signalling and detoxification (Dintner *et al.*, 2011). In addition to BceRS-AB, there are two further paralogous peptide-sensing and detoxification (ABC transporter) modules present within the *B. subtilis* genome i.e. YxdJK-LM and PsdRS-AB (formerly YvcPQ-RS). The YxdJK-LM system responds to the human antimicrobial peptide LL-37 (Pietiainen *et al.*, 2005) and the PsdRS-AB system responds primarily to lipid II-binding lantibiotics, such as nisin, gallidermin, subtilin, and actagardine (strongest inducer) as well as the lipid II-binding lipopeptide, enduracidin. Other lantibiotics, including Pep5, mersacidin sublancin and duramycin, as well as for lipid II-binding antibiotics vancomycin and ramoplanin, are not inducers (Staron *et al.*, 2011). On the basis of these findings, and the different lipid II-associated motifs that these peptides target, it would seem that PsdRS induced expression is linked to antimicrobials with an N-terminal lipid II-binding motif, referred to as a pyrophosphate cage. A range of TCSs were characterised in *L. casei* BL23, insertional mutations identified that of these TC09

and TC12, which are homologous to the three paralogous TCS of *B. subtilis*, BceRS, PsdRS, and YxdJK were responsible for nisin resistance. Of the remaining mutated TCSs identified, some showed more or even greater sensitivity to nisin and one (T04) showed more resistance (Alcantara *et al.*, 2011).

BraRS of S. aureus

A BceRS-like (IM-HK) TCS associated with *S. aureus*, BraRS, has been shown to be essential for resistance to bacitracin, nisin and nukacin ISK-1 (Hiron *et al.*, 2011; Kawada-Matsuo *et al.*, 2013b; Kolar *et al.*, 2011; Yoshida *et al.*, 2011). As a consequence of having been identified in three separate studies, this TCS is also referred to as BceRS (Yoshida *et al.*, 2011) and NsaRS (Blake *et al.*, 2011). The name BraRS (bacitracin resistance associated) is representative of its characteristics and so has been chosen for further referral (Hiron *et al.*, 2011). Through use of a *lacZ* reporter–gene fusion, it has been demonstrated that *braRS* expression is up-regulated by a variety of cell-envelope-damaging antibiotics, including phosphomycin, ampicillin, nisin, gramicidin, carbonyl cyanide *m*-chlorophenylhydrazone and penicillin G (Kolar *et al.*, 2011). Immediately downstream of *braRS* are the genes which encode the ABC transporter BraDE (an example of the BceAB type transporters discussed above), which is also of key importance to bacitracin and nisin resistance through sensing and signalling through BraRS. BraRS also activates transcription of the detoxification ABC transporter, *vraDE* (Hiron *et al.*, 2011) (Fig. 3). Highly conserved imperfect palindromic sequences have been identified upstream from the *braDE* and *vraDE* promoter sequences that are essential for transcriptional activation by BraRS (Hiron

et al., 2011). Microarray analysis revealed that the transcription of 245 genes is altered in a Δ*braS* mutant (the majority being down-regulated). These 245 genes include several that are involved in transport, drug resistance, cell envelope synthesis, transcriptional regulation, amino acid metabolism and virulence (Kohler *et al.*, 2009). Thus BraRS has an important resistance role in *S. aureus* and functions to reprogram gene expression to modify cell envelope architecture, facilitating adaptation and survival.

The extent of the contribution of BraRS to antimicrobial resistance, relative to other TCSs, has been assessed through the creation and analysis of a number of mutants. When a *S. aureus* Δ*braRS* mutant was co-cultured with a nukacin ISK-1 or nisin A producer, a significant decrease in numbers was observed, which was not apparent for the parental *S. aureus* strain. In contrast, Δ*vraSR* and Δ*graRS* (GraRS is yet another TCS) mutants were more moderately affected, thus establishing the greater importance of BraRS, and its associated transporters (Kawada-Matsuo *et al.*, 2013b). Interestingly, through screening of a bank of nisin resistant mutants, a single mutation was identified in *braS* (A20E) that conferred an 8-16 fold increase in nisin resistance (Blake *et al.*, 2011). It is thus apparent that changes to BraRS can provide a means via which *S. aureus* can attain enhanced resistance to lantibiotics.

GraRS of S. aureus

Overexpression of a second IM-HK identified in *S. aureus* and its associated response regulator, named GraRS, has been linked with the vancomycin-intermediate *S. aureus* (VISA) phenotype. This TCS is also referred to as *vraCA* (Li *et al.*, 2007a)) and closely resembles *bceRS* of *B. subtilis* (Cui *et al.*, 2005). GraRS

induces expression of the adjacently located genes *vraFG* that encode an ABC transporter (Meehl *et al.*, 2007), which is known to be overexpressed in strains exhibiting increased vancomycin resistance (Kuroda *et al.*, 2000). This transporter is revealed to be involved in antimicrobial sensing and signalling through GraSR (Falord *et al.*, 2012). A recent study identified an upstream gene whose product, GraX, appears to be a cytosolic accessory protein which acts by signalling through the GraS kinase and also effectively interacts with VraF and VraR. GraXRS-VraFG has been defined as a five-component system involved in cationic antimicrobial peptide sensing and signal transduction to promote resistance to these peptides in *S. aureus* (Fig. 3) (Falord *et al.*, 2012). It has also been suggested that GraRS may regulate more than just *vraFG*. Analysis of *graR* and *graS* mutants highlighted alterations in overall surface charge, with charge becoming more negative than that of wild-type cells (Meehl *et al.*, 2007). It has also been discovered that these cell surface changes are due to the regulation of *dlt* and *mprF* by GraRS (Li *et al.*, 2007a; Matsuo *et al.*, 2011). GraRS are also thought to contribute to tolerance of high temperatures, survival upon exposure to oxidative stress and the regulation of pathogenicity (Falord *et al.*, 2011).

With respect to lantibiotic resistance/sensitivity, it has been noted that a *graRS* negative mutant exhibits a greater susceptibility to nukacin ISK-1 than nisin A, while no susceptibility to bacitracin is observed (Kawada-Matsuo *et al.*, 2013b). A previous study found a *graR* mutant had increased susceptibility to vancomycin and polymyxin B. Interestingly single or multiple amino acid changes within GraR have been associated with enhanced resistance, such changes include S197N (Cui *et al.*, 2005; Meehl *et al.*, 2007). Expression of *graR* transcripts were not found to increase

upon exposure of the cell to nukacin ISK-1 or nisin (Kawada-Matsuo *et al.*, 2013b). This supports the model proposed in the same study which details the co-ordinated effort achieved by GraRS and BraRS, upon exposure of *S. aureus* to a relatively high level of nisin A or nukacin ISK-1 or such cationic antimicrobials. That is, GraRS is seen as important for resistance until significant induction of VraDE expression by BraRS occurs. In conclusion *S. aureus* is defined to possess three distinct class-I-bacteriocin-resistance systems, implemented through the independent action of VraSR (an LiaRS-like TCS) and the co-ordinated function of the two TCS mentioned above (Fig. 3) (Kawada-Matsuo *et al.*, 2013b). *S. epidermidis* also harbours a GraRS equivalent named ApsRS that has been found to be induced by nisin and other antimicrobials (Li *et al.*, 2007b).

AnrAB Transporter of L. monocytogenes

AnrAB was named on the basis of its role; i.e. *A*BC transporter involved in *n*isin *r*esistance. The permease component of the transporter was discovered as a result of screening a *mariner* transposon bank of *L. monocytogenes* EGD-e mutants for nisin sensitivity and was found to be a BceAB-like transporter. Examination of a subsequently generated Δ*anrB* deletion mutant also revealed an associated increased sensitivity to the lantibiotic gallidermin, to bacitracin and to a large number of β-lactam antibiotics (Collins *et al.*, 2010a). Based on these results it was proposed that AnrAB is a multidrug resistance (MDR) transporter that contributes to the innate antimicrobial resistance of *L. monocytogenes*. The TCS VirRS and RpoN (σ^{54}) are responsible for regulation of *anrAB* (Arous *et al.*, 2004; Mandin *et al.*, 2005). It has been proposed that Lmo1746-47 (a BceAB-like ABC transporter

downstream from VirR), along with AnrAB and VirRS, may form an antimicrobial sensing and detoxification module similar to the VraDE-BraRS-BraDE network in *S. aureus* (Gebhard & Mascher, 2011).

Interestingly, AnrAB, which is a BceAB-like transporter, is most similar to an ABC transporter in *Streptococcus pneumoniae* D39 (Sp0912-Sp0913), which was found to contribute to the innate resistance of this strain to nisin, bacitracin, gramicidin (all cell envelope-active antimicrobials), and lincomycin (a protein synthesis inhibitor). Furthermore, expression of *sp0912* is induced by up to 13-fold upon treatment with nisin or bacitracin (Majchrzykiewicz *et al.*, 2010) and, more recently, has been found to be regulated by a TCS designated Rr01-Hk01 (Dintner *et al.*, 2011).

2. LiaRS and LiaRS-like Two Component Systems

Most members of the phylum Firmicutes harbour a TCS, generally referred to as LiaRS (formerly *yvqEC*), which is involved in the response to cell envelope stress elicited by inhibitors of the lipid II cycle (Fig. 2(b)). Upon the addition of vancomycin or bacitracin, LiaRS autoregulates the genes *liaIHGFSR,* which are organised across two operons *liaIH* and *Lia(G)FSR*. Across the Firmicutes *liaG* is only present in *Bacillus* spp. closely related to *B. subtilis* (Wolf et al., 2010). Systematic deletion of the operon has revealed the functions of each gene product. LiaF is a potent negative regulator of LiaR-dependent gene expression. As the sequence and genomic location of the *liaF* gene is conserved in the Firmicutes, it would seem that LiaFRS is a common three component system, with both positive and negative feedback loops that sense and respond to cell envelope stress signals. LiaH, a

homolog of *E. coli* phage shock protein A, responds through damage sensing. *In silico* analysis could not predict the role of *liaI* or *liaG*, though the fact that they harbour two or one trans-membrane domains, respectively, is indicative of membrane localization. However, LiaI seems to be involved in sensing and counteracting membrane damage (Jordan *et al.*, 2006; Wolf *et al.*, 2010). In addition to characterisation of the genes within the operon the promoter of the *lia* operon and the cis-acting sequences necessary for antibiotic-inducible gene expression were also identified (Mascher *et al.*, 2004). This promoter responded strongly to a subset of cell wall-active antibiotics that interfere with the lipid II cycle, such as bacitracin, ramoplanin, vancomycin and nisin (Mascher *et al.*, 2004). A detailed analysis strongly suggests that the stimulus sensed by the LiaRS system is some aspect of interference with cell wall biosynthesis by such antimicrobials, rather than directly sensing their presence. In that instance LiaRS acts as a damage-sensing and signal-transducing system, and thus contrasts with BceRS-AB which is thought to function through direct drug sensing and signal transduction (Wolf *et al.*, 2012).

The role of LiaRS in *L. monocytogenes* has been investigated (Collins *et al.*, 2012; Fritsch *et al.*, 2011; Gravesen *et al.*, 2004). LiaRS-mediated signal transduction is also negatively regulated by LiaF in a similar fashion to that described for *B. subtilis* (Fritsch et al., 2011). LiaFRS in *B. subtilis* is seen to regulate expression of itself and *liaIH* (Wolf *et al.*, 2010), and as seen in *L. monocytogenes* sv1/2a EGD this occurs in response to exposure to the cell wall-active antibiotics vancomycin and bacitracin. In *L. monocytogenes*, however, the expression of 27 genes (Fritsch *et al.*, 2011), including penicillin-binding protein (PBP) (Gravesen *et al.*, 2001) and *telA*,

which encodes a protein involved in toxic ion resistance are also regulated (Collins *et al.*, 2010b). Collins *et al*. have reported that a Δ*liaS* strain grew more successfully than the corresponding wild-type in the presence of nisin. A mutant lacking another histidine kinase, Δ*lisK*, which also has a nisin-resistant phenotype, was previously reported to alter *liaS* and PBP expression. With this in mind, the nisin resistance of 3 mutants Δ*liaS,* Δ*liaS/*Δ*lisK* and Δ*lisK* was compared and it was discovered that the Δ*lisK* mutant is 2-fold more resistant than the wild-type and that the Δ*liaS* and Δ*liaS/*Δ*lisK* mutants are a further 2-fold more resistant. On the basis of the nisin-resistant phenotype of these mutants, it was postulated that these phenotypes were attributable to the increased production of the corresponding PBP, the expression of which was also increased in the Δ*liaS* mutant (Collins *et al.*, 2012). In contrast, mutation of the corresponding response regulator, Δ*liaR*, in seven wild-type strains of *L. monocytogenes* resulted in an average decrease in cell density of 5.2 ± 0.7 $\log_{10}$ CFU ml^{-1} after 24 hr for the 7 Δ*liaR* strains compared to the wild-type on exposure to nisin (Bergholz *et al.*, 2013). Furthermore, this study established that *lmo1746*, a gene downstream from *virR* (a response regulator) encoding an ABC transporter permease, contributed to LiaR-mediated nisin resistance (Bergholz *et al.*, 2013). A Group B *Streptococcus* Δ*liaR* mutant has also been found to be more susceptible to cell wall-active antibiotics (vancomycin and bacitracin) and to antimicrobial peptides (polymyxin B, colistin, and nisin) than the parent strain. In the absence of LiaR this strain also became avirulent, no longer causing sepsis and pneumonia in mouse models (Klinzing *et al.*, 2013). The different consequences of mutating LiaS and LiaR most likely reflect the inactivation of different members of

the three-component system, with deletion of LiaS potentially resulting in the cross-activation of LiaR by other histidine kinases.

The LiaFSR system of *S. mutans* has also been characterised and is known to regulate genes encoding membrane- and cell wall-associated proteases and chaperones as well as genes encoding proteins involved in cell envelope biogenesis and remodelling (Suntharalingam *et al.*, 2009). LiaRS has also been identified to upregulate *dltA* expression in *Streptococcus gordonii* (McCormick *et al.*, 2011). CesSR (also referred to as TCS-D (Mascher *et al.*, 2004; O'Connell-Motherway *et al.*, 2000)) is a LiaRS homologue found in *L. lactis*. Upstream of *cesSR* is *llmg1650,* a *liaF* homologue. A *L. lactis* Δ*cesR* mutant was found to be approximately twofold more sensitive to nisin, plantaricin C and bacitracin than the corresponding parent strain. It has also been established that the bacteriocins lactococcin 972 and plantaricin C, as well as bacitracin, and vancomycin, act as inducers of the *llmg1650* promoter in a concentration-dependent manner, but that nisin did not induce this promoter (Martinez *et al.*, 2007).

VraSR of S. aureus

To date four two-component systems involved in both cell wall synthesis and drug resistance have been identified in *S. aureus*; WalKR, BraRS, GraRS and VraSR. While WalKR is more specifically involved in cell wall metabolism and its regulation, the other three TCSs contribute significantly to antimicrobial resistance (Dubrac *et al.*, 2007; Mascher, 2006). The *vraSR* genes are homologous to *liaRS* of *B. subtilis*. VraSR, vancomycin resistance associated sensor/regulator, contributes to resistance to cell-wall-targeting agents such as lantibiotics, and has been shown to

protect against cell damage by modulating components of the peptidoglycan biosynthesis pathway. Through microarray analysis of *S. aureus* strain N315 and its *vraSR* null mutant, Kuroda *et al*. discovered that of 139 transcripts induced by exposure of the parent strain to vancomycin, 46 were no longer transcribed in the mutant strain (Kuroda *et al.*, 2003). Among the genes regulated by VraSR are those encoding SgtB (a monofunctional glycosyltransferase involved in the polymerization of peptidoglycan), PBP2 (penicillin-binding protein 2 also involved in peptidoglycan polymerization) and MurZ (UDP-*N*-acetylglucosamine enolpyruvyl transferase required for murein monomer precursor synthesis); all involved in cell-wall biosynthesis. A number of genes previously associated with β-lactam, as well as glycopeptide resistance in *S. aureus* have also been identified to be under the control of VraSR. Among them is *vraDE,* which encodes an ABC transporter that is linked with bacitracin and nisin resistance. This transporter is also regulated by another *S. aureus* TCS, BraRS (Hiron *et al.*, 2011). It has been demonstrated that overproduction of PBP2 significantly increases resistance to the lipid II cycle disrupting antibiotic teicoplanin (Hanaki *et al.*, 1998). Therefore, the significant reduction in teicoplanin resistance observed in *vraSR* null mutants agrees well with the loss of PBP2 induction. Konuda *et al*. also revealed that over-expression of *vraSR* via a high-copy-number plasmid alone did not increase transcription of *pbp2* and *sgtB (Kuroda et al., 2003).* Induction thus requires exposure to the cell-wall synthesis inhibitors in the presence of VraSR. In other words, resistance is dependent on attack in the form of inhibition of cell-wall synthesis, which in turn activates the VraS sensor kinase. In addition, VraSR is independently activated upon inhibition of cell wall biosynthesis, such as was observed through the action of

mersacidin, daptomycin and vancomycin (Kawada-Matsuo *et al.*, 2013b; Sass *et al.*, 2008). However, a study by Muthaiyan *et al*., revealed that unlike daptomcin and vancomycin in the previous study, nisin treatment did not induce *vraSR* expression (Muthaiyan *et al.*, 2008). This was confirmed by a second study that demonstrated that the lantibiotic nukacin-ISK1 had such induction properties; this difference was attributed to their differing structures and modes of action (Kawada-Matsuo *et al.*, 2013b).

In the absence of VraSR, *S. aureus* shows a significant increase in susceptibility against various cell wall synthesis inhibitors and antimicrobials, including nukacin-ISK1, oxacillin, cefoxitin, tunicamycin, cycloserine daptomycin, vancomycin and bacitracin (Kawada-Matsuo *et al.*, 2013b; Kuroda *et al.*, 2003; McCallum *et al.*, 2011). The *vraSR* genes have been found to be more highly expressed in some vancomycin intermediately resistant *S. aureus* (VISA) compared to vancomycin susceptible *S.* aureus (VSSA). In fact, VraSR has been proposed to be involved in the VISA-type resistance mechanism via contribution to cell wall thickening which prevents antimicrobials from reaching their target molecules, as is the case for vancomycin and lipid II (Fig. 1(d)). Although mersacidin also has the same target molecule, it does not bind to the D-alanyl-D-alanine terminus. In the case of the thickened cell wall, increased free amounts of false such target sites are present, leading to reduced diffusion velocity of vancomycin through the cell wall. Resistance to mersacidin is not hindered in such a manner but is still observed to induce the VraSR TCS (Kuroda *et al.*, 2000; McAleese *et al.*, 2006; Sass *et al.*, 2008).

3. Other Two Component Systems

LisRK of L. monocytogenes

LisRK is a TCS associated with a significant role in the virulence potential of *L. monocytogenes*. Originally identified in the strain LO28 it has since been revealed that in addition to its major contribution in responding to ethanol, pH, and hydrogen peroxide stresses, LisRK is involved in the cell's tolerance to antimicrobials including nisin and the cephalosporin family of antibiotics (Cotter *et al.*, 1999; Kallipolitis & Ingmer, 2001). A Δ*lisK* mutant showed a large reduction in the expression of three genes. One is thought to encode a penicillin-binding protein that shows homology to PBP2a of *Streptococcus pneumoniae* and PBP1a of *Bacillus subtilis*, both of which are high-molecular-weight PBPs possessing glycosyltransferase and transpeptidase domains (Cotter *et al.*, 2002). The other LisK-regulated genes encode another histidine kinase that is homologous to LiaR of *B. subtilis* and a protein of unknown function with homology to *B. subtilis* YvlB (Cotter *et al.*, 2002).

VirRS of L. monocytogenes

In almost all cases, genes encoding two-component systems are contiguous on the chromosome. This is not the case for *virR* (regulator) and *virS* (HK), which are separated by three genes. *L. monocytogenes* transcriptomic analysis of both the *virR*::Tn*917* and Δ*virS* mutants revealed that VirR positively controlled 12 genes and that, while VirS also regulated these genes, it also regulated a further 108 genes in both a positive and a negative manner. The simplest explanation of this difference would be that the VirS histidine kinase is able to interact with one or

several other response regulators, leading to the activation/repression of the transcription of these genes (Mandin *et al.*, 2005). The putative VirR DNA-binding site, a palindromic region of 16 bases, was found upstream of transcriptional units commonly regulated by VirR and VirS. The palindrome was highly conserved between the different promoter regions and showed a common organization (Mandin *et al.*, 2005).

The VirR/VirS system was originally identified as being involved in *L. monocytogenes* virulence, with *in vivo* up-regulation of VirR mediated by the major virulence regulator PrfA (Camejo *et al.*, 2009). With respect to genes known to contribute to innate nisin resistance, it is notable that *dltA*, *mprF* and *anrB* have been shown to be VirRS-regulated in *L. monocytogenes* (Collins *et al.*, 2010a; Mandin *et al.*, 2005). This strongly suggests that a significant role of the VirR/VirS system is to regulate resistance of *L. monocytogenes* to cationic peptides.

CprRK of C. difficile

The CprK sensor kinase and an orphan response regulator CprR, encoded elsewhere on the chromosome, are responsible for the resistance of *Clostridium difficile* to cationic antimicrobial peptides, including lantibiotics such as nisin and gallidermin (Suarez *et al.*, 2013). The lantibiotics nisin, gallidermin, subtilin, mutacin 1140 and cinnamycin were found to activate the CprK-CprR system, while sublancin, cytolysin, and actagardine did not. It was proposed by Suarez *et al*. that a combination of the (methyl-)lanthionine groups along with proline and glycine residues form a motif that interacts with CprK and leads to activation of the CprK-CprR two-component system. CprK and CprR are both expressed at low levels prior

to lantibiotic exposure, but CprK expression is markedly induced upon activation, while CprR expression is not (Suarez *et al.*, 2013). The separate locations of the *cprR* and *cprK* transcripts may explain the fact that they are not co-ordinately regulated. The genes encoding the ABC transporter CprABC are regulated by CprK-CprR and found adjacent to *cprK* on the genome. Insertional disruption of one of the transporter genes resulted in significant decreases in resistance to both nisin and gallidermin (McBride & Sonenshein, 2011b). Hence, this TCS and ABC transporter pair, contribute to the resistance of *C. difficile* to many lantibiotics.

NsrRS and LcrRS of S. mutans

In *S. mutans*, two novel two-component systems, NsrRS and LcrRS, are associated with resistance to nisin A, lacticin 481 and nukacin ISK-1. NsrRS regulates the expression of NsrX, a protein which shares homology with several acetyltransferases, including those of the TraX family, which is associated with F pilin acetylation in *E. coli.* Nisin-binding studies revealed that more nisin bound to cells that had a complemented Δ*nsrX* mutation than the mutant. Thus, it was determined that NsrX (or an as-yet-unidentified factor modified through NsrX) is involved in binding nisin A and preventing its interaction with lipid II. LcrRS regulates the expression of the ABC transporter LctFEG. Mutation of the genes *lcrRS* and *lctFEG* results in increased susceptibility to nukacin ISK-1 (and lacticin 481 with respect to Δ*lcrRS*) (Kawada-Matsuo *et al.*, 2013a).

Other Resistance Elements

Biofilms

A biofilm is a population or community of bacteria living in organized structures at a liquid interface. Within a biofilm, each bacterium occupies a specific microenvironment, which is determined by surrounding cells, proximity to a channel (both of which determine the pH and availability of nutrients and oxygen) and the extracellular polymeric substance (EPS) matrix. The structuring of biofilms in micro-colonies and water channels has been shown to be influenced by fluid flow, nutrient composition and intercellular small messenger molecules, or quoromones (acylated homoserine lactones, AHLs), that are used for bacterial communication. Cells in biofilms are more resistant to antimicrobials than planktonic (free-living) cells, providing protection against many antimicrobials, including lantibiotics, and against protozoan grazing and host defences (Anderson & O'Toole, 2008; Mah & O'Toole, 2001; Matz & Kjelleberg, 2005).

All biofilms contain an extracellular matrix that holds cells together. This matrix is often composed of a polysaccharide biopolymer along with other components such as proteins or DNA (Branda *et al.*, 2005). The nature of the matrix exopolysaccharide greatly varies depending on growth conditions, medium and substrates, and also varies amongst bacterial strains. Cells in biofilms also employ adhesive proteins. For instance, *S. aureus* matrix harbours *B*iofilm-*A*ssociated *P*roteins (termed Bap) that are required for biofilm formation. Anchored to the cell wall of *S. aureus,* these proteins serve to hold cells together within the biofilm, probably by interacting with other proteins on the surface of adjacent cells. In addition to the exopolysaccharides and proteins, extracellular DNA (eDNA) also

provides structural integrity to the biofilm. *S. aureus* biofilms, for example, have eDNA in the matrix, providing added stability to the biofilm. It is thought that this eDNA is caused by cell lysis and the release of genomic DNA (Rice *et al.*, 2007).

Increased resistance to environmental stresses as observed in biofilms appears to be as a result an increase in the portion of persister cells, which neither grow nor die in the presence of bactericidal agents, and thus exhibit multidrug tolerance (Spoering & Lewis, 2001). Persister cells have been proposed to be protected from the action of antibiotics because of their dormant nature and the requirement of active targets by antibiotics. Persister cells express toxin–antitoxin systems which lead to this dormant state. The toxin typically is a protein that inhibits important cellular functions such as translation or replication, and forms an inactive complex with the antitoxin (for review see (Hayes & Van Melderen, 2011)). Overproduction of chromosomally-encoded "toxins" such as RelE, an inhibitor of translation, or HipA, the first validated persister/multi drug tolerence gene, causes a sharp increase in persisters and an increase in antimicrobial tolerance (Correia *et al.*, 2006; Keren *et al.*, 2004). Deletion of the *hipBA* module produces a sharp decrease in persisters in both stationary and biofilm cells (Keren *et al.*, 2004). Extracellular matrices also act as a diffusion barrier to small molecules. The reduced diffusion of nutrients, vitamins, or cofactors in biofilms results in the presence of metabolically inactive cells within the bacterial community. In addition, as a consequence of being confined to a restricted space, the rate of bacterial growth is limited (Stewart & Franklin, 2008). Hence, bacteria in biofilms are naturally in a stationary phase of growth, resulting in the production of secondary metabolites such as antibiotics, pigments, and other small-molecules (Martin & Liras, 1989).

These secondary metabolites also function as signalling molecules to initiate the process of biofilm formation (Lopez & Kolter, 2010). Thus the physical protection provided by biofilms, their compact nature and the reduced rates of cellular growth, bacteria within biofilms are less susceptible to the action of antimicrobials. Indeed, cells in biofilms have been shown to exhibit a 10- to 1000- fold greater antimicrobial resistance than the corresponding cells when grown planktonically and with notably great cell densities (Davies, 2003). A study of the antibiotics amoxicillin, doxycycline and metronidazole revealed that the MICs increased markedly when planktonic populations of *Porphyromonas gingivalis* were tested at cell densities equal to those found in biofilm populations (10^7–10^8 cells/ml) (Larsen, 2002).

The resistance of biofilms to lantibiotics has been investigated and it is clear that, like many other antimicrobials, lantibiotics can be effective in preventing the formation of biofilms but are less successful at killing microorganisms present in established biofilms. In one instance, the lantibiotic gallidermin and the well-studied biofilm-forming *S. aureus* SA113 and *S. epidermidis* O47 strains were the subject of investigation (Saising *et al.*, 2012). When used at MIC levels as determined with planktonic cells, gallidermin was completely unable to form a biofilm. In the case of *S. aureus*, a biofilm-inhibiting effect was evident even when in the sub-MIC range. It was particularly notable that the transcription levels of a gene involved in primary adhesion (the major autolysin, *atl*) and another involved in exopolysaccharide production (the intercellular adhesin, *ica*) were significantly decreased in the presence of gallidermin. In contrast, when biofilm-associated staphylococci were treated with 8 × MIC of gallidermin, colony forming units (CFUs)

were decreased by 3 logs in the 24hr biofilms and by only 1 to 2 logs in the 5-day biofilms (Saising *et al.*, 2012). The activity of the two peptide lantibiotic, lacticin 3147, against *S. mutans* in biofilms has also been tested. At 2 X MIC (6.25µmol l^{-1}), lacticin 3147 reduced *S. mutans* biofilm formation by ~90%. However, when 50 µmol l^{-1} lacticin 3147 was tested against 1-day old biofilms of *S. mutans*, only a 24-50% reduction was apparent (Dobson *et al.*, 2011). It is worth noting that many *S. mutans* strains are capable of producing lantibiotics and/or other bacteriocins, known as mutacins. The production of these antimicrobials may allow these strains to compete within the dental biofilm and to persist when nutrients become limited in the dental plaque (Nguyen *et al.*, 2009). Similarly, nisin has been shown to prevent biofilm formation of 25 *S. aureus* strains at minimum growth inhibitory concentrations, however at subinhibitory concentrations biofilm formation was not prevented (Sudagidan & Yemenicioglu, 2012).

A recent study compared the potential of lantibiotics nisin A, nukacin ISK-1 and the class II bacteriocin lacticin Q to inhibit 24 hr established methicillin-resistant *S. aureus* biofilms (Okuda *et al.*, 2013). Despite targeting lipid II, nukacin ISK-1 has a bacteriostatic mode of action only, inhibiting cell wall synthesis but not forming pores (Asaduzzaman *et al.*, 2009). Lacticin Q functions by forming toroidal pores in target membranes (Yoneyama *et al.*, 2009). From this study, it would appear that pore formation leading to ATP efflux is important for bactericidal activity against biofilm cells. Of the antimicrobials tested, nisin A most closely fits this requirement, with ATP efflux observed through the action of nisin A at 0.25× MIC (0.625 µM), whereas 1× MIC (5.0 µM) lacticin Q was required. Nukacin ISK-1 had no effect on ATP efflux even at 8× MIC (96 and 80 µM) (Okuda *et al.*, 2013).

Nisin has been encapsulated within liposomes (nisin-liposome) in order to prolong the inhibition of *S. mutans* strain 10449b glucan-biofilms on microplates. While unencapsulated nisin lost its potency within 6 hr, only 76% of the nisin within nisin-liposomes was released within this time, thus prolonging the inhibitory effects (Yamakami *et al.*, 2013). Nisin has also been combined with sodium fluoride to provide a stronger bactericidal effect on *S. mutans.* The observed synergy revealed through checkerboard and survival assays was also more effective in preventing biofilm formation over 4 hr and 16 hr as compared with sodium fluoride alone (Tong *et al.*, 2011). Nisin also functions synergistically with daptomycin and ciprofloxacin with respect to inhibiting established (24 hr) MRSA biofilms. Time-kill assays using the minimum biofilm-eradication concentrations of the antimicrobials demonstrate a 3-log reduction in CFU of an MRSA control and two clinically obtained MRSA biofilms occurred even within 4 hr when used in combination, the individual antimicrobials never obtaining such level of kill even at 24 hr post application (Dosler & Mataraci, 2013).

The immobilization of nisin, via a poly(ethylene glycol) (PEG(1000)) linker, to multi-walled carbon nanotubes significantly enhanced the antimicrobial and anti-biofilm properties of the nanotubes by up to 7-fold (Qi *et al.*, 2011). Similarly, nisin has been covalently bound to stainless steel surfaces to the same effect, reducing adhesion of bacteria and thus biofilm formation (Hequet *et al.*, 2011). Incorporating different concentrations (0.1%, 0.5% and 1%) of nisin into poly-ethylene-co-vinyl acetate (EVA) films reduced biofilm formation on these surfaces, with the beneficial effects being more evident against a strain of *S. epidermidis* than against representative *L. monocytogenes* or *S. aureus* strains (Nostro et al., 2010). A very

recent breakthrough involves acyldepsipeptides, which have been shown to effectively assist in the kill of persisters as a result of uncontrolled activation of a subunit (ClpP) of the protease enzyme Clp. This activation causes a dramatic increase in the size of the central pore in ClpP allowing access for peptides and proteins to the proteolytic chamber, resulting in an increase in protein degradation and ultimately cell death (Conlon *et al.*, 2013).

Nisin Resistance Protein

A 35-kDa *n*isin *r*esistance *p*rotein (NSR) has been found to be produced by some strains of *L. lactis.* Originally *nsr* was observed as a specific nisin resistance gene located on a 60-kb plasmid in the nisin non-producer *L. lactis* subsp. *diacetylactis* DRC3 (Froseth & McKay, 1991). Since then, several groups have isolated nisin-resistant lactococcal strains containing *nsr* on a plasmid (Liu *et al.*, 1997; Sun *et al.*, 2009; Yun *et al.*, 2006). The mechanism by which NSR confers resistance is proposed to involve the proteolytic degradation of nisin, mediated by a C-terminal conserved tail-specific protease (TSPc) domain at the C terminus of NSR (Chatterjee *et al.*, 2005; O'Driscoll *et al.*, 2006). TSPc efficiently cleaves substrates that have apolar residues and a free alpha-carboxylate at the C terminus and have been identified and characterised in a range of species, including *E. coli* (Keiler *et al.*, 1995). Such proteases contain a conserved PDZ domain adjacent to the TSPc domain, which is indispensable for binding of the TSPc with nonpolar C-termini of its peptide substrates and thus for the catalytic activity (Beebe *et al.*, 2000; Spiers *et al.*, 2002). However, NSR does not possess a PDZ domain. NSR also differs from other TSPc containing proteins in that it harbours charged and polar residues (Lys,

His, and Ser) at the C terminus (Keiler & Sauer, 1996). More detailed analyses have revealed that NSR and purified NSRSD (a version of NSR without the predicted N-terminal signal peptide sequence) proteolytically inactivates nisin by cleaving the peptide bond between MeLan^{28} and Ser^{29}. This truncated nisin (nisin$^{1-28}$) showed a noticeably reduced affinity for the lactococcal membrane, a significantly reduced effectiveness in pore formation in the target membrane and a 100-fold-lower bactericidal activity against *L. lactis* MG1363, compared to whole nisin (Sun *et al.*, 2009). An *nsr* gene has also been identified in *S. agalactiae* ATCC 13813, the gene product of which is referred to as SaNSR. A plasmid expressing this resistance gene was shown to confer a 20-fold increase in resistance to the host strain. *In silico* screening for *nsr*-like genes revealed their presence across a range of species including *Corynebacterium casei* and *Corynebacterium ammoniagenes*, and various strains of *Leuconostoc mesenteroides, Leuconostoc carnosum, Enterococcus faecium, Staphylococcus epidermis, Streptococcus ictaluri, Streptococcus sanguinis*, *Streptococcus dysgalactiae* and *Streptococcus agalactiae*. These genes were found within operons that also contained genes encoding an ABC transporter, named NsrFP and a TCS NsrRK. It is proposed that these genes confer nisin resistance in these strains (Khosa *et al.*, 2013).

Nisinase

In a study dating back to 1967, anti-nisin activity was detected in extracts of bacilli and endospores of organisms that had a lytic mechanism for rupture of the spore coat (type L), which was not detectable in spores which ruptured the spore-coat mechanically (type M). Further investigation of cell-free extracts of *Bacillus*

cereus and *B. polymyxa* revealed that the anti-nisin phenotype was not as a result of proteolytic activity, was somewhat specific as it did not affect polymyxin, gramicidin or bacitracin, but did inactivate nisin and subtilin (Jarvis, 1967). Nisinase was isolated from several *Bacillus* sp. and was shown to be a dehydropeptide reductase, as it specifically reduced the C-terminal dehydroalanyl-lysine of nisin to alanyl-lysine (Jarvis, 1970). Nisinase activity has also been associated with *Lactobacillus plantarum* (Kooy, 1952), *S. thermophilus* (Alifax & Chevalier, 1962), *C. botulinum* (Rayman *et al.*, 1983), *L. lactis* subsp. *cremoris*, *E. faecalis* and *S. aureus* (Carlson & Bauer, 1957).

Gad System

In *L. monocytogenes*, several systems help to withstand low pH stress, but the glutamate decarboxylase (GAD) system is probably the most important (Cotter *et al.*, 2001a; Cotter *et al.*, 2001b; Cotter *et al.*, 2005b). Of the five *gad* genes, *gadD1*, *gadD2*, and *gadD3* encode decarboxylases that catalyse the conversion of glutamate to γ-amino butyrate (GABA) and carbon dioxide (CO_2). *gadT1* and *gadT2* encode antiporters, which import glutamate and export GABA. Deletion of *gadD1* impairs the ability of a strain to tolerate exposure to both sublethal and lethal levels of nisin (Begley *et al.*, 2010). It was discovered that the intracellular ATP levels were reduced in the Δ*gadD1* mutant, being only approximately 60% of those of the parent, suggesting that GadD1 contributes significantly to ATP pools and hence tolerance of nisin (Begley *et al.*, 2010).

arc *Operon*

The *arc* genes are involved in the breakdown of arginine via the arginine deiminase pathway. This pathway is responsible for the breakdown of arginine into ornithine, ammonium, and carbon dioxide. Arginine deiminase (ArcA), ornithine carbamoyltransferase (ArcB), and carbamate kinase (ArcC) are the three enzymes responsible for this degradation process (Poolman *et al.*, 1987). The *arc* operon is thought to contribute to acquired nisin resistance as a fourfold overexpression of the genes *arcAC1C2DT2* was observed in a *L. lactis* nisin resistant strain compared to the corresponding wild-type strain. The conversion of arginine into ammonium might result in a locally less acidic pH at the outer side of the cytoplasmic membrane, preventing nisin from attaching to the lipid II molecule (Kramer *et al.*, 2006).

TelA

The tellurite resistance gene, *telA*, was identified from the screening of a *mariner* random mutant bank for nisin-sensitive *L. monocytogenes* mutants. The *telA* gene encodes a toxic ion resistance transporter that also plays a role in resistance to cell wall-acting antibiotics**.** LiaR has been reported to regulate *telA* expression under salt-induced nisin resistance conditions (Bergholz *et al.*, 2013). The *telA* gene also forms a two-gene operon with *xpaC*, which encodes a putative member of the halogen hydrol superfamily. This same genetic organization is conserved across many members of the Firmicutes and Proteobacteria phyla. The creation of a nonpolar deletion mutant of *telA* confirmed its involvement in resistance as the strain was found to be 4-fold-more sensitive to nisin and, in

addition, the Δ*telA* strain was also 8-fold-more susceptible to gallidermin and 2-fold-more susceptible to bacitracin, cefuroxime, cefotaxime, and tellurite (Collins *et al.*, 2010b).

PurR

Three mutations within the gene *purR* (encoding a purine operon repressor) were identified in a nisin resistant mutant of *S. aureus* SH1000. However, this gene does not seem to be essential for resistance as it is intact in other cases of spontaneous nisin resistance in *S. aureus* (Blake *et al.*, 2011). The fact however, that the three mutations ($G_{228}E$, $V_{229}G$ and $E_{184}STOP$) were retrieved from independently selected nisin resistant mutants of SH1000 strongly suggests that this locus is involved in resistance in this strain.

IreK-IreP-IreB

One of mechanisms employed by *E. faecalis* to survive therapeutic concentrations of cephalosporins may also be involved in determining resistance to lantibiotics. This means of resistance involves a signalling system comprised of a eukaryotic-like serine/threonine kinase (IreK) and phosphatase (IreP) pair that antagonistically regulate cephalosporin resistance (Kristich *et al.*, 2007). A third protein, IreB, is modulated by IreK-dependent phosphorylation and, although its role is unknown, it has been found to contribute to negative regulation of resistance and to be an endogenous substrate of both IreK and IreP (Hall *et al.*, 2013). Homologues of these three proteins are found in the genomes of nearly all Gram positive bacteria. A strain bearing a deletion in *ireK* exhibited reduced

resistance to sodium cholate (a detergent found in bile) and to nisin (Hall *et al.*, 2013; Kristich *et al.*, 2007). In addition, a double mutant lacking *ireK* and *ireB* showed enhanced resistance to both these bactericidal agents (Hall *et al.*, 2013; Kristich *et al.*, 2007).

Sigma Factors

One important mediator of the stress response in many Gram positive bacteria is the alternative sigma factor SigB. In *B. subtilis* SigB activity is regulated by a complex network of protein-protein interactions governed by a variety of environmental or metabolic stresses such as heat shock, osmotic shock, ethanol treatment, or entry into stationary growth phase (Boylan *et al.*, 1993). The growth of a *L. monocytogenes* EGDe *sigB* mutant is noticeably reduced in the presence of nisin or lacticin 3147 relative to its parental strain (Begley *et al.*, 2006). It was hypothesised that SigB regulates general stress proteins or proteins involved in extrusion of antimicrobials out of the cell. Indeed, SigB binding sites are located upstream of *htrA* (lmo0292), which encodes a putative molecular chaperone shown to be involved in tolerance of penicillin G (Stack *et al.*, 2005). Another SigB binding site is upstream of *mdrL* (lmo1409), encoding an antibiotic efflux pump (Mata *et al.*, 2000).

Another alternative sigma factor, SigL, also contributes to antimicrobial resistance. Nisin survival assays show that both SigB and SigL affect the sensitivity of *L. monocytogenes* 10403S to nisin in broth survival assays. Contrary to the findings of Begley et al. (2006), the Δ*sigB* mutant was seen to be more resistant to nisin. The differences observed were attributed to class of peptide, the strain, initial

number of bacteria, growth phase, and the assay used for evaluation (Palmer *et al.*, 2009). Interestingly, a *sigB* null mutation was revealed to sensitize the cell in a Δ*sigL* background, leading to reduced nisin resistance (Palmer *et al.*, 2009). In addition, Butcher *et al*. have proposed that the extracytoplasmic function (ECF) sigma factors σ^M, σ^X and σ^W all contribute to antibiotic resistance in *B. subtilis* (Butcher & Helmann, 2006). A *B. subtilis sigX* deletion mutant has been found to be more sensitive to nisin than the wild-type. Furthermore, most genes under σ^X control processes contribute to the biosynthesis or metabolism of the cell envelope, and include the *dlt* operon and *pbpX* (penicillin-binding protein) amongst many others (Cao & Helmann, 2004). σ^M was found to contribute to bacitracin resistance via regulation of the *bcrC* (*ywoA*) gene, encoding a putative bacitracin transport permease (Bernard *et al.*, 2005; Cao & Helmann, 2002). With respect to nisin resistance, the major contribution of σ^M is expression of *ltaSa*, encoding a stress-activated lipoteichoic acid synthase. Lipoteichoic acid (LTA) adsorbs cations from the environment, including antimicrobials such as nisin. Binding of such cations to LTA may inhibit their transit through the cell wall, and thereby increase resistance (Kingston *et al.*, 2013). Together, σ^M and σ^X regulate cell envelope structure to decrease access of nisin to its lipid II target. SigW has been linked with increased rigidity and decreased fluidity of the membrane (Kingston *et al.*, 2011; Lee *et al.*, 2012) and has been directly linked with lantibiotic resistance. The σ^W-dependent operons, such as the *B. subtilis* resistance module *yqeZ-yqfAB,* are seen as critical for sublancin resistance. The membrane-integrated protease encoded within this operon, YqeZ, is thought to degrade lantibiotics that integrate into the membrane (Butcher & Helmann, 2006). In terms of nisin resistance, the σ^W associated SppA

appears to be the only signal peptide peptidase in *B. subtilis* that makes a contribution. In addition, σ^W regulates expression of the phage shock proteins PspA and YvlC, which enhance membrane stability and thus provide resistance specifically against the membrane-perturbing and pore forming activity of nisin (Kingston *et al.*, 2013). Tellurite resistance related proteins (YceGHI) are also regulated by σ^W, with the protein YceG homologous to TelA found in *L. monocytogenes* (Collins *et al.*, 2010b; Kingston *et al.*, 2013). These self-protective mechanisms also provide resistance to other lantibiotics such as mersacidin, gallidermin and subtilin (Kingston *et al.*, 2013).

Immune Mimicry

Lantibiotic self-immunity mechanisms are the means by which lantibiotic producers protect themselves against their own bactericidal agents. Immunity specific proteins (LanI) or specific self-protection ABC transporters (LanFE(G)) are found alone or in combination, providing specific resistance to lantibiotic self-producers. Cross immunity is extremely rare between lantibiotic producers and these immunity mechanisms are specific to the particular lantibiotic produced by the cell (for review see (Draper *et al.*, 2008)). An unusual means of resistance has been recently revealed, in which non-lantibiotic producing bacteria were shown to possess genes homologous to the lantibiotic immunity genes. With respect to lacticin 3147, functional immunity homologues were found in *E. faecium DO* and *B. lichiniformis* DSM 13. The *B. licheniformis* BliI protein has 37% identity with the lacticin 3147 immunity protein LtnI, and when *bliI* is expressed constitutively in a lacticin 3147-sensitive strain, *L. lactis* MG1363, the resistance of the strain was

increased by 20-fold (Draper *et al.*, 2009). A similar result was seen when genes encoding the LtnFE ABC transporter homologue, EfdFE, from *E. faecium* DO were also expressed in the MG1363 background. The protection provided was specific in that no resistance to the distantly related one peptide lantibiotic nisin was apparent (Draper *et al.*, 2009). This phenomenon appears to extend beyond lacticin 3147 resistance as genes encoding immunity homologues have been found in *Streptococcus infantarius* subsp. *infantarius* BAA-102. The genes, *spiFEG*, encode an ABC transporter with >50% identity to that associated with immunity in the nisin U operon. Interestingly genes homologous to the individual components of the nisin two-component system known as NisRK (or NsuRK in the case of nisin U), referred to as *spiRR'K* are co-located with the *spiFEG* genes. While these regulatory genes did not confer a nisin U resistant phenotype when co-expressed with *spiFEG*, protection was provided when *spiFEG* was expressed constitutively in the nisin U sensitive *Lactococcus lactis* subsp. *cremoris* HP (Draper *et al.*, 2012).

Conclusions

The emergence of antimicrobial resistance represents a Grand Challenge to humanity; understanding how and by what means bacteria become resistant facilitates the development of new therapeutic treatments. In tandem it allows for the reassessment of current antimicrobials and the potential to improve on current therapeutic regimes. With low levels of naturally occurring lantibiotic resistance, these modified peptides serve as a credible alternative to commercial antibiotics. As some lantibiotics have a dual mode of action by forming pores and also inhibiting cell wall biosynthesis they represent a significant challenge to the target cell. A detailed classification of the known means of lantibiotic resistance is summarised in Table 1. Such and future characterisation may allow for further tailoring of genetic variants of lantibiotic peptides that could circumvent resistance issues, and will inform the development of appropriate therapeutic regimes to overcome such issues if and when lantibiotics are deployed in clinical settings.

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Tables and Figures

Table 1.

Cell wall/membrane altering mechanisms

Name	Species	Regulated by	Resistance to lantibiotics
Mprf	*S. aureus* *L. monocytogenes*	VirRS/GraRS	Nisin, Gallidermin
DltA	*Streptococcus sp.* *Staphylococcus sp.* *L. monocytogenes* *L. lactis* *B. subtilis* *C. difficile*	VirRS/Sigma factors	Nisin, Gallidermin
gal operon	*L. lactis*		Nisin
Penicillin-binding protein	*L. monocytogenes* *L. lactis* *S. aureus*	LiaRS LisRK, VraSR, Sigma factors	Nisin
Lipid composition incl. the *fab* operon	*L. monocytogenes* *L. lactis*		Nisin

Miscellaneous resistance mechanisms

Name	Species	Regulated by	Resistance to lantibiotics
Nisin resistance protein*	*L. lactis* *Corynebacterium sp.* *Leuconostoc sp.* *Enterococcus sp.* *Streptococcus sp.*		Nisin
Nisinase*	*S. thermophilus* *Lb. plantarum* *C. botulinum* *L. lactis* *E. faecalis* *S. aureus* *Bacillus sp.*		Nisin, Subtilin
Gad	*L. monocytogenes*		Nisin
NsrX	*S. mutans*	NsrRS	Nisin
arc operon	*L. lactis*		Nisin
PurR	*S. aureus*		Nisin
IreK-IreP-IreB	*E. faecalis*		Nisin
Sigma factors	*L. monocytogenes* *B. subtilis*		Nisin, Sublancin, Mersacidin, Subtilin Gallidermin

Table 1. (continued)

Transporters

Name	Species	Regulated by	Resistance to lantibiotics
VraFG	*S. aureus*	GraXRS	Nisin, Nukacin ISK-1
CprABC	*C. difficile*	CprRK	Nisin, Gallidermin
AnrAB	*L. monocytogenes*	VirRS	Nisin
VraDE	*S. aureus*	BraRS, VraSR	Nisin
YsaBC	*L. lactis*	LlrG/KinG	Nisin
BraDE	*S. aureus*	BraRS	Nisin
Lmo1746-47	*L. monocytogenes*	VirRS, LiaRS	Nisin
BceAB	*B. subtilis*	BceRS	Actagardine, Mersacidin
PsdAB	*B. subtilis*	PsdRS	Nisin, Gallidermin, Subtilin, Actagardine
Sp0912-Sp0913	*S. pneumoniae*	Rr01-Hk01	Nisin
LctFEG	*S. mutans*	LcrRS	Nukacin ISK-1, Lacticin 481
NsrFP	*Corynebacterium sp.* *Leuconostoc sp.* *Enterococcus sp.* *Streptococcus sp.*	NsrRK	Nisin
TelA	*L. monocytogenes*	LiaRS	Nisin, Gallidermin
EfdFE*	*E. faecium*		Lacticin 3147
SpiFEG*	*S. infantarius*	SpiRR'K ?	Nisin

Two Component Systems

Name	Species	Resistance to lantibiotics
TC09 & TC12	*L. casei*	Nisin
GrsRS	*S. aureus*	Nisin, Nukacin ISK-1
BraRS	*S. aureus*	Nisin, Nukacin ISK-1
PsdRS	*B. subtilis*	Nisin, Gallidermin, Subtilin, Actagardine
LlrG/KinG	*L. lactis*	Nisin
ApsRS	*S. epidermidis*	Nisin
BceRS	*Bacillus sp.*	Actagardine, Mersacidin
LiaRS	*Bacillus sp.* *Streptococcus sp.* *L. monocytogenes*	Nisin
NsrRS	*S. mutans*	Nisin, Lacticin 481, Nukacin-ISK1
LcrRS	*S. mutans*	Nukacin ISK-1, Lacticin 481
VraSR	*S. aureus*	Nisin, Mersacidin, Nukacin-ISK1
LisRK	*L. monocytogenes*	Nisin
CesSR	*L. lactis*	Nisin, Plantaricin C
VirRS	*L. monocytogenes*	Nisin
CprRK	*C. difficile*	Nisin, Gallidermin, Subtilin, Mutacin 1140, Cinnamycin

Table 1. Summary of lantibiotic resistance methods. Listed are the strains in which lantibiotic resistance has been observed and the lantibiotics to which this resistance is conferred. Not included in the table are the general resistance mechanism inferred by the Gram negative cell wall and that of spore and biofilm formation. Lantibiotic specific resistance methods are indicated with an asterisk.

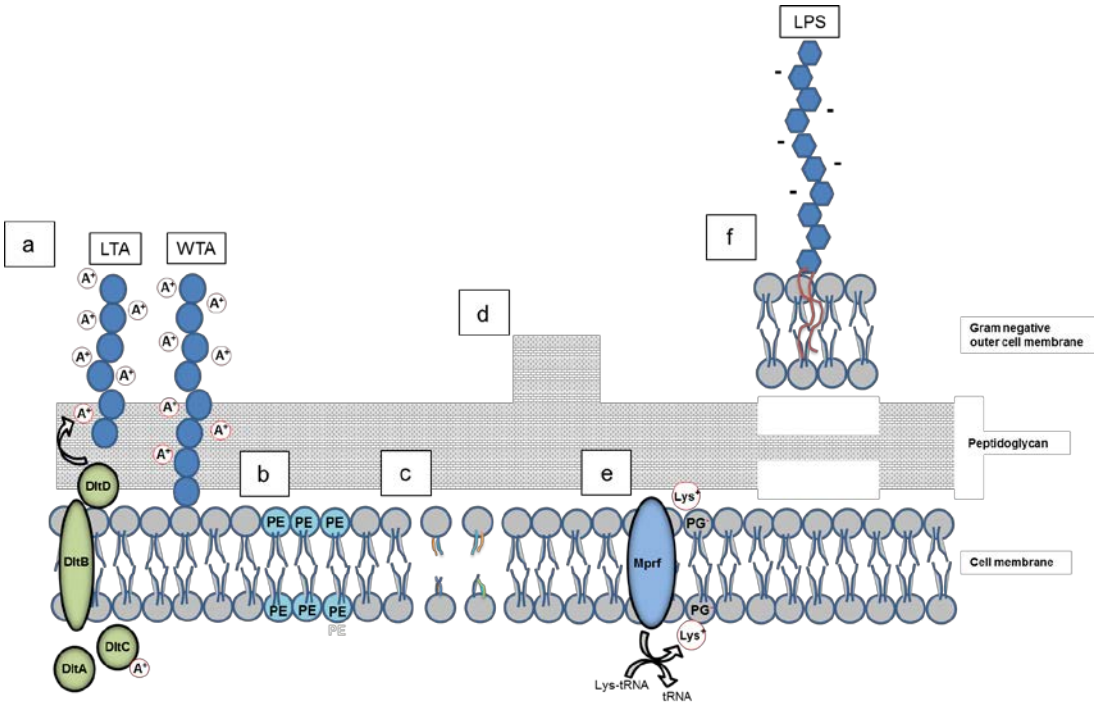

Fig. 1. Mechanisms of lantibiotic resistance which relate to the cell wall and membrane. D-alanylation of lipoteichoic acids (LTA) and wall teichoic acids (WTA) by the dltABCD operon which infers a positive charge (a); changes in phospholipid composition (b); changes in membrane fatty acid composition (c); cell wall thickening (d); lysine esterification of one of the two hydroxyl groups of phosphatidylglycerol (PG) by Mprf (e); the Gram negative outer cell membrane containing lipopolysaccharide (LPS) (f).

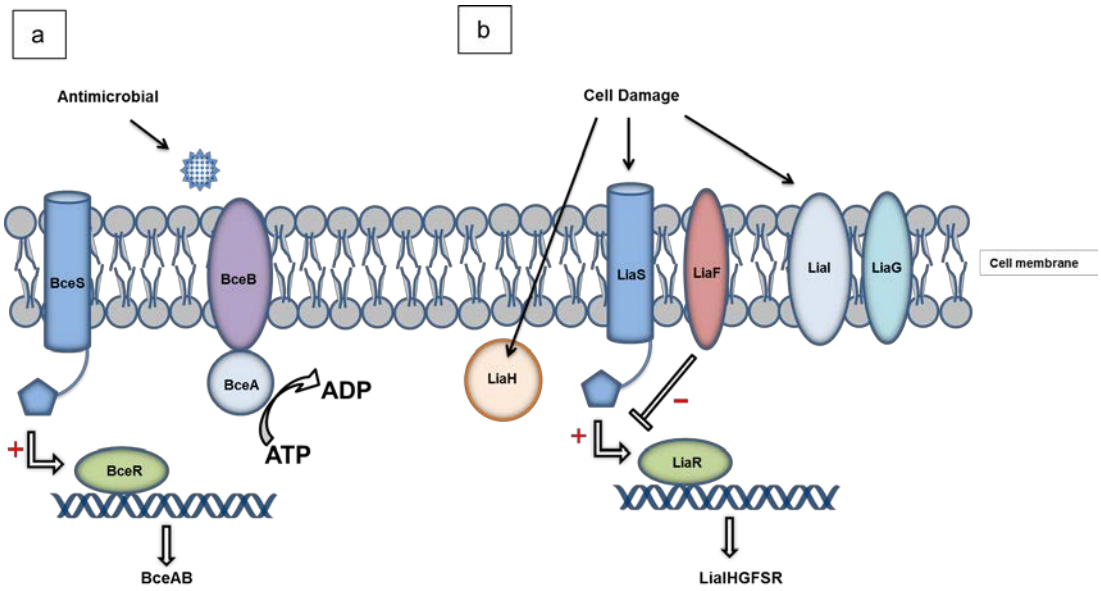

Fig. 2. Two main types of two component systems (TCS) are responsible for lantibiotic resistance. These incorporate the Bce-like TCSs (a) and the Lia-like TCSs (b). The presence of antimicrobials such as lantibiotics or the cell damage incurred as a result of their presence causes these TCSs to mediate the transcription of genes whose products infer a resistance phenotype.

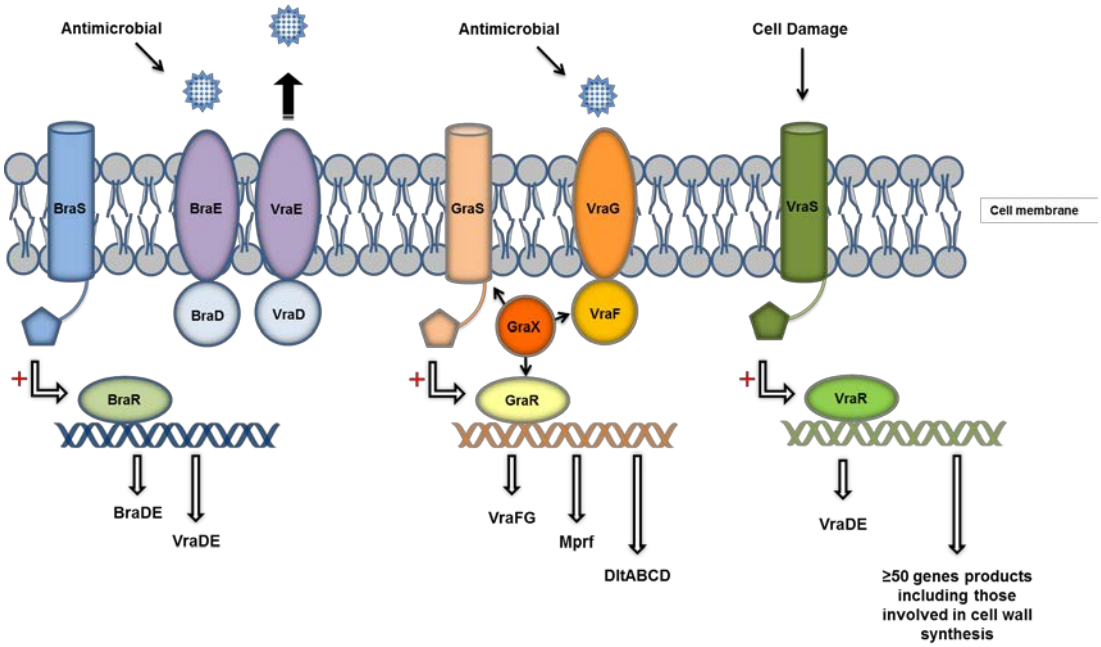

Fig. 3. In *S. aureus* three main two component systems (TCS) are responsible for lantibiotic/antimicrobial resistance. These include two Bce-like TCSs, BraRS and GraRS and a Lia-like TCSs, VraSR. A co-ordinated resistance effort results from the action of these TCSs, causing upregulation of genes whose products alter the composition of the cell wall and membrane, and also encode ABC transporters which expel antimicrobials from the cell.

CHAPTER II

The two peptide lantibiotic lacticin 3147 acts synergistically with polymyxin to inhibit Gram negative bacteria.

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Summary

The emergence of bacterial drug resistance encourages the re-evaluation of the potential of existing antimicrobials. Lantibiotics are post-translationally modified ribosomally synthesised antimicrobial peptides with a broad spectrum antimicrobial activity. Here, we focussed on expanding the potential of lacticin 3147, one of the most studied lantibiotics and one which possesses potent activity against a wide range of Gram positive species including many nosocomial pathogens. More specifically, our aim was to investigate if lacticin 3147 activity could be enhanced when combined with a range of different clinical antibiotics. Initial screening revealed that polymyxin B and polymyxin E (colistin) exhibited synergistic activity with lacticin 3147. Checkerboard assays were performed against a number of strains, including both Gram positive and Gram negative species. The resultant fractional inhibitory concentration (FIC) index values established that, while partial synergy was detected against Gram positive targets, synergy was obvious against Gram negative species, including *Cronobacter* and *E. coli*. Combining lacticin 3147 with low levels of a polymyxin could provide a means of broadening target specificity of the lantibiotic, while also reducing polymyxin use due to the lower concentrations required as a result of synergy.

Introduction

The challenge presented by the emerging problem of antibiotic resistance is a significant one. One approach has been to identify new bactericidal agents while another has involved a re-examination of the potential of previously identified antimicrobials. With this latter route in mind, there has been a particular focus on assessing and enhancing the benefits of applying lantibiotics in clinical settings (Cotter *et al.*, 2013; Piper *et al.*, 2009a). Lantibiotics are ribosomally synthesised antimicrobial peptides that are subjected to post-translational modification, resulting in the presence of unusual amino acids including intramolecular lanthionine and β-methyl lanthionine bridges. These bridges are formed through a two-step process that is initiated by the dehydration of serine and threonine residues to dehydroalanine (Dha) and dehydrobutyrine (Dhb), respectively. The subsequent reaction of these modified amino acids with intrapeptide cysteines results in the formation of lanthionine (Ala-S-Ala; in the case of dha) or β-methyl-lanthionine (Abu-S-Ala; in the case of dhb) bridges (for review see (Bierbaum & Sahl, 2009; Chatterjee *et al.*, 2005; Suda *et al.*, 2012)). Lacticin 3147 is a two peptide lantibiotic which exhibits broad spectrum activity against Gram positive targets. The two lacticin 3147 peptides, Ltnα and Ltnβ, work synergistically in a 1:1 ratio (Morgan *et al.*, 2005; Suda *et al.*, 2012). Ltnα first binds to the precursor of peptidoglycan production, lipid II, with Ltnβ subsequently interacting with this complex. The net effect is the inhibition of peptidoglycan synthesis and the formation of a membrane depolarising pore (Wiedemann *et al.*, 2006).

Some lantibiotics are active at single nanomolar levels against particular targets and several lantibiotics inhibit drug–resistant Gram positive pathogens, including methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE) (Cotter *et al.*, 2013; Piper *et al.*, 2009a). Lantibiotics are highly stable, resistance is rare and activity can be enhanced through genetic alteration and, thus, they are considered to be viable alternatives to traditional antibiotics (Cotter *et al.*, 2013). Lacticin 3147 inhibits many Gram positive pathogens including *Listeria monocytogenes*, *Staphylococcus aureus* and *Clostridium difficile* as well as a variety of streptococci, enterococci and mycobacteria (Carroll *et al.*, 2010; Iancu *et al.*, 2012; Rea *et al.*, 2007). However, to date, the inhibition of Gram negative species by lacticin 3147 has not been reported. This is most often attributed to the presence of the outer membrane, which prevents access of the lantibiotic to the cytoplasmic membrane.

There are many potential benefits associated with identifying antibiotics that function synergistically with lacticin 3147. While antibiotic resistance has become a major obstacle, significant resistance to lacticin 3147 has yet to be reported and thus the use of antibiotic-lacticin 3147 combinations may prevent/overcome the emergence of resistance. Furthermore, certain antibiotic-lacticin 3147 combinations may allow for a broader range of species to be targeted. Here we assess the impact of combining lacticin 3147 with a variety of clinical antibiotics and establish that lacticin 3147 exhibits synergistic activity in combination with either polymyxin B or polymyxin E.

Methods

Cultures and growth conditions

Salmonella Typhimurium UK1, *Salmonella* Typhimurium LT2, *Escherichia coli* 0157:H-, *E. coli* EC101, *E. coli* DH5α (University College Cork (UCC) culture collection) and *Cronobacter sakazakii* 6440 (Dairy Products Research Centre (DPC) culture collection) were grown in Luria-Bertani (LB) broth and agar at 37°C, while *Bacillus cereus* 8079 (DPC culture collection) and *Enterococcus faecium* strains DO (Arduino *et al.*, 1994), EC538, EC295 and EC725 (British Society for Antimicrobial Chemotherapy (BSAC)) were grown in Brain Heart Infusion (BHI) broth and agar at 37°C. *Staphylococcus aureus* strains ST528, ST523, ST530, ST291, ST534 (BSAC) and 5247 (DPC culture collection) was also grown at 37°C but with aeration in cation supplemented Mueller Hinton broth and Mueller hinton agar. *Lactococcus lactis* MG1363 (UCC culture collection) was grown at 30˚C without aeration in M17 broth (Oxoid Ltd., Basingstoke, Hampshire, England) supplemented with 0.5% (wt/vol) glucose (GM17).

Antimicrobials

Cefoperazone, cefaclor, teicoplanin, bacitricin, colistin sulphate (polymyxin E), polymyxin B, oxacillin and fusidic acid antimicrobial susceptibility discs were purchased from Oxoid. Polymyxin B and colistin sulphate (polymyxin E) were obtained from Sigma-Aldrich while lacticin 3147 was purified using the following procedure: TYG media (tryptone, 2.5 g l^{-1}; yeast extract, 5.0 g l^{-1}; glucose, 10 g l^{-1}; β-glycerophosphate, 19.0 g l^{-1}; $MgSO_4$ x $7H_2O$, 0.25 g l^{-1}; $MnSO_4.4H_2O$, 0.05 g l^{-1}) was

passed through 500 g XAD-16 beads (Sigma-Aldrich Company Ltd., Dorset, England) in order to remove all hydrophobic components. An overnight culture of *L. lactis* MG1363.pMRC01.pOM02 (Cotter *et al.*, 2006) was then used to inoculate 1 L of the modified TYG broth (1% inoculum) and incubated at 30˚C overnight. The cells were subsequently harvested by centrifugation (7,000 g for 20 min) and resuspended in 250 ml 70% propan-2-ol, pH2.0 (adjusted to pH2.0 with addition of conc. HCl). Following stirring at 4˚C for four hours the cell debris was removed by centrifugation and the supernatant was subjected to rotary evaporation (50 mbar at 40˚C) to reduce the volume to ~60 ml via removal of propan-2-ol. The resultant preparation was applied to a 10 g 60 ml^{-1} Strata $C_{-18}E$ Giga-Tube (Phenomenex, Cheshire, UK) after pre-equilibration with 60 ml methanol followed by 60 ml water. The column was subsequently washed with 120 ml of 30% ethanol and the lantibiotic was then eluted from the column via addition of 100 ml of 70% propan-2-ol, pH2.0. From the 100 ml preparation, 20 ml volumes were subjected to rotary evaporation in order to reduce them to ~1.7 ml through removal of propan-2-ol. Aliquots of 1,800 µl were then applied to a Phenomenex (Phenomenex, Cheshire, UK) C_{12} reverse phase (RP)-HPLC column (Jupiter 4µ 90Å 250×10.0 mm, 4 µm) previously equilibrated with 25% propan-2-ol containing 0.1% trifluoroacetic acid (TFA). The column was then developed in a gradient of 30% propan-2-ol containing 0.1% TFA to 60% propan-2-ol containing 0.1% TFA in 4 to 40 min at a flow rate of 1.2 ml min^{-1}. Fractions containing Ltnα and Ltnβ were collected after each HPLC run and stored under nitrogen gas. The Ltnα and Ltnβ containing fractions were pooled separately and subsequently subjected to rotary evaporation to remove all propan-

2-ol before freeze-drying of the peptides. The Ltnα and Ltnβ peptides were weighed in μg quantities using a Mettler UMT_2 micro-balance.

Antibiotic disc-based assessment of antimicrobial sensitivity and synergy

The sensitivities of *S*. Typhimurium LT2, *C. sakazakii* 6440, *S. aureus, and E. faecium* strains to a variety of antibiotics were determined by antibiotic disc diffusion assays as described previously (Collins *et al.*, 2010). Briefly, stationary-phase cultures (16 h) were diluted to 10^7 CFU ml^{-1} and swabbed onto Mueller Hinton, LB or BHI agar plates. Six mm antibiotic discs (Oxoid) infused with specific antibiotics were placed on the agar plates. On the same plate lacticin 3147 (1.2, 1.9 or 2.5 μg) was added to a second antibiotic-containing disc and to a blank disc (control). Following overnight incubation (16 hr) at 37°C, the resultant zones of inhibition were measured. The antibiotic discs employed included cefotaxime, novobiocin, cefoperazone, teicoplanin, ceftazidime, cefaclor, cephradine, cefaclor (30 μg), bacitracin, imipenem, fusidic acid (10 μg), penicillin G (5 μg), and oxacillin (1 μg), colistin sulphate (polymyxin E) (25 μg) and polymyxin B (300 U).

Minimum inhibitory concentrations

Minimum inhibitory concentration (MIC) determinations were carried out in triplicate in 96 well microtitre plates as described by Wiedemann *et al*., 2006. Briefly, bacterial strains were grown overnight in the appropriate conditions and medium, subcultured into fresh broth and allowed to grow to an OD_{600} of ~0.5. Serial two-fold dilutions of the lacticin 3147, polymyxin B or colistin sulphate were made in the growth medium of the respective strain. Bacteria were then added

diluted to a final concentration of 10^5 cfu ml^{-1} in each a 0.2 ml MIC test well. After incubation for 16 hr at 37°C, the MIC was read as the lowest peptide concentration causing inhibition of visible growth.

Checkerboard assay for combining antimicrobials

In order to analyse combinations of two different antimicrobials (e.g. X and Y), the minimum inhibitory concentration of each antimicrobial has to be defined against a specific strain. Once this is known a 2-fold serial dilution of X is made horizontally in 50 µl in a microtitre plate beginning at 8 x MIC for X. In a second microtitre plate, a similar dilution of Y is created and then 50 µl of this is added vertically to the original microtitre plate containing the dilution of X. Bacteria were then added in the same fashion as performed for to the singular peptide minimum inhibitory assays described previously. Fractional inhibitory Concentration (FIC) index is defined by the following equation: $FIC = [FIC_X + FIC_Y] = [(X/MIC_X) + (Y/MIC_Y)]$. Where (X) is the lowest level of antimicrobial X in combination with another to achieve an inhibitory effect, while (MIC_x) is the MIC of that antimicrobial alone for the bacterial strain under investigation. FIC index results are interpreted as follows: FIC ≤ 0.5 is synergy, 0.5 <FIC ≤ 0.75 is partial synergy, 0.75< FIC ≤ 1.0 is additive, FIC >1.0 is indifferent and FIC > 4 is antagonistic (Neu, 1977).

Results

Sensitivity of bacteria to lacticin 3147 and antibiotics in combination.

To determine whether lacticin 3147 could work synergistically with a variety of clinically utilised antibiotics, we used antibiotic discs assays to assess the potency of individual antibiotics (cefotaxime, novobiocin, cefoperazone, teicoplanin, ceftazidime, cefaclor, cephradine, cefaclor (30 μg), bacitracin, imipenem, fusidic acid (10 μg), penicillin G (5 μg), oxacillin (1 μg), colistin sulphate (polymyxin E) (25 μg) and polymyxin B (300 U)), in the presence and absence of lacticin 3147. It was evident that lacticin 3147 had the ability to enhance the activity of a number of the antibiotics tested (data not shown) but the benefits of combining lacticin 3147 with polymyxin B or polymyxin E were particularly obvious (Fig. 1). In the case of the representative Gram positive and negative strains, *E. faecium* DO and *E. coli* EC101, the diameters of the zones of inhibition were increased by over 180% and by over 121%, respectively. Indeed, in the case of *E. faecium* DO, combining sub-inhibitory concentrations of the individual antimicrobials resulted in the formation of a zone of clearing (Fig. 1). Based on these preliminary experiments it was apparent that the benefits of combining lacticin 3147 with either polymyxin B or E merited further examination. We used broth based microtitre plate assays to determine MICs and combined FICs against a range of Gram negative and representative Gram positive strains (Table 1). It was apparent that a combination of lacticin 3147 and polymyxin B or E had an indifferent effect (FIC = 1.25 and 1.125 respectively) against *Salmonella* Typhimurium UK1 and an antagonistic effect (FIC > 4) was observed in the case of the LT2 strain. However, combining these antimicrobials against other

targets gave more positive results. Indeed, a high level of synergy was observed against *Cronobacter sakazakii* strain 6440, with an FIC index corresponding to 0.250 for a lacticin 3147 and polymyxin B combination and 0.062 for a lacticin 3147 and polymyxin E combination. FIC values here were determined on the basis of the reduction in MIC values for the polymyxins alone as an MIC value for lacticin 3147 could not be determined as it is not active against *C. sakazakii*, even at the highest level tested (924 μg ml^{-1}). However, it can be established that the FIC is <0.312 for lacticin 3147 in combination with polymyxin B and <0.125 when combined with polymyxin E.

Corresponding studies with three *E. coli* strains again revealed synergism between lacticin 3147 and the polymyxins. An FIC index value of 0.248 was obtained when lacticin 3147 and polymyxin B were combined against 0157:H- while the corresponding lacticin 3147 and polymyxin E FIC value was 0.188. When lacticin 3147 and polymyxin B were combined against *E. coli* DH5α and EC101 FIC indices of 0.188 and 0.5 were obtained, respectively. In addition an FIC index of 0.188 was determined when lacticin 3147 and polymyxin E were combined for these two target strains.

A number of additional assays were carried out in order to determine if the benefits of combining lacticin 3147 and the polymyxins in broth extended to Gram positive targets. For this purpose *Bacillus cereus* 8079, *Enterococcus faecium* DO and *Staphylococcus aureus* 5247 were selected as representative indicator strains. It was established that, while some partial synergy between lacticin 3147 and polymyxin B was observed with respect to *B. cereus* 8079 and *S. aureus* 5247 (FIC =

0.62 and 0.75, respectively), the other combinations resulted in an additive or indifferent outcome.

Given that the most notable outcome from the study was the synergistic activity of lacticin 3147 and the polymyxins against some Gram negative targets, further investigations were carried out to determine how the respective components of lacticin 3147, i.e. Ltnα and Ltnβ, perform individually in the presence of polymyxin B/E. Selecting the sensitive strain *E. coli* 0157:H- as a target, we were able to evaluate the contribution of the individual α and β peptides to this phenomenon (Table 3). Taking into consideration the molecular weights and 1:1 ratio at which α and β are combined, we can derive the relative amount (μg ml^{-1}) of each individual peptide present when lacticin 3147 (Ltnα and Ltnβ combined in a 1:1 ratio) is synergistic with polymyxin B/E. With this information we can compare the action of α and β alone to the same amount of each peptide present in whole lacticin 3147 in each case of synergy. Although various degrees of synergy exist due to the different combinations and concentrations assessed, only those that yielded the greatest synergy with respect to lacticin 3147 are listed in Table 1. Obtaining such a high degree of synergy was not possible with the single peptides, Ltnα and Ltnβ. For this reason additional synergy values/FIC data for lacticin 3147 in combination with polymyxin B and E has been included in Table 2. This provides a means by which the contribution of the individual lacticin 3147 components can be derived by focusing on a fixed level of polymyxin B/E in each case of synergy. Hence, it is apparent that, when combined with a set concentration of polymyxin B and E, 6 times more Ltnα alone is required to achieve the level of synergy obtained when both Ltnα and Ltnβ are present. In contrast, only 4.7 times Ltnβ alone is

required to achieve a corresponding level of activity in the absence of Ltnα. Interestingly the reverse is seen when you consider the individual action of Ltnα and Ltnβ alone, in the absence of polymyxin. In this situation only 1.5 times the amount of Ltnα is required, while 4.7 times Ltnβ is needed to achieve an MIC relative to their contribution when both lacticin 3147 peptides are present.

Discussion

We undertook a series of investigations to determine whether lacticin 3147 acts synergistically with a range of clinically important antibiotics. Antibiotics encompassing many families and modes of action were chosen, including cephalosporins, polypeptides, glycopeptides, carbenems, and quinolones. Following this initial screen, it became clear that lacticin 3147 and the polymyxins acted synergistically.

Polymyxins are a group of polypeptide antibiotics that exclusively target Gram negative microorganisms. The five distinct members of this group, polymyxin A-E, were discovered in 1947 and are produced non-ribosomally by different *Bacillus polymyxa* species (Storm *et al.*, 1977). Polymyxin B and polymyxin E, also referred to as colistin, have been used in clinical practice for decades in otic and ophthalmic solutions (Nakajima, 1965; Ohzawa, 1965). Polymyxins are decapeptide antibiotics which consist of a heptapeptide ring, with polymyxin E differing from polymyxin B only by the presence of D-Leu *in lieu* of a D-Phe. This ring is linked to a tripeptide side-chain which carries an aliphatic chain attached via an amide bond to the amino terminus (Velkov *et al.*, 2010). The polymyxins carry five positive charges due to the presence of L-α-γ-diaminobutyric acids (Storm *et al.*, 1977) and it has been established that the amphiphilic nature of this molecule gives it the ability to interact, bind and traverse the Gram negative outer membrane. The target molecule is lipopolysaccharide (LPS) (Vaara, 1992), and specifically the lipid A component (Morrison & Jacobs, 1976; Srimal *et al.*, 1996). The polymyxins dissociate protective divalent cations from their association with anionic LPS. This

displacement permeabilises the Gram negative outer membrane to allow the polymyxins, or other cationic peptides, to form pores (Hancock, 1997). It should be noted, however, that the use of polymyxins in clinical settings has been restricted to use only where drug resistant pathogens have been encountered. This is due to the toxicity, primarily nephro- and neuro-toxicity, associated with its use (Falagas & Kasiakou, 2006), although this toxicity has been suggested to be dose dependent (Evans *et al.*, 1999). Nonetheless, the polymyxins are, in many cases, the only antibiotics capable of overcoming specific drug resistant pathogens, such as *Pseudomonas aeruginosa* and *Acinetobacter baumannii* in cystic fibrosis patients (for review see (Falagas & Kasiakou, 2005; Yuan & Tam, 2008; Zavascki *et al.*, 2007). For this reason the polymyxins cannot be ignored, but strategies that could reduce the dose needed for these antibiotics to be effective are highly desirable.

A numbers of studies have investigated the consequences of combining various antibiotics with polymyxins. Antimicrobial agents such as miconazole (Pietschmann *et al.*, 2009), rifampicin (Giamarellos-Bourboulis *et al.*, 2001; Giamarellos-Bourboulis *et al.*, 2003) meropenem, ampicillin-sulbactam, ciprofloxacin, piperacillin-clavulanic acid, imipenem, amikacin, and gentamicin (Kasiakou *et al.*, 2005) ciprofloxacin (Hoiby *et al.*, 2005) trimethoprim, trimethoprim-sulfamethoxazole, and vancomycin (Vidaillac *et al.*, 2012), to name but a few, have been the focus of studies to assess if they can work synergistically with polymyxins (also see Yahav *et. al*., for a review of compounds synergistic with polymyxin E (Yahav *et al.*, 2012)). To date the only lantibiotic to have been investigated in this way is nisin, which displays synergy with polymyxin B and polymyxin E against *Listeria* and *E. coli* (Naghmouchi *et al.*, 2010; Naghmouchi *et al.*,

2011). Nisin has also been shown to function synergistically when combined with polymyxin E (and clarithromycin) against *Pseudomonas aeruginosa* (Giacometti *et al.*, 1999). Combination studies have also recently revealed that lacticin 3147 and the lactoperoxidase system (LPOS) successfully inhibited growth of *Cronobacter* spp. in rehydrated infant formula (Oshima *et al.*, 2012). Lacticin 3147, like nisin, is a food grade bactericidal agent obtained from the GRAS organism *Lactococcus lactis.* Notably, however, it differs from nisin with respect to its target specificity and its greater potency against a number of species (Iancu *et al.*, 2012). Also the mechanism of action contrasts from the single nisin peptide, in that it requires the interaction of two peptides, Ltnα and Ltnβ, for optimal bactericidal activity.

Here we report the first study to investigate whether synergy can occur between polymyxin(s) and a two-component lantibiotic. Not only do we reveal that synergy is apparent against a range of strains tested, we also investigated the individual contributions of Ltnα and Ltnβ. We established that, when combined with polymyxin B/E, the levels of lacticin 3147 required to inhibit Gram negative species are equivalent or lower than the levels of lacticin 3147 alone against many Gram positive targets. Thus, in the presence of 0.3125 μg ml^{-1} polymyxin B/E, the concentration of lacticin 3147 required to inhibit Cronobacter sp. is less than the lacticin 3147 MIC for *Mycobacterium avium* subsp. *paratuberculosis* (MAP) ATCC 19698 or *Mycobacterium kansasii* CIT11/06 (Carroll *et al.*, 2010). Similarly the MIC of lacticin 3147 (alone) against many *S. aureus* (which includes many of the nosocomial pathogens: methicillin-resistant *S. aureus* (MRSA), *S. aureus* with intermediate resistance to vancomycin (VISA), *S. aureus* with heterogenous vancomycin intermediate resistance (hVISA)) (Iancu *et al.*, 2012; Piper *et al.*,

2009b), is greater than that required to inhibit *E. coli* species when in the presence of a polymyxin. It is also important to note that synergy with lacticin 3147 may provide a means of reducing the dose of polymyxins required to inhibit specific targets, thereby addressing polymyxin-associated toxicity issues. For example, 8-fold and 16-fold lower levels of the polymyxins are required to inhibit *E. coli* and *Cronobacter* when in the presence of lacticin 3147. Furthermore a recent study by Naghmouchi *et al*., has shown that in addition to its role in providing synergy with polymyxin E, the lantibiotic nisin appears, at certain concentrations, to eliminate its toxicity, as seen in Vero cell lines (Naghmouchi *et al.*, 2013). Having established the role lacticin 3147 has in polymyxin synergy, further investigations are warranted in order to ascertain if such toxicity preventing attributes are common amongst lantibiotics.

As with previous studies (Gales *et al.*, 2001), the solo activities of polymyxin B and polymyxin E against the strains tested here are very similar. With respect to the dual action of lacticin 3147 and polymyxins, it appears that the lacticin 3147-polymyxin B combination has the greater potency against Gram positive targets but that the lacticin 3147-polymyxin E combination has a greater effect against Gram negative strains. Thus, the single amino acid difference between the two polymyxin peptides appears to have an impact on its bactericidal action and target specificity when combined with lacticin 3147. It was also notable that the lacticin 3147 sensitivity of Gram positive microorganisms such as *Enterococcus faecium* DO, which is already highly sensitive to lacticin 3147, is not enhanced by the presence of the polymyxins. However, in the case of the strains that are relatively more lacticin 3147 resistant, the benefits of adding polymyxin B (especially with respect to Gram

positive strains) and polymyxin E (especially for Gram negative strains) is most apparent. It is interesting to note that this phenomenon does not correlate with results obtained during the initial agar based disc assay screen, where the opposite pattern was observed. However, it is acknowledged that the agar-based screen is a much cruder assay, and in that instance polymyxin concentrations were fixed and only lacticin 3147 concentrations were altered. Moreover, no FIC data can be derived and so increased zone sizes may not represent the optimal combination of the antimicrobials as obtained through checkerboard assays. The mechanism by which this synergy occurs with respect to Gram negative targets is presumably based in the action of polymyxin permeabilising the outer membrane to allow lacticin 3147 to gain access to the cytoplasmic membrane and its lipid II target (Hermsen *et al.*, 2003). However, a phenomenon concerning the synergy between polymyxin B/E and the singular peptides Ltnα and Ltnβ is also unveiled during this study. Considering the action of the singular peptides in the absence of polymyxin, a greater quantity of Ltnβ alone, than Ltnα alone, is required to inhibit *E. coli* (4.7 times versus 1.5 times respectively). This is logical in that Ltnα has been shown to have greater solo activity, and can bind to lipid II and prevent peptidoglycan synthesis (Wiedemann *et al.*, 2006). However, in the presence of polymyxin B/E, Ltnα needs to be added at a 6 times greater concentration to bring about an inhibitory effect equal to that achieved by Ltnα:Ltnβ combined. In contrast, Ltnβ only needs to be added at a 4.7 fold greater concentration to compensate for the absence of Ltnα and thus Ltnβ seems more potent than Ltnα in the presence of either polymyxin. It is not clear if this is due to the potency of Ltnα being slightly compromised by the activity of the polymyxins or is a reflection of a particularly

beneficial interaction between these antibiotics and Ltnβ. Additional studies will be required in order to investigate this further.

Regardless of the mechanism involved, this study documents a means by which lacticin 3147 can be combined with polymyxins in order to effectively inhibit some Gram negative species. There are a number of practical implications to these findings but these will require *in vivo* analysis. One outcome may be to ultimately facilitate the use of lower concentrations of polymyxins in situations where the levels currently employed are of concern from a toxicity perspective. Alternatively, enhancing the spectrum of lacticin 3147 to include Gram negative targets could have benefits with respect to, for example, the treatment of bovine mastitis. While lacticin 3147 has been established as being effective with respect to controlling bovine mastitis caused by Gram positive microorganisms, reducing levels of *S. aureus*, *Streptococcus dysgalactiae* or *Streptococcus uberis* (Klostermann *et al.*, 2010; Ryan *et al.*, 1998), mastitis can also be caused by Gram negative species and in particular by *E. coli* species (Schukken *et al.*, 2012; Shpigel *et al.*, 2008), against which lacticin 3147 has limited efficacy. *E. coli* can be considered the quintessential environmental pathogen with respect to mastitis. Infections tend to result in acute and often severe clinical mastitis and account for as many as 30% to 40% of clinical mastitis cases (Hogan & Smith, 1987). Combining lacticin 3147 with low levels of a polymyxin could provide a means of broadening target specificity, for example in the treatment of mastitis, while keeping the concentrations of antimicrobial employed to a minimum.

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Organism	MIC (μg ml^{-1})						
	Lacticin 3147	**Polymyxin B**	**Polymyxin E**	**Lacticin 3147/ Polymyxin B**	**FIC**	**Lacticin 3147/ Polymyxin E**	**FIC**
Salmonella Typhimurium UK1	924	0.0586	0.0586	924/0.015	1.25[d]	924/0.0073	1.125[d]
Salmonella Typhimurium LT2	231	0.3125	0.4688	No MIC	>4[e]	No MIC	>4[e]
Cronobacter sakazakii DPC 6440	>924	0.3125	0.3125	57.75/0.0781	0.250 (<0.312)*[a]	57.75/0.0195	0.062 (<0.125)*[a]
E. coli 0157:H-	231	0.0586	0.0781	28.875/0.0073	0.250[a]	28.875/0.0049	0.188[a]
E. coli DH5α	462	0.0781	0.0781	28.875/.0098	0.188[a]	28.875/0.0098	0.188[a]
E. coli EC101	462	0.0781	0.0781	14.4375/.0391	0.5[a]	28.875/0.0098	0.188[a]
E. faecium DO	0.9625	>375	>375	0.9625/23.4375	1[c]	0.9652/23.4375	1[c]
B. cereus 8079	3.85	187.5	375	1.925/23.4375	0.62[b]	3.85/375	2[d]
S .aureus 5247	15.4	187.5	>375	7.7/46.875	0.75[b]	15.4/23.4375	1[c]

Table 1. MIC data for lacticin 3147, polymyxin B and polymyxin E alone and in combination. FIC figures have been calculated as a result of triplicate experiments and indicate [a]synergy, [b]partial synergy, [c]additive effects, [d]indifference, and [e]antagonism between the combined antimicrobials. *FIC index which includes the reduction in lacticin 3147 MIC from the highest level tested to that which achieves an MIC in the presence of polymyxin.

***E.coli* 0157:H-**

MIC (μg ml^{-1})						
Lacticin 3147	**Polymyxin B**	**Polymyxin E**	**Lacticin 3147/ Polymyxin B**	**FIC**	**Lacticin 3147/ Polymyxin E**	**FIC**
231 (37.5 μM) (α :124.74, B: 106.26)	0.0586	0.0781	28.875/0.0073 28.875 / 0.0147*	0.250[a] 0.376*[a]	28.875/ 0.0049 14.4375 / 0.0195*	0.188[a] 0.312*[a]
Ltnα/	**Polymyxin B**	**Polymyxin E**	**Ltnα/ Polymyxin B**	**FIC**	**Ltnα/ Polymyxin E**	**FIC**
187.11 (56.25 μM) (**1.5** X Ltnα)	0.0586	0.0781	93.555 / 0.0073 (**6.0** X Ltnα in combin.)	0.625[b]	46.7775/ 0.0195 (**6.0** X Ltnα in combin.)	0.500[a]
Ltnβ/	**Polymyxin B**	**Polymyxin E**	**Ltnβ/ Polymyxin B**	**FIC**	**Ltnβ/ Polymyxin E**	**FIC**
495.88 (175 μM) (**4.7** X Ltnβ)	0.0586	0.0781	61.9850 / 0.0147 (**4.7** X Ltnβ in combin.)	0.376[a]	30.9925 / 0.0195 (**4.7** X Ltnβ in combin.)	0.313[a]

Table 2. MIC data for lacticin 3147, and its individual peptides Ltnα and Ltnβ, polymyxin B and polymyxin E alone and in combination. FIC figures have been calculated as a result of triplicate experiments and to indicate [a]synergy and [b]partial synergy effects.*Alternative MIC and FIC data that allows for fixed levels of polymyxin across antimicrobial combinations, thus allowing for the calculation of the involvement of Ltnα and Ltnβ in synergy with polymyxin.

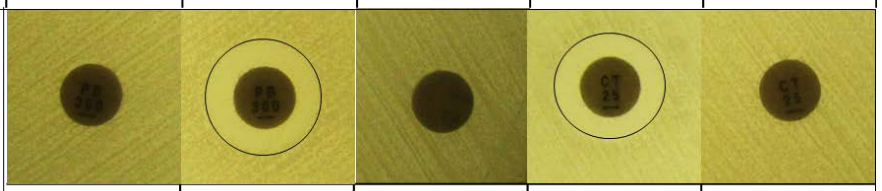
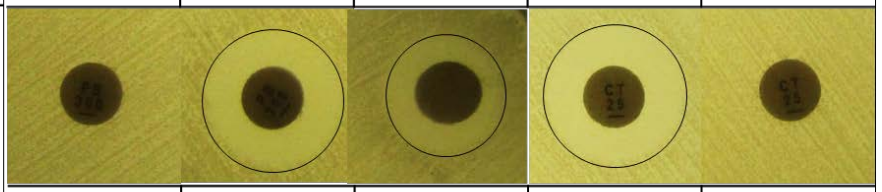
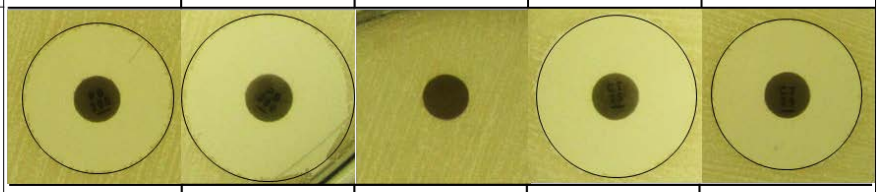
	Polymyxin B alone	Lacticin 3147 + Polymyxin B	Lacticin 3147 alone	Lacticin 3147 + Polymyxin E	Polymyxin E alone
***E. faecium* DO** (1.2µg lacticin 3147)					
	-	90.81±0.5	-	77.91±0.5	-
***E. faecium* DO** (1.9µg lacticin 3147)					
	-	139.14 ±0.26	81.55 ±0.31	152.40 ±0.30	-
***E. coli* EC101** (2.5µg lacticin 3147)					
	291.64 ±0.15	355.32±1.16	-	352.99±1.16	299.87 ±0.05

Fig. 1. Antibiotic disc-based assessment of lacticin 3147 and polymyxin B/E sensitivity and synergy. Antibiotic discs infused with polymyxin B and polymyxin E were placed on agar plates swabbed with *E. faecium* DO and *E. coli* EC101. Lacticin 3147 (1.2, 1.9 or 2.5 µg) was added to additional discs containing the respective polymyxins and to blank, non-polymyxin containing, controls. Results are the outcome of duplicate experiments and are expressed as total area of inhibitory zone expressed in mm^2.

CHAPTER III

Insights into Lantibiotic Immunity Provided by Bioengineering of LtnI.

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Summary

The lantibiotic lacticin 3147 has been the focus of much research due to its broad spectrum of activity against many microbial targets, including drug-resistant pathogens. In order to protect itself, a lacticin 3147 producer must possess a cognate immunity mechanism. Lacticin 3147 immunity is provided by an ABC transporter, LtnFE, and a dedicated immunity protein, LtnI, both of which are capable of independently providing a degree of protection. In the study described here, we carry out an in-depth investigation of LtnI structure-function relationships through the creation of a series of fusion proteins and LtnI determinants that have been the subject of random and site-directed mutagenesis. We establish that LtnI is a transmembrane protein that contains a number of individual residues and regions, such as those between amino acid 20 and 27 and amino acid 76 and 83, which are essential for LtnI function. Finally, as a consequence of the screening of a bank of 28,000 strains producing different LtnI derivatives, we identified one variant (LtnI I81V) that provides enhanced protection. To our knowledge, this is the first report of a lantibiotic immunity protein with enhanced functionality.

Introduction

Lantibiotics are post-translationally modified antimicrobial peptides produced by Gram positive bacteria. Many lantibiotics are active in nanomolar concentrations and have a broad spectrum of activity against many bacteria, including drug-resistant pathogens (Chatterjee *et al.*, 2005; Cotter *et al.*, 2005a; Guder *et al.*, 2000; McAuliffe *et al.*, 2001; Pag & Sahl, 2002). As a consequence, lantibiotics have been the subject of much investigation with respect to clinical and/or food applications (Breukink & de Kruijff, 2006; Brumfitt *et al.*, 2002; Cotter *et al.*, 2005a; Galvin *et al.*, 1999; Kruszewska *et al.*, 2004; Wiedemann *et al.*, 2001). Because of the potency of lantibiotics, each producer must provide immunity against its own lantibiotic. Lacticin 3147 is a type II lantibiotic produced by rare strains of *Lactococcus lactis* (Suda *et al.*, 2011). The lacticin 3147 producer employs two systems to provide immunity (Draper *et al.*, 2008; Draper *et al.*, 2009; McAuliffe *et al.*, 2000). One system is comprised of an ABC transporter complex designated LtnFE, thought to function through the extrusion of lacticin 3147 from the cytoplasmic membrane. Such immunity transporters have been identified in other lantibiotic producers and are generically designated LanFE(G) (Guder *et al.*, 2002; Otto *et al.*, 1998; Peschel & Gotz, 1996; Stein *et al.*, 2003; Stein *et al.*, 2005). Immunity to lacticin 3147 is also provided by a dedicated immunity protein, LtnI. Generically designated LanI, these heterogeneous proteins/lipoproteins can provide protection against an associated lantibiotic alone or in combination with LanFE(G) (Klein & Entian, 1994; Kuipers *et al.*, 1993; McAuliffe *et al.*, 2000; Okuda *et al.*, 2005; Pag *et al.*, 1999). Immunity to a number of other lantibiotics, including Pep5, epicidin 280, lactocin S and

cytolysin, is provided solely by the corresponding immunity proteins, PepI, EciI, LasI and CylI, respectively (Coburn *et al.*, 1999; Heidrich *et al.*, 1998; Skaugen *et al.*, 1997).

Relatively little regarding the mechanism by which LtnI provides protection to lacticin 3147 is known. Although this 116-amino-acid (aa) protein is predicted to be membrane associated on the basis of hydrophobicity profiling (McAuliffe *et al.*, 2000), to date, other insights into LtnI function have had to be inferred from what is known about other LanI proteins. NisI and SpaI, proteins associated with immunity to nisin and subtilin, respectively, differ from LtnI in that they are lipoproteins that are linked to the membrane by a lipid moiety. These proteins have been investigated in some depth. For example, a series of C-terminally truncated NisI proteins were created and expressed in *L. lactis* in order to identify the region of NisI that interacts with nisin. A 21-amino-acid C-terminal deletion resulted in the retention of just 14% of the protective effect provided by native NisI, whereas longer deletions (up to 74 aa) had no additional effect. When the corresponding 21-aa region of SpaI was replaced with that of NisI and expressed in *L. Lactis,* the SpaI'-'NisI fusion protein provided immunity to nisin, confirming the nisin-specific protective capabilities of these C-terminally located amino acids (Takala & Saris, 2006).

Similar investigations have been carried out to identify essential domains within PepI, a LanI protein associated with Pep5 immunity (Meyer *et al.*, 1995), and its homologue, EciI, which is responsible for epicidin 280 immunity and cross-immunity to Pep5 (Heidrich *et al.*, 1998). The introduction of charged amino acids into the N-terminal hydrophobic 20-amino-acid stretch of PepI impacted on the membrane

localisation of the protein. One such mutant protein, PepI-I17R, conferred substantially reduced immunity to Pep5. The addition of an F13D change in this background, slightly increased immunity levels compared to I17R alone, but also resulted in an enhanced susceptibility to proteolysis (Hoffmann *et al.*, 2004). To investigate the importance of the C-terminal domain of PepI, a truncated protein, $PepI_{1-65}$ that lacked the four C-terminally located charged amino acids was created. The immunity provided by this truncated version was greatly reduced (Pag *et al.*, 1999). A further study focused on three other C-terminally truncated versions of PepI; $PepI_{1-63}$, $PepI_{1-57}$ and $PepI_{1-53}$. As each segment consisting of two positively charged residues next to one negatively charged amino acid was removed, the level of protection was further reduced. The negative impact on immunity was evident, despite the fact that these proteins remained located in the membrane, thereby suggesting that the C terminus of PepI is also involved in target recognition. The importance of charge distribution within this C-terminal region was also apparent from the negative impact on immunity arising from the creation of a PepI-K59T derivative (Hoffmann *et al.*, 2004).

Finally, the structure and function of the LanH protein associated with immunity to the type II lantibiotic nukacin ISK-1, NukH (92 amino acids), have been extensively investigated (Aso *et al.*, 2005). NukH, although distinct from LanI proteins in that it functions as an accessory protein to the ABC transporter immunity system NukFEG, has a transmembrane (TM) location. Through the creation of truncated versions of NukH fused to an alkaline phosphatase (PhoA) reporter and by evaluation of their sensitivity to proteinase K, it was established that NukH contains 3

transmembrane domains (TMDs). The PhoA fusion sites of NukH from amino acid 1 to 33 [NukH(1-33)-PhoA] and amino acids 1 to 92 [NukH(1-92)-PhoA] were shown to be extracellularly located, in that they were subject to proteinase K degradation, whereas the PhoA domain of NukH(1-64)-PhoA was not, thereby supporting *in silico* predictions that this corresponded to a transmembrane domain (Okuda *et al.*, 2005). To identify functional domains within NukH, amino acid substitutions, deletions and truncated versions were created. Deletion of either the N terminus (position 1 to 6) or the C terminus (position 89 to 92) of NukH did not have any effect on its nukacin ISK-1 binding capabilities or immunity function. However, substituting the amino acids of the internal or external loop for alanines abolished NukH function. It was revealed that the external loop was of greatest importance with respect to target binding and that while deletion of the transmembrane regions abolished immunity completely, the truncated protein was still capable of binding its target (Okuda *et al.*, 2005).

Here, to address a lack of knowledge with respect to the topology and functional domains of LtnI, or indeed, type II immunity proteins in general, a series of fusion proteins and site-directed derivatives were created. We also created the first bank of randomly mutated LanI proteins and identified the first LanI variant that provides enhanced lantibiotic protection.

Materials and Methods

Growth Conditions

The strains and plasmids utilized during this study are found in Table 1. Lactococci were routinely grown at 30˚C without aeration in M17 broth (Oxoid Ltd., Basingstoke, Hampshire, England) supplemented with 0.5% (wt/vol) glucose (GM17), GM17 supplemented with K_2HPO_4 (36 mM), KH_2PO_4 (13.2 mM), sodium citrate (1.7 mM), $MgSO_4$ (0.4 mM), $(NH_4)_2SO_4$ (6.8 mM) and 4.4% glycerol (GM17 freezing buffer) without aeration; or GM17 agar unless otherwise stated. *Escherichia coli* was grown in Luria-Bertani (LB) broth (Sambrook *et al.*, 1989) at 37˚C with vigorous agitation. Antibiotics were used, where indicated, at the following concentrations: Ampicillin (Amp) was used at a concentration of 100 μg ml^{-1} for *E. coli* and chloramphenicol at a concentration of 10 μg ml^{-1} for *E. coli* and 5 μg ml^{-1} for *L. lactis*.

General molecular biology techniques

Plasmid DNA was isolated from *E. coli* strains using the High Pure plasmid isolation kit as recommended by the manufacturer (Roche Diagnostics, Mannhein, Germany). Plasmids isolated from *L. lactis* were isolated in the same way following treatment with protoplast buffer (5mM EDTA, 50 U ml^{-1} mutanolysin, 10 mg ml^{-1} lysozyme, 0.75M sucrose, 20mM Tris-HCl pH7.5). Total cell DNA was isolated using Roche High Pure PCR template preparation kit (Roche Diagnostics, Mannheim, Germany). Chemically competent *E. coli* Top10 was used as an immediate host for the plasmids pNZ44 following manufacturer's guidelines for transformation. *L. lactis* strains

were made electrocompetent following the procedure described by Holo and Nes (Holo & Nes, 1995). In both cases electrotransformation was performed with an Electro cell manipulator (BTX-Harvard apparatus). PCR was performed according to standard procedures using BioTaq DNA (Bioline), Vent polymerase (New England biolabs), *KOD* DNA polymerase (Novagen) and *Pwo* DNA polymerase (Roche Diagnostics). For colony PCR, genomic DNA was accessed through lysis of cells in 10% Igepal CA-630 (Sigma-Alrich) at 94˚C for 10 mins. Extraction of DNA from agarose gels were performed using the KeyPrep Spin Gel DNA Clean Up Kit (Anachem, Bedfordshire, UK) as recommended by the manufacturer. DNA ligations were executed according to established procedures using T4 ligase supplied by Roche Diagnostics. Restriction enzymes were also used to manufacturer's guidelines and were supplied by Roche Diagnostics. DNA sequencing was performed by MWG Biotech AG or Beckman Coulter genomics.

Random mutagenesis of ltnI

Plasmid DNA was isolated from *E. coli* Top10 pNZ44*ltnI* (Draper *et al.*, 2009) using the Maxiprep plasmid kit (Qiagen) to a concentration of approximately 428 ng μl^{-1}. pNZ44*ltnI* was then used as template for the Genemorph II random mutagenesis kit (Stratagene) according to manufacturer's guidelines. To introduce an average of 1 base pair change in the 488-bp cloned fragment, amplification was performed in a 50-µl reaction mixture containing approximately 500 ng of target DNA (pNZ44*ltnI*), 2.5 units Mutazyme DNA polymerase, 1 mM dNTPs and 200 ng each of primers LtnIRMFor and LtnIRMrev. The reaction was preheated at 96°C for 1 min, then

incubated for 25 cycles at 96°C for 1 min, 52°C for 1 min and 72°C for 1 min, and then finished by incubating at 72°C for 10 min. Amplified products were purified by gel extraction and reamplified with *KOD* DNA polymerase before being digested with *Kpn*I and *Xba*I, ligated with similarly digested and shrimp alkaline phosphatase-treated pNZ44, and introduced into *E. coli* Top 10. To determine if the correct rate of mutation had been achieved, recombinant plasmid DNA was isolated from selected clones and sequenced. Transformants were pooled and stored in 80% glycerol at −20°C. Plasmid DNA isolated from the mutant bank was used to transform *L. lactis* MG1363. Transformants (approximately 28,000) were isolated from Q trays using a Genetix QPIX II-XT colony-picking robot, inoculated into 384-well plates containing GM17 freezing buffer, incubated overnight and subsequently stored at −20°C.

Construction of lacZ- and phoA- ltnI gene fusions.

pMRC01 was used as the template to amplify, by PCR, C-terminally truncated *ltnI* fragments using LtnIxbaF (containing the ribosomal binding site and start codon for *ltnI*) as the forward primer for all constructs. The respective reverse primers are listed in Table 2. All *lacZ* reverse primers (LtnI21B to LtnI114B) contain a *Bam*HI restriction site to facilitate in-frame fusion with the *lacZ* gene of pRMCD70 (Daniels *et al.*, 1998), whereas reverse primers for *phoA* fusions (LtnI21H to LtnI114H) contain a *Hind*III site to facilitate in-frame fusion with *phoA* of pRMCD28 (Daniels *et al.*, 1998). All constructs were electroporated into *E. coli* CC118, and transformants were selected on LB plates containing ampicillin and initially checked using a phoA or lacZ check primer situated

upstream of the cloned *ltnI* gene fragment in conjunction with the appropriate reverse primer used to make the constructs. The integrity of the constructs was subsequently confirmed by DNA sequencing.

Creation of truncated LtnI proteins

Plasmid pMRC01 was used as a template to facilitate the creation of truncated *ltnI* genes. The primer 5562R was used to generate all N-terminal deletion mutant constructs in combination with the forward primers Nterm14F, Nterm20W and Nterm28Y (all containing a *Pst*I site; Table 2). Primers 5563F and Cterm83, Cterm90 and Cterm109 were used to generate the C-terminal deletion mutant. In all cases the PCR products were digested with the appropriate restriction enzymes, introduced downstream of the constitutive P_{44} promoter in pNZ44 and transformed into *E. coli* Top10 cells. Transformants were selected on LB-Cm plates, further analysed by PCR and sequenced to ensure their integrity. Plasmids were then electroporated into *L. lactis* MG1363 to assess the level of immunity provided.

Site-directed mutagenesis in LtnI

Amino acids in *LtnI* were changed using the site-directed mutagenesis strategy (QuikChange, Stratagene) as described previously (Cotter *et al.*, 2005b) using pNZ44*ltnI* as the template and the primers listed in Table 2. That is, the primers LtnIL1AF/R and LtnIL2AF/R were used for constructing pNZ*ltnI*L87A and pNZ*ltnI*L94A, respectively; and pNZ44*ltnI*D57A, pNZ44*ltnI*R59A were created using the primers LtnID57AF/R and

LtnIR59AF/R, respectively, for the single mutants. A double mutant (pNZ44*ltn*D57A-R59A) was created using one pair of primers (LtnID57/R59F/R) encompassing both mutations. The QuikChange procedure was used according to the manufacturer's instructions with the exception that *E. coli* Top10 were used as the cloning host. Putative mutants were selected on LB-Cm plates, confirmatory PCRs were carried out using an appropriate check primer in conjunction with pNZR or 5562R, successful mutation was confirmed with DNA sequencing and the immunity provided when these plasmids were introduced into MG1363 was assessed.

Agar-based lacticin 3147 sensitivity tests

Using the Genetix QPIX II-XT colony-picking robot, the mutant bank was stamped onto Q trays containing GM17 seeded with various concentrations of a skimmed milk-based preparation of lacticin 3147 (lacticin 3147 fermentation, Teagasc Moorepark) a 1 mg ml^{-1} solution of which corresponds to 640 Activity Units (AU) ml^{-1} against the lacticin 3147-sensitive target *L. lactis* HP. Sensitivity to lacticin 3147 was indicated by a failure to grow in the presence of 3 mg ml^{-1} lacticin 3147 powder. Enhanced resistance to lacticin 3147 was screened for through exposure to 12 mg ml^{-1} (7,680 AU ml^{-1}) lacticin 3147 powder. Sensitivity to lacticin 3147 was also assessed using a gradient agar sensitivity test (Hoffmann *et al.*, 2004). Briefly, square petri dishes (100x100mm) were filled with 25 ml of GM17 containing 15 mg ml^{-1} lacticin 3147-milk powder, placed at approx a 3^{o} angle and allowed to set. The petri dish was then placed in a horizontal position and an additional 25 ml of GM17 was added resulting in the

creation of a lacticin 3147 concentration gradient. Diluted bacterial cultures (0.5 McFarland units) were applied with a sterile cotton swab along the lacticin 3147 gradient and incubated overnight at 30°C.

Broth-Based lacticin 3147 sensitivity assays

Broth based growth assays were performed by inoculating *L. lactis* MG1363.*pNZ44ltnI* strains to give a final inoculum of 10^5 CFU ml^{-1} in a volume of 0.2 ml in GM17 containing 1.1 µM lacticin 3147 and monitoring optical density at 600 nm (OD_{600}) with a Spectromax 340 spectrophotometer (Molecular Devices, Sunnyvale, California) over a 16 hr period. Minimum inhibitory concentration (MIC) assays were performed as described previously (Draper *et al.*, 2009).

β-Galactosidase and alkaline phosphatase assays.

β-Galactosidase and alkaline phosphatase assays were carried out as described by Miller (Miller, 1972) and Manoil (Manoil, 1991), respectively. Briefly, for LacZ constructs, *E. coli* were grown in 10 ml LB broth until the OD_{600nm} reached ~0.5, collected by centrifugation and resuspended in 1 ml LacZ buffer. Cells were permeabilised with 0.1% SDS and chloroform as described by (Israelsen *et al.*, 1995). Subsequently, 4 mg ml^{-1} 2-nitrophenyl β-D-galactopyranoside (ONPG) was mixed with the permeabilised cells, the mixture was incubated at 30°C until the development of a yellow pigment and the reaction was stopped with 1M sodium carbonate ($NaCO_3$). Enzymatic activities were calculated using the following formula [(522 × OD_{420nm} of

reaction mixture)/(OD_{600nm} of culture × volume per ml of culture used × time of reaction)]. Miller activity per ml of culture represents the average of three triplicate experiments. To assay PhoA activity, *E. coli* were grown as for β-galactosidase assays, resuspended in 1M Tris pH 9.0, permeabilized as before; phosphatase substrate (10 mg ml^{-1}) was added; and samples were incubated at 37°C until the development of a yellow pigment; the reaction was stopped with 10M sodium hydroxide (NaOH). PhoA activity was calculated using the formula; [(1000 × OD_{420nm} of reaction mixture)/(OD_{600nm} × volume of culture × time of reaction)].

Results

In silico analysis of LtnI.

It was previously reported by McAuliffe *et al*. that LtnI (116 amino acids in length) is likely to be an integrated membrane protein, based on Kyte and Doolittle hydrophobicity plots that predict three highly hydrophobic domains (McAuliffe *et al.*, 2000). We can now report a more extensive bioinformatic analysis, using the TMHMM (Krogh *et al.*, 2001), HMMTOP (Tusnady & Simon, 2001), SPLIT (version 4.0) (Juretic *et al.*, 2002), SOUSI (Hirokawa *et al.*, 1998) and TMpred (Hofmann & Stoffel, 1993) servers, which also strongly predict that these hydrophobic regions correspond to three trans-membrane domains (TMDs). All predictions suggest that the N terminus of LtnI is located outside of the cell, that TMD1 and TMD3 have an outside-to-inside orientation, that TMD2 has an inside to outside orientation and that the C terminus has a cytoplasmic location (Fig. 1). A study analyzing the accuracy of 13 TM helix prediction methods, including those used in this study, has highlighted the accuracy of TMHMM2 and SPLIT (version 4.0) (Cuthbertson *et al.*, 2005), and thus, the structures predicted by TMHMM2 and SPLIT (version 4.0) were used the templates (Fig. 1(c)) to design all subsequent experimentation.

In silico analysis (PSI-BLAST; http://blast.ncbi.nlm.nih.gov/) has also facilitated the identification of genes encoding LtnI-like proteins from within microorganisms whose genomes have been sequenced (Fig. 2). This includes a *blil* gene that has previously been shown to provide protection against lacticin 3147 when expressed heterologously (Draper *et al.*, 2009). An alignment of the putative amino acid

sequences of the LtnI-like proteins facilitates the identification of conserved regions that potentially correspond to regions that are essential to the function of these proteins. One notable feature relates to the fact that the putative cytoplasmic loop is characterized by a large number of what are mostly charged amino acids that are conserved across homologues, represented in LtnI by D57, E58, R59 and T60. Furthermore, while previous *in silico* investigations have predicted the presence of a leucine zipper in LtnI (McAuliffe *et al.*, 2000), the identification of leucine zippers can be easily assigned incorrectly when the correct orientation of leucines alone is employed. Notably, when reassessed using 2ZIP, a server for leucine zipper prediction (Bornberg-Bauer *et al.*, 1998), it becomes apparent that LtnI lacks other essential coiled coil segments, thus casting doubt over the existence of a leucine zipper domain. Finally Batch CD search (a conserved domain search tool) (Marchler-Bauer *et al.*, 2011) fails to annotate functional protein domains within LtnI.

Analysis of LtnI membrane topology in E. coli using ltnI-lacZ and ltnI-phoA gene fusions.

We used β-galactosidase (*lacZ*) and alkaline phosphatase (*phoA*) gene fusions to experimentally probe LtnI membrane topology (Manoil, 1991). The aforementioned bioinformatic analysis was used as the basis for constructing a series of constructs whereby each of the predicted inside/outside domains, including the 3 TMDs (Fig. 1(c)), was fused to LacZ and PhoA. Fusions of this nature can reveal the location of individual domains based on the premise that enzymatically active LacZ is only achieved if it is

located in the cytoplasm and active PhoA hybrids are only observed if the enzyme is located outside the cytoplasmic membrane. The low-copy-vectors pRMCD28 (*phoA*) and pRMCD70 (*lacZ*) were utilized to fuse truncated forms of *ltnI* to *phoA* or *lacZ* genes lacking the first eight codons. These were under the control of a *lacI* promoter. Fourteen hybrids were generated. In these hybrids, LacZ and PhoA were fused to the C-terminal amino acids A21, F45, K55, L75, G85, C100 and D114 of truncated LtnI proteins. β-Galactosidase and phosphatase assays showed that the K55 hybrid had a LacZ-positive and PhoA-negative phenotype, indicating that, as predicted, K55 is located in the cytoplasm (Fig. 3). Similarly, the G85 fusions behaved as expected (LacZ-negative, PhoA-positive), indicating that this region is located outside the cytoplasmic membrane. A21 fusions have a LacZ- and PhoA-negative phenotype, which may be due to the production of a non-functional hybrid protein as a consequence of its small size, or may indicate that A21 may be located in the membrane. The C-terminal region D114 fusions had a PhoA-negative phenotype and a slightly LacZ-positive phenotype, suggesting that the C terminus of LtnI is located in the cytoplasm but is in close contact with the membrane, in a manner that results in lower LacZ activity. Fusions made within putative TM regions (F45, L75 and C100) all have a LacZ- and PhoA- negative phenotype, thus indicating that these amino acids are indeed embedded in the membrane (Fig. 3).

Design and analysis of N-terminal and C-terminal deletions of LtnI.

To further investigate the importance of different regions, truncated versions of LtnI were created. Initially, the N terminus, a region highly conserved between LtnI-like proteins, was truncated to exclude residues 1 to 13 of LtnI (abbreviated */tnI*ΔNT1-13), while a second mutant that lacks this region as well as residues 14 to 19, and a third lacking residues 1 to 27 were generated (Fig. 2). The construct pNZ44/*tnI*ΔNT1-13 was cloned into MG1363 and was initially tested by well diffusion assay for an immunity phenotype. The level of immunity provided by MG1363.pNZ44/*tnI*ΔNT1-13 was comparable with that of MG1363.pNZ44/*tnI*, as was MG1363.pNZ44/*tnI*ΔNT1-19, a fact that was confirmed by MIC studies (Table 3). In contrast, MG1363.pNZ44/*tnI*ΔNT1-27 was as sensitive as the control host strain MG1363, thus demonstrating that the region between W20 and N27 inclusive of the N terminus of LtnI is essential for its functionality. In contrast, it was apparent that the absence of the C-terminal region of LtnI pNZ44/*tnI*ΔCT110-116 did not alter the immunity phenotype, with an MIC of 1.25μM (identical to that of MG1363.pNZ44/*tnI*). While the removal of larger regions of the C terminus (facilitated by the creation of pNZ44/*tnI*ΔCT90-116 and pNZ44/*tnI*ΔCT83-116) resulted in decreased immunity, strains expressing these proteins remained quite resistant (MIC 62.5nM) relative to the sensitive host MG1363 (Table 3).

Creation and analysis of site-directed mutants in LtnI

In order to investigate a role for the previously identified leucine zipper-associated residues in LtnI, residues L87 and L94 (the 1st and 2nd leucines within the motif) were individually converted to an alanine through manipulation of pNZ44/*tnI* and expression in MG1363. A number of conserved residues corresponding to residues D57, E58, R59 and T60 in LtnI were also invetigated. One positively charged amino acid (R59) and one negatively charged amino acid (D57) within the conserved, putatively cytoplasmically-located domain were converted to alanine to assess their importance. A double D57A-R59A change was also made (Fig. 4). In all cases, immunity levels were equal to those provided by pNZ44/*tnI* as determined by well diffusion assays (data not shown). Subsequently, more sensitive MIC determination tests were carried out. However, it was again evident that the changes made did not impact on the level of immunity provided (Table 3), thereby revealing that neither the putative leucine zipper, nor the two charged residues in the cytoplasmic domain, are essential for immunity.

Random mutagenesis of ltnI and identification of essential residues and domains

Given the failure of rational site-directed mutagenesis to identify residues that are important with respect to the provision of LtnI-mediated immunity, a random mutagenesis-based strategy was developed. More specifically, the plasmid pNZ44/*tnI* was isolated and utilized as a template for a GeneMorph II PCR-based reaction that was carried out in a manner designed to result in the introduction of at least a 1-bp change

in the *ltnI* amplicons. Ligation of these amplicons into pNZ44 and heterologous expression in *L. lactis* MG1363 led to the creation of a bank of 28,000 strains expressing randomly mutated forms of *ltnI*. Spotting of the bank onto GM17 infused with 3 mg ml^{-1} (1,920 AU ml^{-1}) lacticin 3147-skim milk powder revealed more than 200 strains that were unable to grow despite PCR confirmation that a copy of the *ltnI* gene was present. Ninety-five representative mutants were selected for DNA sequencing to identify the changes responsible for the dysfunction of LtnI immunity (Table 4, Fig. 4). In 18 cases, disruption of immunity was as a consequence of a single amino acid substitution. These represented 12 distinct mutants, as I6N, E11D and N29S variants were each detected on two occasions while D79N was recovered four times (Table 4). Of the 12 single amino acid substitutions, 5 occur at the N terminus between amino acid positions 4 and 13. The E4G change alters the overall charge of the external N terminus of LtnI from a net charge of -2 to -1 by replacing glutamic acid with glycine. Conversion of the isoleucine at position 6 to an asparagine replaces a hydrophobic residue with a hydrophilic one. While the F9I mutation maintains a hydrophobic moiety at this position, it does however, involve the loss of an aromatic ring at this position. Similarly, the mutation E11D does not alter the overall charge but does result in the loss of a carbene group. The final amino acid to be altered in the external N-terminal stretch in LtnI is leucine at position 13, which is converted to a proline.

Within the transmembrane region there are a total of six changes resulting in a complete loss of immunity. The changes in the first membrane spanning domain include N29S, G33D, F36I and F36L. The change at N29S maintains the hydrophilic

nature of the region but altering this essential amino acid to a serine reduces the immunity phenotype. The second change, G33D, introduces a negative charge into the native TMD region. Two different changes occur at phenylalanine 36: conversion to an isoleucine and conversion to a leucine. Within the second transmembrane domain, two mutations, D79N and F82S, resulted in the elimination of activity,. Replacing the aspartic acid at position 79 with an arginine represents the loss of a negative charge, while replacement of phenylalanine at position 82 and with a hydrophilic serine results in the loss of the hydrophobic moiety of this region. The final single amino acid change identified related to K110I, whereby the positively charged lysine is replaced by isoleucine, thus altering the net charge of the protein at the membrane.

Thirty-two strains expressing mutated forms of *ltnI* were sensitive as a consequence of frameshift mutations (Table 4; Fig. 4). These represented 16 distinct mutations, as M1, Y15, E19, S24, L32 and K56 were each altered on multiple occasions. Notably, all detrimental frameshift mutations occurred within codons corresponding to regions between residue 1 and 65 but not in more C-terminally located residues. Stop codons were introduced in 17 instances, corresponding to 9 different positions as stop codons at positions corresponding to K2, E22, K55 and Y69 were identified on more than one occasion. In all cases these stop codons occurred within the region between residues 2 and 69. It is also noteworthy that while frameshifts will have multiple effects, they are comparable to the effects of stop codons, as they indicate that the region after the frameshift must have been important if activity has been eliminated. In combination, the location of the detrimental ‘frameshift’ and ‘stop’ mutations provides

further evidence that the provision of LtnI is not dependent on the presence of intact C-terminal domains.

Finally of the 95 mutants sequenced, there were 12 incidences (10 of which were distinct) of 2 to 4 changes, changes that resulted in amino acid changes, frameshifts and/or the introduction of stop codons; and thus the specific change responsible for inactivity was not apparent. Finally, there were 16 cases where ≥20 changes were identified in the genes that resulted from excessive mutagenesis of *ltnI*.

Identification of an LtnI derivative that provides enhanced protection

An agar-based screening strategy was employed to screen for *ltnI* derivatives that provide enhanced protection resulting in the ability of the host strain to grow in the presence of 12 mg ml^{-1} (7,680 AU ml^{-1}) lacticin 3147-milk powder. A single mutant with an ability to survive in the presence of increased levels of lacticin 3147 was identified. To ensure that this enhanced protection was as a consequence of the *ltnI*-associated change, rather than a spontaneous change within the host's genome, the associated pNZ44/*ltnI* plasmid was isolated, re-introduced into a fresh MG1363 background and found to again provide enhanced protection. The corresponding *ltnI* gene was sequenced and a mutation predicted to result in an I81V change was identified. Residue 81 of LtnI is predicted to be part of the second transmembrane domain, close to the interface of the extracellular membrane. To further assess the enhanced protection provided by this change, MG1363.pNZ44/*ltnI* and

MG1363.pNZ44*ltnI*(I81V) were grown in the presence and absence of 1.1µM lacticin 3147. This confirms the enhanced resistance of LtnI(I81V) to the lantibiotic (Fig. 5).

Discussion

The dedicated immunity proteins associated with lantibiotic production are a heterogeneous group of proteins of differing size, composition and structure. They are target molecule-specific and are highly efficient in their action. NisI, SpaI and PepI are anchored to the cytoplasmic membrane, and unusually, NisI has also been found as a lipid-free form, presumably scavenging for exogenous nisin (Hoffmann *et al.*, 2004; Koponen *et al.*, 2004; Stein *et al.*, 2003; Stein *et al.*, 2005). In contrast, it was previously recognized that LtnI was very likely to traverse the membrane (McAuliffe *et al.*, 2000) in a manner similar to that predicted for CylI (Coburn *et al.*, 1999) and established for NukH (Okuda *et al.*, 2005). Notably, however, none of these proteins show any homology to each other.

In this study, the assessment of LtnI topology suggests that LtnI contains three transmembrane regions. This is consistent with the SPLIT (version 4.0) and TMHMM algorithms, which have previously been established to be 85% and 83% accurate, respectively, in predicting the location of residues (Cuthbertson *et al.*, 2005). The biological and *in silico* data predict the existence of an internal loop between TMD 1 and TMD 2 and an external loop between TMD 2 and TMD 3. The predicted cytoplasmic loop has a large number of positively charged residues, and in general such residues act as strong topogenic signals, influencing the conformation of membrane proteins in prokaryotes and eukaryotes (Sipos & von Heijne, 1993; von Heijne, 1989; von Heijne, 2006). On the basis of an extensive study comparing integral membrane proteins from 107 genomes (both prokaryotic and eukaryotic) in which the distribution

of the positively charged amino acids, lysine and arginine was analyzed, it was suggested that this trend is true for all TM proteins (Nilsson *et al.*, 2005). Our biological assessment of topology relied on assays carried out in *E. coli*. It is thus important to note that it has previously been demonstrated that topological data derived from *E. coli* are highly reflective of the situation in lactic acid bacteria (Johnsborg *et al.*, 2003). Therefore, it is likely that the topology of LtnI predicted is a true reflection of the situation in a lacticin 3147-immune *L. lactis* isolate.

Our targeted mutagenesis focused on specific residues and domains within LtnI. The importance of two charged amino acids within a highly conserved region of the cytoplasmic loop of LtnI was assessed by their substitution, both singly and in combination. Interestingly, unlike that observed for PepI, where loss of charged residues in the C terminus reduces immunity function (Hoffmann *et al.*, 2004) , here in neither case was there a detrimental impact on the associated immunity phenotype, establishing that neither of these residues have a role in the immunity function of LtnI. While it may be the case that other positively charged residues in this region and/or the other topological signals within LtnI are sufficient to ensure the retention of functionality, it was notable that random mutagenesis did not reveal essential residues within this internal loop. The leucine zipper-like motif located near the C terminus of LtnI was also subjected to mutagenesis to investigate if this unusual feature had a role in the functionality of LtnI. Site-directed mutagenesis of either the 1^{st} or 2^{nd} leucine had no effect on the immunity phenotype, suggesting the zipper motif has no functional role in immunity. It appears that predicting the presence of a leucine zipper solely on

the basis of bioinformatic tools identifying a distinct pattern of leucines cannot be made with confidence, and our findings would seem to indicate that the presence of the leucine repeats in LtnI may be coincidental. Truncation of the N and the C terminus of LtnI revealed that the extended N terminus plays a key role in the immunity phenotype, but that the latter part of the C terminus, as also seen in the case of NukH (Okuda *et al.*, 2005), is not essential. Furthermore, the removal of TMD 3 alone or in conjunction with the external loop from the C terminus, results in a reduction of immunity but does not eliminate immunity. This is a phenomenon also observed for NukH, whereby the TMD3 is essential only for a full immunity level (Okuda *et al.*, 2005). Interestingly, the last 7 amino acids of LtnI would not appear to make any contribution to protection, as pNZ44/*tnI*ΔCT110-116 provided the same level of immunity as pNZ44/*tnI*. However, it is noteworthy that removal of a positive charge within this region (K110I) has a more dramatic impact. Such an alteration may have a knock-on effect on other, nearby, regions, and/or negatively impact on the native structure of the protein. This is also true for the N terminus of LtnI where removal of the negative charge at position 4 results in loss of function. In contrast, although truncation within the extended N terminus of LtnI results in inactivity (MG1363.pNZ44/*tnI*ΔNT1-27 is not immune but MG1363.pNZ44/*tnI*ΔNT1-20 is), no immunity-eliminating amino acid substitutions were identified between position 20 and 27, suggesting that the region as a whole, rather than specific amino acids, is important. In contrast, substitutions involving charge (E4G) or the loss (F9I) or gain (L13P) of large or secondary structure-distorting amino acids within the region from amino acids 1 to 13 in the N terminus

have a greater impact than the deletion of this entire region. The first of two absolutely essential domains identified in this study is therefore within the N terminus, and contains a region between W20 and N27 (inclusive) that is essential for immunity and notably contains conserved amino acids amongst homologues.

With respect to the transmembrane regions, it was observed that changes resulting in loss of immunity were in most cases found close to the membrane and, in all cases, were confined to TMD1 and TMD2. In this regard, the use of a level of 3 mg ml^{-1} (1,920 AU ml^{-1}) lacticin 3147 in agar was effective in identifying only strains in which LtnI activity was completely eliminated. From analysis of the various LtnI derivatives that have been generated in this study, it is clear that a second defined region of LtnI between I76 (where a frameshift eliminates immunity) and P83 (after which a truncated derivative retains 50% activity) is of essential importance. It can also be inferred that although TMD 3 (F91 to L109) appears to be tolerant of change, its presence is essential for optimal activity. As previously mentioned, the TMD3 region of NukH is also essential for a full immunity phenotype, and another region of significance is the external loop (Okuda *et al.*, 2005). Here we found that it is a region adjacent to this loop that appears to be essential for lacticin 3147 immunity.

Finally, and perhaps most notably, we have identified an alteration that provides an immunity protein (LtnI I81V) with an enhanced ability to protect against its cognate lantibiotic. Modified lantibiotic immunity proteins with enhanced activity have not previously been described. This observation is of significant interest. The bioengineering of lantibiotic producers to generate overproducing strains or strains

that produce lantibiotic derivatives with enhanced antimicrobial activity has been the focus of much attention in recent years (Field *et al.*, 2010). Bacteria producing these proteins need to be protected from the bactericidal agent that they are producing, and thus, self-protection may ultimately become a limiting factor. It is thus anticipated that mechanisms to enhance immunity may in turn facilitate enhanced production.

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Tables and Figures

Table 1. Strains and plasmids

Strains and Plasmids	Description	Source/reference
E. coli CC118	Δ*phoA20*	(Manoil, 1991)
E. coli Top10	Intermediate cloning host	Invitrogen
L. lactis MG1363	Plasmid free, lacticin 3147 sensitive	(Gasson, 1983)
L. lactis MG1363.pMRC01	MG1363 with lacticin 3147 producing plasmid	(Cotter *et al.*, 2003)
pRMCD28	*E. coli phoA* in pWSK29;Amp^r	(Daniels *et al.*, 1998)
pRMCD70	*E. coli lacZ* in pWSK29;Amp^r	(Daniels *et al.*, 1998)
pNZ44	*L. lactis* P_{44} promoter in pNZ8048;Cm^r	(McGrath *et al.*, 2001)
pNZ44*ltnI*	pNZ44 containing *ltnI*	(Draper *et al.*, 2009)

Table 2. Primers used for truncation and site-directed mutagenesis of LtnI and to construct LacZ and PhoA fusions.

NAME	Sequence (5' to 3')[a]
5563F	AT**GCATGC**AACTATACACCTTCTT
5562R	TAT**AAGCTT**TACCATGTGCTATTGAT
Nterm14F	GGG**CTGCAG**ATGTTTTACTCATTAAAAGAGTGGGCG
Nterm20W	GG**CTGCAG**ATGTGGGCGGAAGGTTCAGCAAAC
Nterm28Y	GGG**CTGCAG**ATGTATAATATACTTTTAGGCTTAAGT
Cterm83	G**TCTAGA**TTAAAATATTATGTCACATATAATTAG
Cterm90	G**TCTAGA**TTATGGTTGAATTAAATATCC
Cterm109	GGG**AAGCTT**TAAAAGACAATAAATCCCACC
LtnID57AF	CCACAAAGATTGGGAAAAAAGGCAGAAAGAACAACTAAAATAAGTT
LtnID57AR	AACTTATTTTAGTTGTTCTTTCTGCCTTTTTTCCCAATCTTTGTGG
LtnID57Ach	CAAAGATTGGGAAAAAAGCA
LtnIR59AF	CCACAAAGATTGGGAAAAAAGATGAAGCAACAACTAAAATAAGTT
LtnIR59AR	AACTTATTTTAGTTGTTGCTTCATCTTTTTTCCCAATCTTTGTGG
LtnID57/R59F	CCACAAAGATTGGGAAAAAAGCAGAAGCAACAACTAAAATAAGTT
LtnID57/R59R	AACTTATTTTAGTTGTTGCTTCTGCTTTTTTCCCAATCTTTGTGG
LtnIL2AF	CTTTATGGCAAAATATGGTTTTTCTGGCCTTGTTGGTGGG
LtnIL2AR	CCATATTTTGCCATAAAGAATGGTTGAATTAAATATCC
LtnIL2Ach	CAACCATTCTTTATGGCA
LtnIL1AF	CCCAAAGGATATGCAATTCAACCATTCTTTATGTTAAAATATGG
LtnIL1AR	CCATATTTTAACATAAAGAATGGTTGAATTCGATATCCTTTGGG
LtnIL1Ach	ATTTCCCAAAGGATATGCA
pNZF	CTAATGTCACTAACCTGCCCCGTTAG
pNZR	GGCTATCAATCAAAGCAACACGTG
LtnIRMFor	GG**GGTACC**CTACACCTTCTTTGTTATTG
LtnIRMRev	GC**TCTAGA**GCTTATATTATTTATTATCTTTAATATAT
LtnIxbaF	AAA**TCTAGA**CTGGAGGACATAAGAATGAAGAATGAAAAT
LtnI21B	AAA**GGATCC**CGCCCACTCTTTTAATGAGTA
LtnI45B	AAA**GGATCC**AAAAACTACACTTGACAT
LtnI55B	AAA**GGATCC**TTTCCCAATCTTTGTGGAAATTG
LtnI75B	AAA**GGATCC**TAGAGTAATTAAAACACA
LtnI85B	AAA**GGATCC**TCCTTTGGGAAATATTAT
LtnI100B	AAA**GGATCC**GCAAGAAAAACCATATTT
LtnI114B	AAA**GGATCC**ATCTTTAATATATTTTAA
LtnI21H	AAA**AAGCTT**CGCCCACTCTTTTAATGAGTA
LtnI45H	AAA**AAGCTT**AAAAACTACACTTGACAT
LtnI55H	AAA**AAGCTT**TTTCCCAATCTTTGTGGA
LtnI75H	AAA**AAGCCT**TAGAGTAATTAAAACACA
LtnI85H	AAA**AAGCTT**TCCTTTGGGAAATATTAT
LtnI100H	AAA**AAGCTT**ATCTTTAATATATTTTAA
LtnI114H	AAA**AAGCTT**ATCTTTAATATATTTTAAAAGAC
Pho/lac check	GCACCCCAGGCTTTACAC

[a]Underline represents restriction sites.

Table 3. MIC of N- and C-terminally truncated derivatives of LtnI.

Construct expressed in *L. lactis* MG1363	MIC
MG1363 (control)	19.5nM
pNZ44/*tnI*	1.25µM
pNZ44/*tnI*D57A	1.25µM
pNZ44/*tnI*R59A	1.25µM
pNZ44/*tnI*D57A-R59A	1.25µM
pNZ44/*tnI*ΔNT1-13	1.25µM
pNZ44/*tnI*ΔNT1-19	1.25µM
pNZ44/*tnI*ΔNT1-27	19.5nM
pNZ44/*tnI*ΔCT83-116	62.5nM
pNZ44/*tnI*ΔCT90-116	62.5nM
pNZ44/*tnI*ΔCT110-116	1.25µM

Table 4. Results from sequencing of 96 randomly mutated LtnI proteins that are susceptible to lacticin 3147.

Mutant	Change	Mutant	Change	Mutant	Change
1	Start codon disruption	33	F9I	65	>20CHANGES
2	N29S	34	G33D	66	D57FRAMESHIFT
3	I6N	35	I80T, F82C	67	S24FRAMESHIFT
4	K56FRAME SHIFT	36	>20CHANGES	68	T8P, V47FRAMESHIFT
5	K56FRAME SHIFT	37	K56FRAME SHIFT	69	N7Y,A21V
6	K110I	38	>20CHANGES	70	S10FRAMESHIFT
7	E22STOP	39	D79N	71	>20CHANGES
8	E11D	40	K2STOP	72	W20STOP
9	K56R, T74S, C107R	41	Start codon disruption	73	N29S
10	E11D	42	Y15FRAMESHIFT	74	I6N
11	>20 CHANGES	43	K55STOP	75	Y15FRAMESHIFT
12	D79N	44	I76FRAMESHIFT	76	>20CHANGES
13	F45STOP	45	K56FRAME SHIFT	77	Y15FRAMESHIFT
14	E22STOP	46	>20CHANGES	78	>20CHANGES
15	K2STOP	47	E19FRAMESHIFT	79	>20CHANGES
16	E22K,A25P,S42R, F45FRAMESHIFT	48	A21FRAMESHIFT	80	Y15FRAMESHIFT
17	K2STOP	49	Y15FRAMESHIFT	81	>20CHANGES
18	K55STOP	50	K56FRAME SHIFT	82	S24FRAMESHIFT
19	L32FRAMESHIFT	51	>20CHANGES	83	>20CHANGES
20	Y69H, L87STOP	52	D79N	84	K2STOP
21	>20CHANGES	53	T48FRAMESHIFT	85	K2STOP
22	S10FRAMESHIFT	54	A21V, N29S, L32STOP	86	L32STOP
23	K56FRAME SHIFT	55	A21V, N29S, L32STOP	87	Y69STOP
24	F82S	56	V47FRAMESHIFT	88	E19FRAMESHIFT
25	E22G, T51A, M93R	57	Y69STOP	89	L65FRAMESHIFT
26	E4G	58	>20CHANGES	90	K18STOP
27	F36L	59	F36I	91	E19FRAMESHIFT
28	D79N	60	A21STOP	92	N5Y,A25V, D57FRAMESHIFT
29	Y15FRAMESHIFT	61	F46FRAMESHIFT	93	>20CHANGES
30	S16T,M40T	62	K55STOP	94	S24FRAMESHIFT
31	W20FRAMESHIFT	63	L32FRAMESHIFT	95	>20CHANGES
32	L13P	64	A21V, N29S, L32STOP	96	WT (control)

Fig. 1.

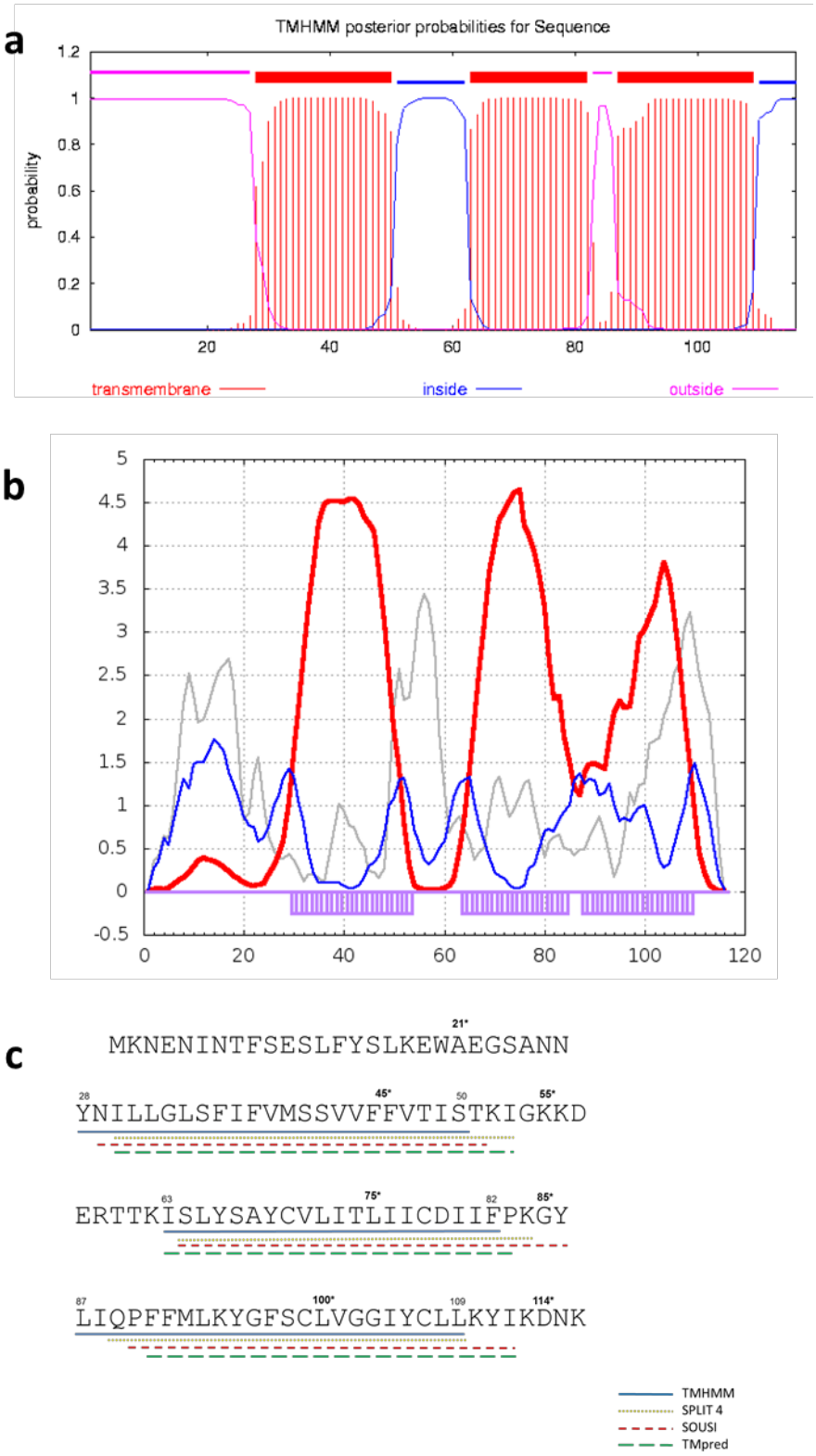
a
TMHMM posterior probabilities for Sequence
probability
transmembrane
inside
outside
b
c
MKNENINTFSESLFYSLKEWAEGSANN
YNILLGLSFIFVMSSVVFFVTISTKIGKKD
ERTTKISLYSAYCVLITLIICDIIFPKGY
LIQPFFMLKYGFSCLVGGIYCLLKYIKDNK
TMHMM
SPLIT 4
SOUSI
TMpred

Fig. 1. LtnI topology predictions

Membrane Topology models of LtnI using Trans-Membrane (TM) prediction sites. TMHMM (a) and SPLIT (version 4.0) (b). (a) Red amino acids predicted to be in membrane; blue, amino acids inside; pink, amino acids outside. (b) Purple hatched boxes indicate amino acids in membrane. (c) On the basis of the study by Cuthbertson *et al*., (Cuthbertson *et al.*, 2005) the findings obtained with different algorithms were compared to indicate LtnI transmembrane regions TMHMM, SPLIT (version 4.0), SOUSI and TMpred. Protein sequence of LtnI also indicates residues fused to reporter enzymes LacZ and PhoA (residues numbered and with asterisks).

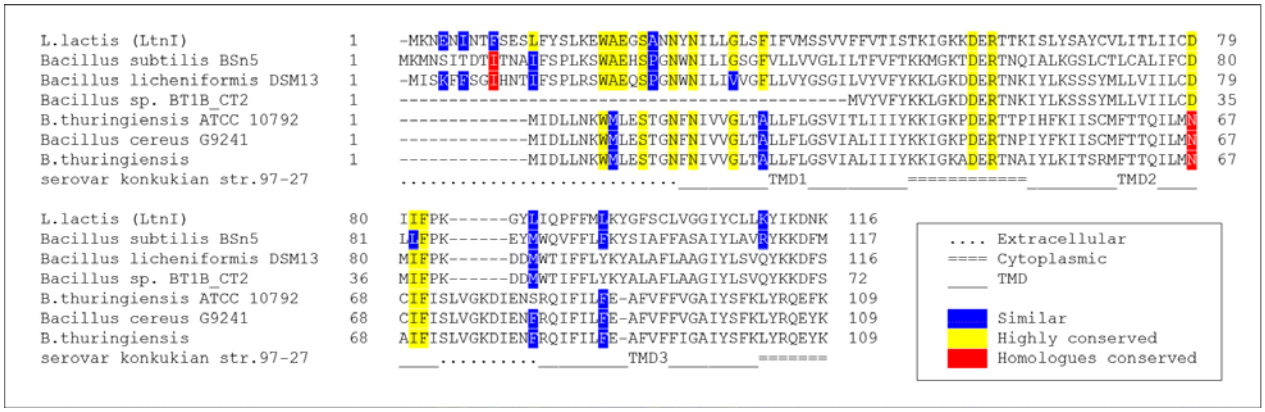

Fig. 2. Investigation of LtnI homologues

LtnI and its closest homologues derived from a variety of *Bacillus* strains. Highlighted are the amino acids that were involved in random or site-directed mutagenesis. The amino acids between W20 and N27 have also been investigated for homology due to the importance of this region in immunity. Where homology is conserved amongst homologues alone (highlighted in red) is of particular interest, as conversion of the amino acid at this position in LtnI to the corresponding conserved amino acid diminishes immunity.

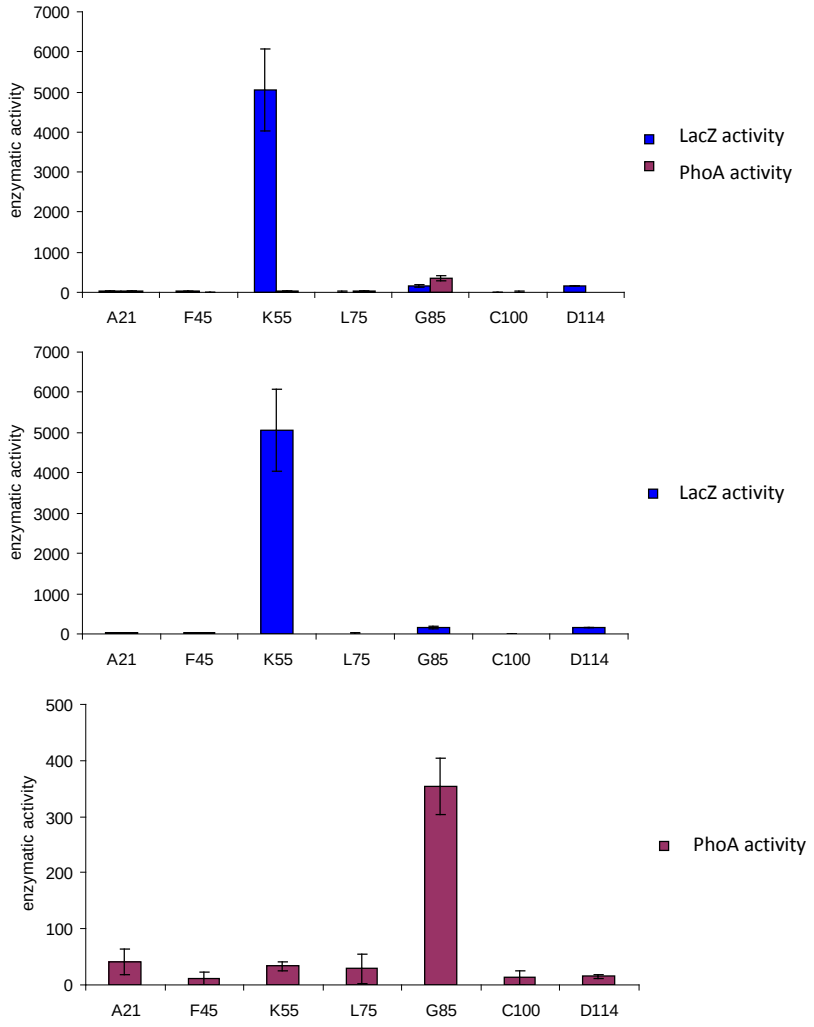

Fig. 3. . Activity of LtnI-LacZ and LtnI-PhoA fusions in *E. coli* CC118.

Activity of LtnI-LacZ and LtnI-PhoA fusions in *E. coli* CC118. A21, F45, etc., represent the residue of LtnI fused to *lacZ* or *phoA*. Activity is representative of the average of three independent triplicate experiments.

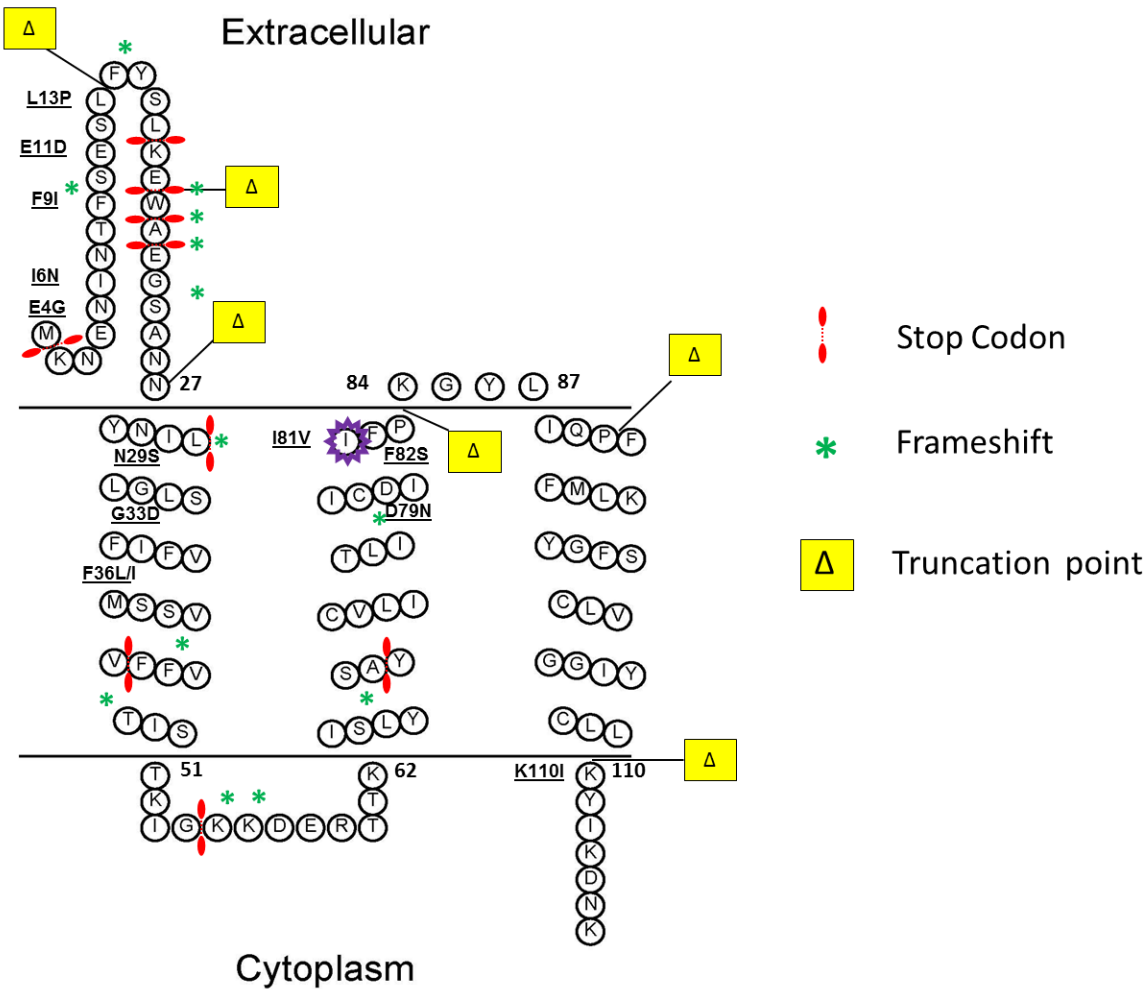

Fig. 4. Prediction of LtnI topology with overview of mutagenesis and truncation positions

Predicted here is the membrane location of LtnI. Indicated are positions at which random mutagenesis due to amino acid changes, frameshifts and stop codon introduction resulted in diminished immunity, or as for I81V, in which the the amino acid change resulted in a functionally enhanced LtnI protein. Positions at which truncated LtnI proteins were created are also present.

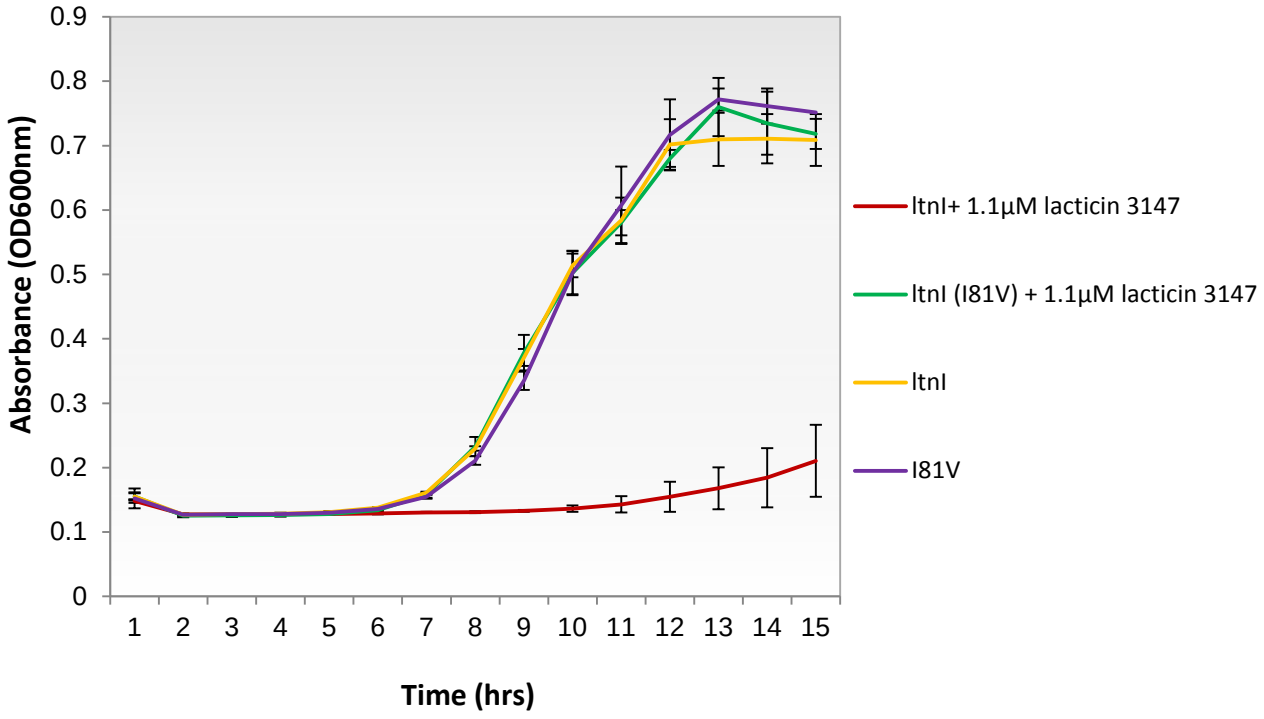

Fig. 5. Growth curve demonstrating the enhanced immunity of LtnI (I81V) MG1363.*pNZ44 ltnI* and MG1363.*pNZ44 ltnI* (I81V) were grown in the presence and absence of 1.1µM lacticin 3147, each strain was grown in triplicate and absorbance readings at 600nm taken hourly.

CHAPTER IV

The *spiFEG* locus in *Streptococcus infantarius* subsp. *infantarius* BAA-102 confers protection against nisin U.

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Summary

Nisin U is a member of the extended nisin family of lantibiotics. Here we identify the presence of nisin U immunity gene homologues in *Streptococcus infantarius* subsp. *infantarius* BAA-102. Heterologous expression of these genes in *Lactococcus lactis* subsp. *cremoris* HP confers protection to nisin U and other members of the nisin family, thereby establishing that the recently identified phenomenon of resistance through immune mimicry also occurs with respect to nisin.

Introduction

Lantibiotics are antimicrobial peptides that have been the focus of intense research in recent years. These ribosomally synthesised peptides undergo post-translational modification, resulting in the presence of unusual amino acids such as the eponymous lanthionine residues, as well as a variety of other modified residues. The nisin family is the most studied of all lantibiotics. Nisin A was initially discovered in 1928 (Rogers, 1928; Rogers & Whittier, 1928) and it has been used commercially as a food preservative for over 50 years (Lubelski *et al.*, 2008). The nisin family has also been investigated for potential applications in clinical and veterinary settings (Cao *et al.*, 2007; Fernandez *et al.*, 2008; Wu *et al.*, 2007) since it is active against a wide range of pathogens, including many drug-resistant strains (Severina *et al.*, 1998). Indeed, it is already commercially employed as an anti-mastitis agent (Sears *et al.*, 1992). To date, seven natural forms of nisin have been identified. Of these, nisin A (Kaletta & Entian, 1989), nisin Z (Mulders *et al.*, 1991), nisin Q (Zendo *et al.*, 2003) and nisin F (de Kwaadsteniet *et al.*, 2008) are produced by *Lactococcus lactis* strains, while nisin U, nisin U2 and nisin U3 are produced by *Streptococcus uberis* (Wirawan, 2007; Wirawan *et al.*, 2006). These variants differ from each other by as many as 11 residues across the 31-34 amino acid peptides (Field *et al.*, 2008). The large differences between the three nisin U's and the other nisins is so significant that it could be argued that they are not, in fact, nisin variants, but are rather members of a distinct lantibiotic subfamily (Piper *et al.*, 2010). As is the case with all lantibiotics, nisin producers possess immunity mechanisms which provide protection from auto-lethality. Despite the diversity of these peptides, the phenomenon of cross-immunity has been observed in some

instances. For example, nisin U-producing strains are immune to nisin U, A and Z (Wirawan *et al.*, 2006). Similarly, the nisin A-producing strain is protected from the activity of nisin U (Wirawan *et al.*, 2006). Lantibiotic immunity is provided by one or more systems consisting of a dedicated immunity peptide, LanI, or an ABC transporter which pumps the lantibiotic out of the cell, designated LanFE(G). A third immunity protein, LanH, is also present in some cases and acts as an accessory protein to the ABC transporter system (reviewed in (Chatterjee *et al.*, 2005; Draper *et al.*, 2008). In the case of the nisin family of lantibiotics, immunity is based on both the action of a LanFEG system and LanI protein. Despite the cross-protection referred to above, cross-immunity between lantibiotic producers is rare, with only a few exceptional examples (Aso *et al.*, 2005; Heidrich *et al.*, 1998). Indeed, cross-immunity between producers of the closely related nisin A and subtilin peptides (63% identity) is not evident. Another unusual phenomenon is immune mimicry, whereby non-lantibiotic-producing strains express functional homologues of lantibiotic immunity systems. In the only study of this phenomenon to date, homologues of immunity genes associated with the lantibiotic lacticin 3147 were identified in *Bacillus licheniformis* DSM 13 and *Enterococcus faecium* DO. It was shown that heterologous expression of these homologues provided protection against lacticin 3147 (Draper *et al.*, 2009). The identification of this phenomenon is a concern and may represent a means by which populations of bacteria could emerge with resistance to specific lantibiotics. Notably, while a number of systems involved in acquired (Gravesen *et al.*, 2001; Kramer *et al.*, 2006) and innate resistance to nisin have been identified (Collins *et al.*, 2010a; Collins *et al.*, 2010b),

systems capable of providing resistance to any of the nisin peptides through immune mimicry have not been identified heretofore.

Here we identify the first incidence of resistance by means of immune mimicry with respect to the nisin family. More specifically, genes encoding a homologue of the nisin U immunity-providing ABC transporter (NsuFEG) were identified within the genome of a non-lantibiotic-producing pathogen *Streptococcus infantarius* subsp. *infantarius* BAA-102. Although the BAA-102 strain was recalcitrant to genetic manipulation, and thus the creation of an knockout mutant was not possible, heterologous expression confirms that SpiFEG and NsuFEG can protect against the action of nisin U and other members of the nisin family.

Results and Discussion

In silico screen for homologs of nisin immunity determinants

Immune mimicry is a recently identified phenomenon and thus far the only examples relate to the protection afforded against lacticin 3147 by homologues of its immunity proteins (Draper *et al.*, 2009). To identify other examples of immune mimicry, a PSI-BLAST search (Altschul *et al.*, 1997) was undertaken using the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov) to determine if genes encoding homologues of the nisin immunity proteins could be identified in strains incapable of nisin production. This screening revealed the presence of the genes predicted to encode the components of an ABC transporter similar to that involved in nisin U immunity (NsuFEG; (Wirawan *et al.*, 2006)) within the genome of *S. infantarius* subsp. *infantarius* BAA-102 (Schlegel *et al.*, 2000). The predicted product of STRINF_01307 (hereafter referred to as *spiF*) resembled NsuF (67% identity, e-value 2e-83) while STRINF_01306 (hereafter annotated as *spiE*) and STRINF_01305 (hereafter referred to as *spiG*) are predicted to encode proteins that resemble NsuE (50% identity, e-value 4e-63) and NsuG (51% identity, e-value of 5e-45), respectively. While the similarity between SpiFE and NsuFE is only marginally greater than to NisFE, SpiG is only 35% identical to NisG. As a result of this discovery, other genes within this region of the BAA-102 genome were subjected to an *in silico* investigation to determine if other lantibiotic-associated genes might be present. Notably, the 3 genes immediately downstream from *spiEFG* all resembled those encoding the individual components of the nisin two-component system, known as NisRK (or

NsuRK in the case of nisin U (Wirawan *et al.*, 2006)), which are responsible for regulating nisin biosynthesis and immunity (Kuipers *et al.*, 1995) (Fig. 1). More specifically, the gene product of STRINF_01304 is 70% identical to the response regulator NsuR, with an e-value of 1e-50 (and is here referred to as *spiR*) and the adjacent gene (STRINF_01303; *spiR'*) also encodes an NsuR-like protein. Although SpiR and SpiR' are 67% identical to NisR, the two proteins are predicted to be quite different to each other, having only 11% identity. Furthermore, the predicted product of STRINF_01302 is 50% identical (e-value 4e-112) to the histidine kinase NisK and 43% identical to NsuK (e-value 7e-101) (Fig. 1). Although none of the nisin-like compounds have multiple LanR proteins associated with their regulation, multiple LanR proteins can be found in the lantibiotic operons of RumA, mersacidin and cytolysin (Cox *et al.*, 2005; Gomez *et al.*, 2002; Guder *et al.*, 2002). The NisR binding motif, a defined sequence of nucleotides by which NisR binds to the promoters of NisF and NisA, is referred to as a *nis*-box (Kleerebezem, 2004). A sequence with high similarity to a *nis*-box is found in the region upstream of *spiFEG*, and theoretically could act as a binding motif for SpiR and/or SpiR'. It was noted that the percent GC content of *spiFEGRR'K* (33.5%) is lower than that observed in the entire BAA-102 genome (37.7%), and thus, the possibility that these genes were acquired through horizontal transfer cannot be discounted. Analysis of up- and downstream genes reveals that none of these possess lantibiotic-associated features. Indeed, further analysis of the BAA-102 genome failed to identify any other lantibiotic-associated genes. Interestingly with respect to SpiF, a conserved domain, the BcrA subfamily (cd03268), was identified, homology between this bacitracin associated transporter and SpiG was also revealed.

The presence of the *spiFEGRR'K* genes in *S. infantarius* subsp. *infantarius* BAA-102 is noteworthy for a number of reasons. *S. infantarius,* referred to as *S. bovis* biotype II/1 before reclassification (Bouvet *et al.*, 1997), has been isolated from the faeces of infants, from clinical specimens associated with endocarditis and from food including dairy products and frozen peas (Abdelgadir *et al.*, 2008; Hoshino *et al.*, 2005; Schlegel *et al.*, 2000). This species can also contribute to the development of cancer, particularly in cases of chronic infection or inflammatory disease where the *S. infantarius* bacterial components interfere with cell function, leading to cell transformation and proliferation (Biarc *et al.*, 2004) and is most frequently associated with non-colonic digestive tract cancers (Corredoira *et al.*, 2008). Notably, nisin U has been shown to be effective against a wide range of disease-associated streptococci, including *Streptococcus pyogenes*, *Streptococcus. salivarius*, *S. uberis*, *Streptococcus agalactiae* and *Streptococcus dysgalactiae*, and thus, the possibility that a potential target such as *S. infantarius* may be resistant to nisin as a consequence of immune mimicry is worthy of note.

Heterologous expression of nsuFEG and spiFEG

S. infantarius BAA-102 is, on the basis of deferred-antagonism assays (for method see (Field *et al.*, 2008)), less sensitive to nisin U than a number of other *Streptococcus* species tested (*S. pyogenes*, *Streptococcus mitis* and *S. agalactiae*, data not shown). However, despite several attempts, we were unable to successfully transform BAA-102 as a prelude to creating isogenic *spiFEG* knockout mutants. As a consequence, we used heterologous expression as a strategy to determine whether the newly identified *spiFEG* genes could encode protection

against nisin U. The corresponding *nsuFEG* genes from *S. uberis* 42 (Jayarao *et al.*, 1991; Wirawan *et al.*, 2006) served as a positive control. To facilitate this, genomic DNA was extracted from *S. uberis* as described previously (Draper *et al.*, 2009). The *nsuFEG* genes were amplified using the primer pairs [AAAACTGCAGAAGTAGCAACTAGAAAG] and [GGGGTACCCTTTTAGGTGGCTAGTATCGC], and the primers for *spiFEG* were [AAAACTGCAGAAAAGTTTGGGACTTCAATG] and [GGGGTACCCCTGTCACCTCAATTGTATTTG], where restriction enzyme sites are underlined. The resulting gene products and the shuttle expression vector pNZ44 (McGrath *et al.*, 2001) were digested with the relevant restriction enzymes, ligated and introduced into electrocompetent *L. lactis* HP (via the intermediate host *Escherichia coli* Top10) as described previously (Draper *et al.*, 2009). Following confirmation of the integrity of the newly created vectors, deferred-antagonism assays were performed to provide an initial insight with respect to the protection provided against the producer of nisin U and other nisins (A, Z, F and Q; (Piper *et al.*, 2010)). These were carried out as described previously, using GM17 and TS agar for *Lactococcus* and *Streptococcus*, respectively (Field *et al.*, 2008). Relative sensitivity was assessed on the basis of zone size (Table 1). No inhibition was apparent when the nisin U producer was overlaid with *L. lactis* HP.pNZ44*nsuFEG*, thus establishing that the nisin U immunity proteins could provide protection when expressed heterologously to the otherwise nisin-sensitive HP strain. Notably, the presence of pNZ44*spiFEG* also provided protection against nisin U, with zone sizes decreasing substantially. The SpiFEG system is thus capable of providing protection through immune mimicry. The ability of pNZ44*nsuFEG* and pNZ44*spiFEG* to protect HP

against the actions of nisin A (produced by *L. lactis* NZ9700; (Kuipers *et al.*, 1993)), nisin F (produced by *L. lactis* NZ9800.pCI372nisF; (Piper *et al.*, 2010)), nisin Z (produced by *L. lactis* NZ9800.pCI372nisZ; (Piper *et al.*, 2010)) and nisin Q (produced by *L. lactis* NZ9800.pCI372nisQ; (Piper *et al.*, 2010)) and nisin U3 (Wirawan, 2007) was also assessed. It was established that heterologous expression of *nsuFEG* in strain HP provides protection against nisin U3 and to a lesser degree against nisin A, nisin F, nisin Z and nisin Q, with zone sizes smaller than those observed when HP was used as a target (Table 1). The presence of pNZ44*spiFEG* also substantially reduced the sensitivity of the HP strain to nisin Z and nisin U3 (Table 1). To further assess the level of protection, the same collection of strains were employed to carry out a series of agarose-based well diffusion assays (Fig. 2) (Derache *et al.*, 2009; Lehrer *et al.*, 1991). In this instance, the antimicrobials were present in the form of cell-free supernatant from overnight cultures of the nisin producers. The benefit of this approach is that the enhanced rate of diffusion of the antimicrobials through agarose (relative to agar) and the use of target cells in early-log-phase provides greater sensitivity. The results from these assays confirm the significantly enhanced resistance of HP.pNZ44*nsuFEG* to all forms of nisin and of HP.pNZ44*spiFEG* to nisins Z and U. However, in this instance, HP.pNZ44*spiFEG* also displayed significantly enhanced resistance to nisins A, F and Q (Fig. 2). These investigations are consistent with those of Wirawan *et al*. who previously noted cross-protection between nisin-producing strains (Wirawan *et al.*, 2006). Given that SpiFEG also resembles transporters involved in bacitracin resistance, the relative resistance of *L. lactis* HP and HP.pNZ44*spiFEG* to this antibiotic was tested via antibiotic disc assays (10 IU, Oxoid) (Collins *et al.*, 2010b). These assays revealed

that *spiFEG* do not provide the HP strains with enhanced resistance to bacitracin (data not shown).

Antimicrobial activity assays with purified nisin U

To better assess the extent to which SpiFEG provide protection from nisin U, broth-based assays with purified nisin U were carried out. To facilitate this, the lantibiotic was purified using an approach previously employed to purify nisin A and derivatives (Field *et al.*, 2008), but with some slight modifications. Specifically, the tryptone-yeast extract-glucose growth medium was supplemented with higher levels of glucose (11 g l^{-1}) and β-glycerophosphate (21 g l^{-1}) before the nisin U present in cell free culture supernatant was isolated by passing it through 60 g XAD-16 beads (prewashed with water), washed with 30% ethanol, and finally eluted with 70% isopropanol. This was combined with the nisin-containing 70% isopropanol from the purification of cell-attached nisin as previously described (Field *et al.*, 2008). Subsequent purification was performed using a 10 g (60 ml) Strata C_{18}-E column (Phenomenex, Cheshire, UK) pre-equilibrated with methanol and water. The columns were washed with 30% ethanol and the inhibitory activity was eluted in 70% isopropanol-0.1% trifluoroacetic acid (TFA). Aliquots (20 ml) were concentrated to 2 ml through the removal of isopropanol by rotary evaporation before being applied to a Phenomenex C_{12} reverse-phase high-performance liquid chromotography (HPLC) column (Jupiter 4µm proteo 90 Å, 250 X 10.0 mm, 4 µm) previously equilibrated with 25% acetonitrile-0.1% TFA. The column was subsequently developed in a gradient of 30% acetonitrile (ACN) containing 0.1% TFA to 60% ACN-0.1% TFA from 10 to 45 min at a flow rate of 2.0 ml min^{-1}. Fractions

containing nisin U were collected post HPLC, acetonitrile removed by rotary evaporation and the protein lyophilised by freeze-drying. Mass spectrometry was performed with an Axima CFR plus matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometer as previously described (Field *et al.*, 2008), which confirmed that the final purified product was nisin U. The purified nisin U was employed in studies to compare the growth of HP, HP.pNZ44*nsuFEG* and HP.pNZ44*spiFEG* in the presence of 416 nM nisin U over 4 hrs in broth (Fig. 3). This was assessed by inoculating 1 x 10^7 CFU ml^{-1} of target cells into fresh broth containing nisin U, incubating at 30°C and at intervals removing aliquots, which were subject to serial dilution in ¼-strength Ringers' solution and plated on GM17 agar. All growth experiments were performed in triplicate from three separate overnight cultures and repeated on at least three different days. These assays revealed that after 4hrs the numbers of HP expressing *nsuFEG* and *spiFEG* were significantly (*P value* < 0·014) greater those of the corresponding HP control (Fig. 3).

Assessing the relative protection provided through heterologous expression of spiFEGRR'K

We postulated that the products of *spiRR'K* may sense and respond to the presence of nisin to further enhance the expression of *spiFEG* and nisin resistance. However, should such a phenomenon exist, it would be mediated through the *nis*-box within the promoter upstream of *spiFEG* (P_{spi}). To investigate this possibility, heterologous expression was again employed. P_{spi}-*spiFEGRR'K* was amplified using the primers [GGGGTACCGAAGGTTGGACAGAAGTTTGG] and [GCTGCAGACCATGTCGTAAATAGTCGTTTTTTC] and digested with the appropriate

restriction enzymes (Fastdigest, Fermentas) (Hayes *et al.*, 1990). This was ligated with similarly digested pCI372 (a shuttle vector which, unlike pNZ44, does not contain a constitutive promoter to drive expression of cloned genes) and transformed into electrocompetent *L. lactis* HP. A phenotypic assay was performed to assess if exposure to sublethal concentrations of nisin A or nisin U enhanced the ability of pCI372P_{spi}-*spiFEGRR'K* to provide protection from a subsequent challenge with concentrated nisin. Specifically, overnight cultures of *L. lactis* HP.pCI372 and HP.pCI372P_{spi}-*spiFEGRR'K* were inoculated (3%) into fresh GM17, incubated until they reached an OD_{600} of 0.3, whereupon 1 ml of cells were exposed to a sublethal concentrations (0.3µM) of nisin A or nisin U for 1 hr in 1.5 ml tubes at 30°C. The cells were then washed in 10mM sodium phosphate buffer and seeded in $^{1}/_{100}$-strength GM17-agarose, into which wells were bored. Approximately 30 µM Nisin A and nisin U were incubated into the wells for 3 hrs and subsequently overlaid with 2X strength GM17-agarose to allow subsequent growth of *L. lactis* strains (see (Derache *et al.*, 2009; Lehrer *et al.*, 1991) for method). Having determined relative sensitivity, on the basis of zone size, we found that in no instance did exposure to sublethal concentrations of nisin significantly enhance the protection provided by pCI372Pspi-*spiFEGRR'K* to subsequent exposure to higher concentrations of nisin (Fig. 4). It should be noted, however, that this does not preclude the possibility that SpiRR'K sense and respond to the presence of nisin in their native background.

As a consequence of the continued emergence of antibiotic-resistant bacteria, the possibility of using ribosomally synthesised antimicrobial peptides such as the lantibiotics as alternative chemotherapeutic agents has received attention (Piper *et al.*, 2009). Despite nisin having been used for over half a century

for food applications, the development of resistance has not become a problem. Nonetheless, it has been established that some bacteria possess innate nisin resistance mechanisms and that others can become resistant upon exposure to nisin in the laboratory (Collins *et al.*, 2010a; Collins *et al.*, 2010b; Kramer *et al.*, 2006). It is thus a concern that the use of nisin and other lantibiotics for clinical applications could also result in the emergence of resistant strains. However, it is hoped that by developing a clearer understanding of the various different mechanisms by which resistance can emerge, it will be possible to develop strategies to counteract such occurrences. The phenomenon of resistance through immune mimicry has been described on only one previous occasion (Draper *et al.*, 2009), and thus, the identification of nisin immunity determinants in the genome of BAA-102 is noteworthy. On the basis of these findings, this phenomenon may be more common than has previously been appreciated, and the possibility that the presence and transfer of such genes could potentially lead to the emergence of lantibiotic-resistant strains needs to be considered carefully.

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Tables and Figures

	Avg zone size (mm) ± SD			
Nisin variant	**HP (control)**	**HP.pNZ44*nsuFEG***	**HP.pNZ44*spiFEG***	***S. infantarius***
A	18.6 (±0.71)	16.3 (±0.71)	17.85 (±1.06)	2.94 (±0.18)
F	20.6 (±1.00)	17.79 (±0.27)	19.67 (±1.00)	5.84(±0.50)
Z	24.4 (±0.36)	20.2 (±0.14)	21.85 (±1.62)	4.9(±0.74)
Q	16.08 (±0.65)	14.8 (±0.28)	15.05 (±1.62)	4.17(±0.75)
U	8.4 (±0.42)	0	4.7 (±1.27)	0*
U3	16.06 (±0.18)	0	12.2 (±2.97)	0*

* denotes no distinct zone but some hazy growth adjacent to nisin producing colony.

Table 1. Deferred-antagonism assay analysis of the protective capabilities conferred by NsuFEG and SpiFEG when expressed in *L. lactis* HP, or the resistance of the natural *S. infantarius* isolate, against the action of a range of natural nisin variant producers. Values are an average of triplicate experiments and represent zone size i.e. diameter of zone minus diameter of bacterial growth.

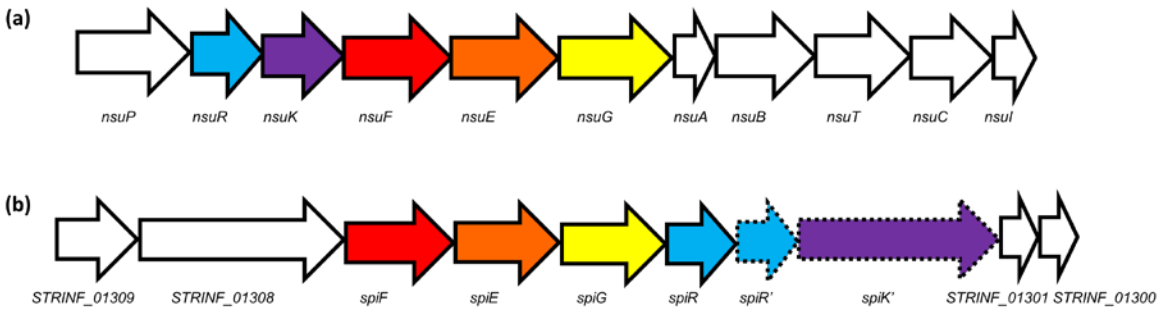

Fig. 1. Genetic orientation of homologues of the nisin U (a) and nisin-associated immunity and regulatory (b) genes found in the strain S. *infantarius* subsp. *infantarius.* The up- and downstream genes are included, and from BlastP analysis, their theoretical functions are as follows; STRINF_01300, UDP-N-acetyl-D-glucosamine 2-epimerase; STRINF_01301, hypothetical protein; STRINF_01308, hypothetical ATP-binding protein; STRINF_01309, hypothetical membrane protein.

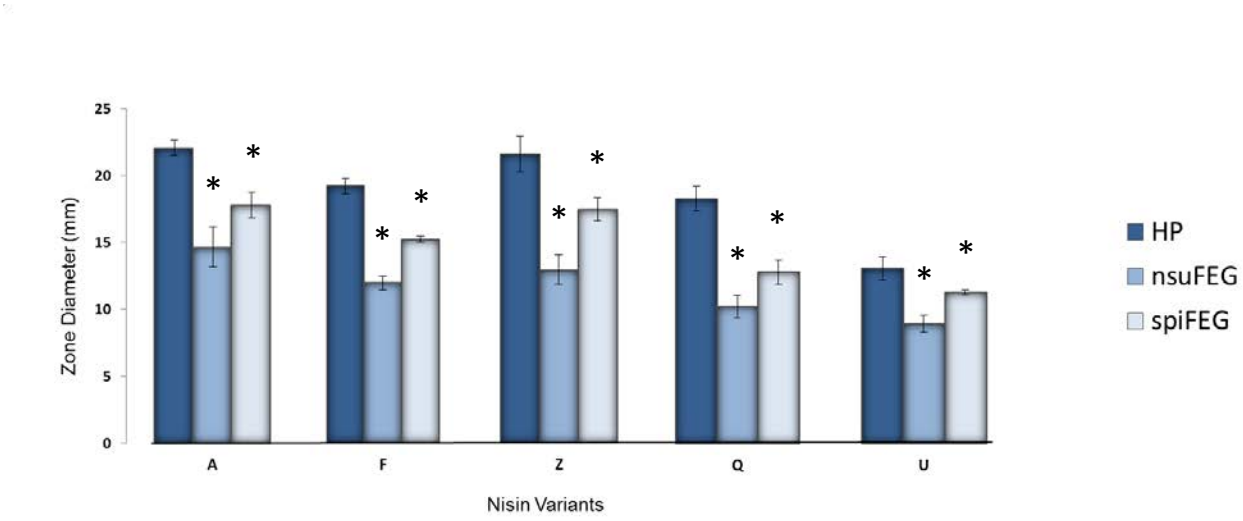

Fig. 2. Agarose well diffusion assay, whereby *L. lactis* HP.pNZ44 and strains expressing *nsuFEG* and s*piFEG* were challenged under adverse growth conditions with nisin A, F, Z, Q and U. Asterisks indicate zone diameters which were significantly smaller by Student's *t*-test ($P < 0.0005$) than that found in *L. lactis* HP, hence implying the protective capabilities of these genes.

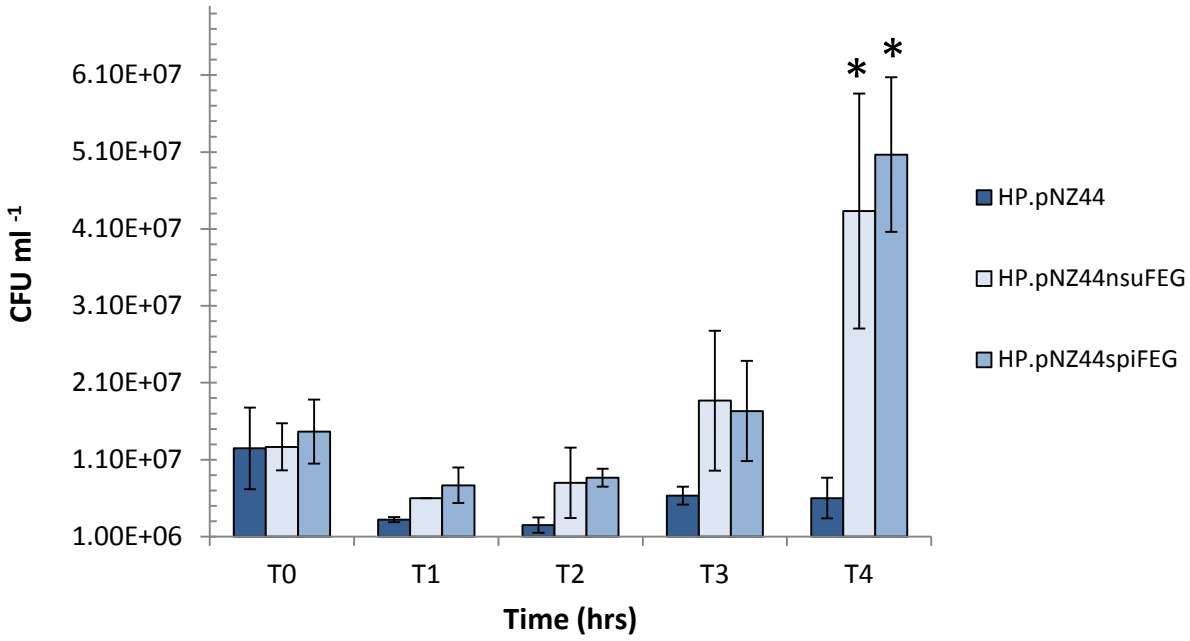

Fig. 3. Survival and growth of the strain *L. lactis* strain HP and strains expressing NsuFEG and SpiFEG when challenged with a sublethal level (416 nM) of nisin U. An asterisk at T4 indicates the difference between HP.pNZ44 and strains expressing immunity genes are statistically significant (Student's *t*-test $P < 0{\cdot}014$).

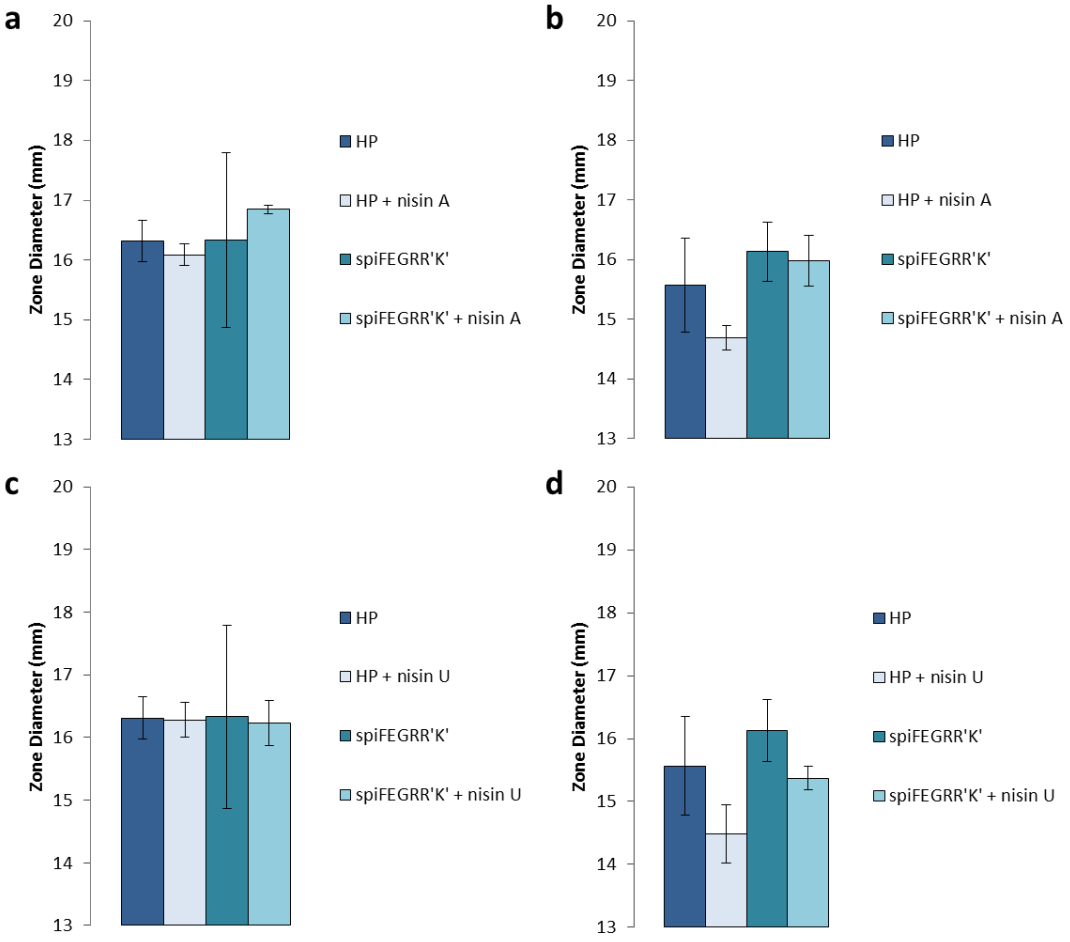

Fig. 4. *L. lactis* HP expressing the genes *spiFEGRR'K* under the control of their native promoter in the vector pCI372 was assessed to discover if the immunity could be induced in the presence of nisin. *L. lactis* HP strains were incubated in the presence of approximately 10 ng of either nisin A (a, b) or nisin U (c, d) prior to being challenged by agarose well diffusion assay with both nisin A (a, c) and nisin U (b, d). *L. lactis* HP.pCI372 alone was included as a control. The data shows that the action of the *spiFEG* genes has not been induced and thus they are not active under these conditions.

CHAPTER V

The lacticin 3147 biosynthetic promoter P_{bac} is regulated by LtnR and *ltnA*$_2$

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Manuscript to be prepared.

Summary

Lacticin 3147, produced by *Lactococcus lactis*, is a member of the lantibiotic class of bacteriocins. Lantibiotics are post-translationally modified antimicrobial peptides that are active against a broad range of Gram positive targets. The genes encoding lacticin 3147 production and immunity are found on two divergent operons; *ltnRIFE*, which is regulated by the promoter P_{imm}, and *ltn*$A_1A_2M_1TM_2J$, controlled by the promoter P_{bac}. We used a green fluorescent protein expression reporter system to establish if any of the lacticin 3147-associated gene products influence P_{bac} expression. It was revealed that LtnR plays a role as an activator of P_{bac}, in addition to acting as a negative regulator of P_{imm}. Moreover, P_{bac} was subject to negative regulation by *ltn*A_1 and *ltn*A_2, and the *ltn*A_2 transcript was shown to play a part in this repression. Thus, fluctuations in the levels of LtnR and *ltn*A_1A_2, may serve to maintain lacticin 3147 expression, modification and export at a homeostatic level within the cell.

Introduction

Lantibiotics are highly potent antimicrobial peptides that target a broad spectrum of Gram positive species, often active in the nanomolar range (Morgan *et al.*, 2005b; Wiedemann *et al.*, 2006). Ribosomally synthesised and subject to post-translational modification that creates unusual residues such as dehydroalanine (Dha), lanthionine and β-methyl lanthionine, these peptides are termed lantibiotics as they are lanthionine-containing peptide antibiotics (for review see (Chatterjee *et al.*, 2005)). Lacticin 3147 is a two-component lantibiotic produced by *Lactococcus lactis*. Both components, Ltnα and Ltnβ, combine in a 1:1 ratio (Morgan *et al.*, 2005a; Wiedemann *et al.*, 2006) and are active against a wide range of targets, including nosocomial pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE) (Cotter *et al.*, 2013; Piper *et al.*, 2009). The immunity and biosynthetic genes associated with lacticin 3147 are located on a 60.2Kb lactococcal plasmid called pMRC01 and consist of two operons *ltnRIFE* (immunity) and *ltn*$A_1A_2M_1TM_2J$ (biosynthesis) (Fig.1) (Dougherty *et al.*, 1998; Draper *et al.*, 2009; McAuliffe *et al.*, 2000a). LtnR shares homology with a number of transcriptional repressors of the PBSX (Xre) family and has been shown to negatively regulate the promoter (P_{imm}) of the *ltnRIFE* immunity operon (McAuliffe *et al.*, 2001). LtnI is an immunity specific transmembrane protein and LtnFE form a self-protecting ABC transporter (Draper *et al.*, 2009; Draper *et al.*, 2012; McAuliffe *et al.*, 2000a). LtnR is thought to bring about this negative regulation through binding to the P_{imm} promoter region. It was established that mutation of the *ltnR* gene in the pMRC01 background resulted in a hyper-immune phenotype, whereas overexpression of *ltnR* resulted in cells with increased

sensitivity to lacticin 3147. The biosynthetic and export machinery are found in the second lacticin 3147 operon (Fig. 1). The genes *ltnA*$_1$ and *ltnA*$_2$, encoding the pre-pro-peptides of Ltnα and Ltnβ, respectively, are found immediately after the promoter P_{bac}. Downstream from these are *ltnM*$_1$ and *ltnM*$_2$, which encode enzymes involved in post-translational modification, catalysing the dehydration/thioether-formation reactions involved in the creation of the mature lantibiotic. Each of the lacticin 3147 pre-peptides is modified by a different modification enzyme, $LtnM_1$ is required to produce mature Ltnα, while $LtnM_2$ is required to produce mature Ltnβ (McAuliffe *et al.*, 2000b). Located between the *ltnM* genes is *ltnT*, encoding an ABC transporter that is involved in leader cleavage and transport of the lacticin 3147 peptides from the cell. The final member of the lacticin 3147 operon is *ltnJ*, encoding a unique enzyme that is responsible for the presence of D-alanine residues in both Ltnα and Ltnβ (Cotter *et al.*, 2005).

Lacticin 3147 biosynthesis and immunity are therefore under the control of two divergent promoters, P_{imm} and P_{bac}. While the negative regulation of P_{imm} by LtnR has been demonstrated, to date the nuances relating to control of the P_{bac} promoter have yet to determined. Random mutagenesis of P_{bac} revealed that the only mutations to disrupt expression were localised to the canonical -10 and -35 regions of the promoter (Field *et al.*, 2007). Additionally, it had been noted that a construct that contained the P_{bac} promoter fused to a β-gal reporter produced levels of β-galactosidase activity comparable to those of a construct that also contained *ltnR*. These results suggested that LtnR did not play a role in the regulation of P_{bac} and hence was thought not to be involved in transcription of the biosynthetic genes (McAuliffe *et al.*, 2001). It was concluded that P_{bac} is

constitutively expressed, with an intragenic rho-independent attenuator in the form of a triple stem loop in *ltnM*$_1$ suggested as a means of limiting the transcription of genes (McAuliffe *et al.*, 2001). This contrasted with the observation that the promoters governing the expression of many lantibiotic structural genes are regulated, frequently *via* a two-component signal transduction system. Regulation of this kind has been evident in the case of the lantibiotics subtilin, produced by *Bacillus subtilis*, and nisin, produced by *L. lactis* (Engelke *et al.*, 1994; Klein *et al.*, 1993). Genes encoding lantibiotic-associated histidine kinases (LanK) and response regulators (LanR) have been identified in producers of these lantibiotics. Signal transduction is achieved by phosphate transfer from LanK, which is autophosphorylated upon recognition of the external signal, to LanR. LanR then binds to specific nisin- and subtilin-specific pentanucleotide direct repeat sequences, called a *nis*- and *spa*-boxes, upstream of the promoter, activating transcription. As the signal molecule in this system is the antimicrobial peptide, production can be said to be positively auto-regulated through a quorum sensing-like mechanism (Kleerebezem *et al.*, 1997). In the case of nisin, these *nis*-box sequences are associated with the promoter P_{nisA}, responsible for transcription of *nisABTCIP,* as well as upstream of the immunity genes *nisFEG*. Similarly, subtilin *spa*-boxes are located upstream of the *spaBTC* promoter P_{spaB}, upstream of P_{spaS} and the gene encoding the subtilin pre-pro-peptide, and upstream of the promoter P_{spaI} of the immunity genes *spaIFEG* (Kleerebezem *et al.*, 2004). Regulation of the gene cluster responsible for production of and immunity to another *B. subtilis*-associated lantibiotic, mersacidin, is through binding of the MrsR1 response regulator to the promoter governing lantibiotic production. In addition, upon

recognition of mersacidin, a two-component system MrsR2/MrsK2 regulates the immunity associates ABC transporter, MrsFGE. MrsR2/MrsK2 are also thought to have a role in mersacidin production to synchronize biosynthesis and immunity (Schmitz *et al.*, 2006). In contrast, the regulatory systems associated with some other lantibiotics involve a single regulatory protein. Such regulatory proteins include MutR (Qi *et al.*, 1999), LasX (Rawlinson *et al.*, 2002) and EpiQ (Peschel *et al.*, 1993). In fact, LasX has a dual role in that it regulates the two divergent operons within the lactocin S gene cluster (*lasAMNTUVPJW* [*lasA–W*] and [*lasXY]*), simultaneously acting as an activator of P_{lasA-W} transcription and as a repressor of P_{lasXY} transcription.

Our understanding of the regulation of lantibiotic biosynthesis has led to the development of the nisin controlled expression (NICE) system (Mierau & Kleerebezem, 2005), highlighting the benefits that can be reaped from in-depth knowledge of promoters and their regulation. Furthermore, since production levels can act as a bottleneck to the commercialisation of lantibiotics, a detailed characterisation of the promoters involved in expression can potentially lead to manipulations that can enhance lantibiotic manufacture. Here we compare the strength of the lacticin 3147 promoter, P_{bac}, with that of a range of known lactococcal promoters and establish that this promoter is regulated by genes within the lacticin 3147 cluster. This improved understanding of P_{bac} regulation has the potential to ultimately facilitate improved lacticin 3147 biosynthesis.

Materials and methods

Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *L. lactis* strains were grown M17 broth (Oxoid) supplemented with 0.5% glucose (GM17) or GM17 agar at 30°C without aeration. *E. coli* was grown in Luria–Bertani (LB) broth with vigorous shaking or agar at 37°C. Antibiotics were used where indicated at the following concentrations: chloramphenicol at 5 μg ml^{-1} for *L. lactis* and 10 μg ml^{-1} for *E. coli;* erythromycin was used at 300 μg ml^{-1} in *E. coli* and was combined with chloramphenicol in *L. lactis* at a concentration of 5 μg m^{-1} l for both antibiotics.

Plasmid creation

Plasmids pNZ25, pNZ32 and pNZbac were created as follows: the high-copy cloning vector pNZ44 was digested with restriction enzymes *Bgl*II and *Nco*I (Roche Diagnostics), sites for which are found either side of the P_{44} promoter, resulting in its removal. The plasmid backbone was subsequently isolated by gel extraction using a GeneJET gel extraction kit (Thermo Scientific) according to manufacturer's guidelines, and treated with shrimp alkaline phosphatase step. Promoters P_{CP25} and P_{32} were created by annealing two complementary oligonucleotides (Table 2). Briefly, 10 μg of each oligo was reconstituted in 50mM Tris-HCl, 10mM MgCl, 100mM NaCl, 1mM dithioerythritol and 1mM EDTA, were combined and heated to 95°C for 5 mins followed by cooling to 25°C over 1hr. Approximately 1 μg of the now annealed oligos were phosphorylated using polynucleotide kinase (Roche) according to manufacturer's guidelines. The promoter P_{bac} was amplified by PCR

from genomic DNA from the strain *L. lactis* MG1363.pMRC01, using *KOD* DNA polymerase (Novagen) using the primers Pbac F and Pbac R (listed in Table 2). Total cell DNA was isolated using an Invitrogen PureLink® Genomic DNA Kit. Promoters were subsequently ligated into the promoterless pNZ44 vector backbone using T4 Ligase supplied by Roche Diagnostics, and transformed into chemically competent *E. coli* Top 10 (Invitrogen) as an intermediate host. Following verification by DNA sequencing (Source BioScience), plasmids were isolated using a High Pure plasmid isolation kit as recommended by the manufacturer (Roche Diagnostics) and digested using the restriction enzymes *Pst*I and *Xba*I (Roche Diagnostics). The *gfp+* gene was amplified from the plasmid pNZ8150*gfp+* using the primers gfp+For and gfp+Rev and was subsequently digested with *Pst*I and *Xba*I and ligated into the vectors pNZ25, pNZ32, pNZ44 and pNZbac and transformed into *E. coli* Top 10. Following verification of DNA sequences, pNZ25*gfp+*, pNZ32*gfp+*, pNZ44*gfp+* and pNZbac*gfp+* were transformed into *L. lactis* strains made electrocompetent according to the procedure described by Holo and Nes (Holo & Nes, 1995).

Gene deletions and their complementation

Deletion of *ltnA$_1$A$_2$* from MG1363.pMRC01 was carried out using a combination of splicing by overlap extension PCR (Horton *et al.*, 1990) and a double-crossover recombination event as described previously (Cotter *et al.*, 2003) using the primers *ltnA1A2*soeA, *ltnA1A2*soeB, *ltnA1A2*soeC and *ltnA1A2*soeD. To express *ltnA$_2$ and ltnR in trans*, the genes were amplified by PCR using *KOD* polymerase using the primers ltnA2 F and ltnA2 R for *ltnA$_2$* and ltnR F and ltnR R for *ltnR* (listed in Table 2). These products were digested with *Pst*I and *Xba*I and ligated

into similarly digested pNZ44 under the control of the P_{44} promoter. The P_{44} promoter and *ltnA*$_2$ or *ltnR* were amplified from pNZ44*ltnA*$_2$ and pNZ44*ltnR* as described previously using primers P44 For and ltnA2 R/ltnR R (listed in Table 2), digested with *Sph*I and *Xba*I and ligated into similarly digested pNZEm (described in Table 3). After transformation into *E. coli* Top 10, plasmid isolation and verification of DNA sequence integrity, the plasmids pNZEmP$_{44}$*R and* pNZEmP$_{44}$*A*$_2$ were electroporated into the corresponding *L. lactis* Δ*ltnR.pNZbacgfp+* and Δ*ltnA*$_1$*A*$_2$.pNZbac*gfp+* strains. To create pNZEmP$_{44}$*A*$_2^{PP-}$, the entire plasmid pNZEmP$_{44}$*A*$_2$ was amplified by PCR using Phusion High-Fidelity DNA polymerase (New England Biolabs) and primers ltnA2^{PP-} F and ltnA2^{PP-} R, which introduced a stop codon immediately after the start codon of *ltnA*$_2$ (for primers see Table 2). PCR products were *Dpn*I treated in order to remove the methylated pNZEmP$_{44}$*A*$_2$ template and the resulting plasmids were transformed into *E. coli* Top 10 prior to DNA sequencing to confirm integrity of the resulting plasmids. pNZEmP$_{44}$*A2*$^{PP-}$ was then electroporated into *L. lactis* MG1363.pMRC01Δ*A*$_1$*A*$_2$.pNZbac*gfp+*.

Growth and fluorescence assays

Broth-based assays were performed by inoculating *L. lactis* strains containing the appropriate plasmids to give a final inoculum of 2X10^7 CFU ml^{-1} in a volume of 0.2 ml in GM17 and monitoring the optical density at 600 nm (OD_{600}) to analyse bacterial growth or to quantify fluorescence (excitation: 435 nm, emission: 520 nm) in a Spectromax 340 spectrophotometer (Molecular Devices, Sunnyvale, CA) over a 23 hr period.

In silico *analysis*

Multiple alignments were performed using the ClustalW2 (Larkin *et al.*, 2007) tool found at the EMBL-EBI website (Goujon *et al.*, 2010). Repeat and palindrome elements of the promoters were identified using Lasergene GeneQuest software version 7.0 (DNASTAR, Madison, WI, USA).

Results

In silico analysis of P_{imm} and P_{bac}

In silico analysis of the P_{imm}/P_{bac} promoter regions revealed a number repeats and palindromes (Fig. 2). These consensus sequences are similar to sites bound by transcriptional regulators other than members of the Xre family, of which LtnR is a member. More specifically, there are two sites that resemble the *E. coli* Fur (ferric uptake regulation protein)-box sequence (Fur1) and another than resembles a *Bacillus subtilis* Per-box sequence (Fur2). In Figure 3 the percentage identity to the proposed operator sequences of P_{imm}/P_{bac} are displayed along with the aligned consensus binding sequence for the transcriptional regulator Xre, the Fur1 and Fur2 boxes referred to above and the two putative operator sequences found in P_{imm} and P_{bac}. Alignments were performed using ClustalW2 (Larkin *et al.*, 2007). The operator sequences of the lacticin 3147 gene cluster more closely resemble Fur consensus sites than the previously reported Xre-like consensus sequence within P_{imm} (84.21% versus 80.00% respectively) (McAuliffe *et al.*, 2001). While it is apparent that LtnR shows greater homology to the consensus sequence of Xre proteins (31.03%) than to that of Fur proteins (16.00%), it is notable that a single Fur protein (locus tag: llmg_1023), which bears 35% identity to this consensus sequence, is present within the strain *L. lactis* MG1363. On the basis of finding multiple binding sites associated within P_{imm}/P_{bac}, further investigations were carried out to determine whether P_{bac} is regulated as suggested by the presence of these various repeat regions.

A comparison of the strength of P_{bac} relative to other lactococcal promoters in MG1363

The P_{bac} promoter associated with lacticin 3147 production was previously suggested to be constitutive in nature (McAuliffe *et al.*, 2001). The strength of P_{bac} activity relative to some known lactococcal constitutive promoters was assessed using green fluorescent protein (GFP) as a reporter. The lactococcal promoters used for this comparison include a promoter similar to P_{CP25} with a *L. lactis* optimised ribosomal binding site (RBS) (Jensen & Hammer, 1998), a truncated version of the promoter P_{32} (van der Vossen *et al.*, 1987) and the P_{44} promoter, as found in the plasmid pNZ44 (McGrath *et al.*, 2001). To facilitate this, the P_{44} promoter in pNZ44 was replaced by P_{CP25}, P_{32} and P_{bac} to create pNZ25, pNZ32 and pNZbac. The *gfp+* gene was cloned into these three plasmids, as well as pNZ44. A comparison of expression levels in *L. lactis* MG1363 revealed that while the presence of the P_{CP25}, P_{32} and P_{44} promoters resulted in the production of GFP+, no GFP+ production was evident from the P_{bac}-containing vector (Fig. 4). Among the vectors that successfully brought about GFP+ production, comparably high levels of expression were observed as a consequence of P_{CP25} and P_{44} driven transcription, while levels were approximately 25% lower when P_{32} was used.

Pbac exhibits strong promoter activity in the presence of pMRC01

As the promoter P_{bac} is naturally found within the lacticin 3147 associated operon, further experiments were carried out to determine if expression from this promoter on pNZbac*gfp+* would be affected in a strain containing the other lacticin 3147-associated determinants present on pMRC01. Thus pNZbac*gfp+*, along with

pNZ25*gfp+*, pNZ32*gfp+ and* pNZ44*gfp+* (for comparative purposes), were electroporated into competent *L. lactis* MG1363.pMRC01 cells. A significant increase in GFP+ production was observed when pNZbac*gfp+* is co-localised with pMRC01. The level of GFP+ expressed by P_{bac} is approximately double that observed for the constitutive promoters P_{CP25} and P_{44} (Fig. 5) when expressed in *L. lactis* MG1363 (Fig. 4). Moreover, the expression of GFP+ from the vectors pNZ25, pNZ32 and pNZ44 is unaltered by the presence of pMRC01 and remains consistent with that observed previously in *L. lactis* MG1363.

Mutation of ltnR or ltnA$_2$ impacts on P$_{bac}$ activity

As production of GFP+ from pNZbac*gfp+* occurred as a consequence of the presence of pMRC01, we hypothesised that there may be elements within the lacticin 3147 operon that regulate P_{bac}. In order to investigate this further, pNZbac*gfp+* was introduced into a series of *L. lactis* MG1363.pMRC01 derivatives in which lacticin 3147-associated genes had been deleted in a non-polar manner. These strains lacked *ltnR, ltnA$_1$, ltnA$_1$A$_2$, ltnM$_1$, ltnM$_2$* or *ltnJ*. The relative GFP+ production from these strains was assessed at 18 hrs (Fig. 6, Fig. 7). These investigations established that the presence of *ltnR* is essential for P_{bac} activity. In addition, P_{bac} is considerably up-regulated in the absence of *ltnA$_1$* and *ltnA$_2$* (Δ*ltnA$_1$A$_2$*). This phenomenon was not as obvious in the absence of *ltnA$_1$* alone, suggesting that the additional absence of *ltnA$_2$* is of key importance (Fig. 6). Deletion of the genes *ltnM$_1$*, *ltnM$_2$* and *ltnJ* did not alter levels of GFP+ relative to those evident in the MG1363.pMRC01 strain (Fig. 7).

Complementation of ltnA$_2$ and ltnR

As the absence of *ltnM$_2$*, which is required for the post translational of Ltnβ, does not impact on P_{bac} activity transcription levels, it was evident that mature Ltnβ is not responsible for the lack of Pbac activity from the Δ*ltnA$_1$A$_2$* strain. This was also apparent when it was established that externally added purified Ltnβ peptide had no effect on expression (data not shown). We speculated that the regulation of P_{bac} was dependent either on the presence of the unmodified pre-pro-peptide, or that the *ltnA$_2$* RNA was involved. In order to investigate this, two *ltnA$_2$*-containing plasmids were generated and their ability to complement the hyper-GFP+ producing phenotype in the Δ*ltnA$_1$A$_2$* background was assessed. One of these contained an intact *ltnA$_2$* gene and the second, *ltnA$_2^{PP-}$*, was designed such that a stop codon was introduced immediately after the start codon. It was established that the presence of the *ltnA$_2$* transcript alone is sufficient to reduce GFP+ production, with no significant difference (Student's *t*-test P <0.4) between *ltnA$_2$* or *ltnA$_2^{PP-}$*(Fig. 6). In contrast, re-introduction of *ltnR in trans* did not complement the Δ*ltnR* phenotype (data not shown).

Discussion

Previous investigations, involving fusion of the promoter to β-gal in the vector pAK80 and expression in *L. lactis* MG1363, indicated that P_{bac} is constitutively expressed (McAuliffe *et al.*, 2001) However with no comparison to any known constitutive promoter(s) and the contrasting use of a of low copy plasmid it makes comparisons to the current study difficult. Here we gain a more in depth insight into lacticin 3147 promoter function via the expression of *gfp+* in the presence of pMRC01 and mutated derivatives.

This study established that LtnR has a dual role in regulation. It was previously known that LtnR has a role in repressing transcription from the immunity associated P_{imm} promoter (McAuliffe *et al.*, 2001), but we have identified a second role as an activator of expression from P_{bac}. This was apparent since there was no transcription from P_{bac} in the *gfp+* containing reporter plasmid in the absence of *ltnR*. Although complementation of this gene was attempted, P_{bac} activity did not change from that observed in the Δ*ltnR* strain. This is consistent with the previous finding that *ltnR* over-expression did not complement the immunity related Δ*ltnR* phenotype (McAuliffe *et al.*, 2001).

Both $ltnA_1$ and $ltnA_2$ also appear to play a role in regulation. In the absence of $ltnA_1$ alone slightly higher levels of GFP+ production are observed, but, when both genes are deleted, GFP+ levels increase dramatically, suggesting that the presence of $ltnA_2$ is particularly important. Notably, exogenously added Ltnβ did not compensate for this phenotype. The absence of $ltnM_2$, and in turn modified Ltnβ, had no impact on expression from P_{bac}. Thus, either the unmodified Ltnβ pre-pro-peptide or the $ltnA_2$ transcript must be involved in regulation. Results of $ltnA_2$

complementation studies revealed that indeed the transcript alone was sufficient to reduce GFP+ expression levels. While further work is required to assess the role of *ltnA*$_1$ and *ltnA*$_2$, the cumulative results of this study suggests that LtnR and at least *ltnA*$_2$ mRNA contribute to co-ordinating expression from the lacticin 3147 gene cluster, maintaining immunity, biosynthesis and the post-translational modification process in equilibrium.

An examination of the P_{bac} promoter revealed a putative operator site, with a set of three heptanucleotide repeats and a set of three hexanucleotide repeats also overlap the two divergent promoters, P_{imm} and P_{bac}. The location of these is such that they overlap the -35 and-10 regions of P_{bac} and thus suggest that they are potential targets of regulation. Regulation via the presence of inverted repeats and palindromes located in the promoter has been reported in many cases (Roy & Falkow, 1991; Yuan & Wong, 1995). Indeed, with respect to bacteriocin regulation, a pair of inverted repeats are thought to be the binding site of SapR, the response regulator component of the SapRK two-component system, involved in the regulation of expression of sakacin A and its immunity genes from two divergent operons (Axelsson & Holck, 1995). A palindrome and series of inverted repeats have also been implicated in the regulation of a newly discovered and as yet unnamed lantibiotic produced by *Bifidobacterium longum* DJO10A (Lee *et al.*, 2011), while a set of heptanucleotide repeats is also associated with LasX-mediated regulation of lactocin S (Rawlinson *et al.*, 2002). Palindromic sequences (operators) have been identified in *B. subtilis* as the binding sequences of Xre repressor proteins (McDonnell & McConnell, 1994), the family of repressors to which LtnR belongs. One such palindromic sequence has previously been identified in P_{imm} (McAuliffe *et*

al., 2001). However, this putative LtnR binding site within P_{imm} more closely resembles that of the core Xre binding consensus sequence (McDonnell & McConnell, 1994) than the palindrome identified in P_{bac}. PBSX/Xre family transcriptional regulators are more typically repressor proteins than activators (Wood *et al.*, 1990). Hochschild and Dove describe how activation and repression through the same DNA-bound regulator and RNA polymerase may elicit different effects at different promoters (Hochschild & Dove, 1998). In one instance, they refer to a study which reveals that the sequence of the promoter (specifically, the degree of similarity of the −35 region sequence to the consensus) determines the regulatory outcome (Monsalve *et al.*, 1997). In contrast to P_{bac}, the -35 region of P_{imm} is seen to greatly diverge from the lactococcal -35 consensus sequence (Fig. 2) (De Vos & Simons, 1994). This may serve to explain how the same LtnR protein can serve as both an activator and repressor while binding at these adjacently positioned promoters.

The intragenic region encompassing the two promoters of the lactocin S gene cluster is characterised by the presence of 3 repeats, thought to act as a binding recognition site for LasX. The co-located promoters P_{imm} and P_{bac} also possess two distinct 3 repeat motifs and in addition, P_{imm} has an Xre consensus-like operator associated with it. Here, we also identify a palindrome within P_{bac} which may also serve as a recognition and binding site for LtnR. In fact another transcriptional Fur-type regulator, found in *Borrelia burgdorferi*, which classically controls expression of genes involved in iron uptake and iron storage by binding to a Fur-box DNA consensus sequence (Andrews *et al.*, 2003), is seen to bind two distinct consensus motifs similar to that proposed for LtnR (Katona *et al.*, 2004). Fur

proteins have also been associated with the regulation of many other genes in addition to those directly related to iron metabolism. It has been revealed that Fur participates in a wide variety of functions including, for example, shock response, defence against oxygen radicals, chemotaxis, metabolic pathways, bioluminescence, swarming, production of toxins and other virulence factors (for review see (Escolar *et al.*, 1999)). Fur has also been associated with regulation of the bacteriocin colicin V (Chehade & Braun, 1988). LtnR does not share any notable homology with Fur proteins to the degree it does with the Xre family of regulators. However, it is still plausible that it may bind, in a manner similar to the Fur-like proteins, to the two such Fur-like binding sequences identified here. With the identification of a Fur protein within *L. lactis* MG1363 it would be of interest to determine whether it has any involvement in lacticin 3147 regulation under circumstances such as responses to shock or oxidative stress.

On overviewing the various constructs designed and investigated in this study, we can reveal, in accordance with the presence or absence of *ltnR* and *ltnA$_1$A$_2$*, a clear rationale of their intercellular roles. We hypothesise that the dual role of LtnR as both an activator and repressor co-ordinates the biosynthesis and immunity of lacticin 3147, and that *ltnA$_1$* and *ltnA$_2$* play a role in modulating biosynthesis so as to maintain lantibiotic production at a homeostatic level. We propose that the cell initiates lacticin 3147 biosynthesis/immunity by initiating expression from $P_{imm,}$ which in fact was previously described as being constitutive in nature in the absence of the regulatory protein LtnR (McAuliffe *et al.*, 2001) (Fig. 8(a)). This allows production of LtnR and the lacticin 3147 immunity proteins, LtnI, F and E. The immune cell is now prepared for the presence of the bactericidal

lantibiotic; LtnR now binds to P_{imm} and represses its own production and that of the immunity genes (Fig. 8(b)). Concomitantly LtnR binds to P_{bac}, stimulating activation of lacticin 3147 biosynthetic, modification and transport gene expression (Fig. 8(c)). In order to prevent an overload of lacticin 3147 pre-pro-peptide within the cell *ltnA*$_2$ (and perhaps to be determined, *ltnA*$_1$) transcript interacts with LtnR or the P_{bac} promoter and reduces activation to a homeostatic level (Fig. 8(d)). This allows the cell sufficient time to complete the essential post-translational modifications characteristic of a lantibiotic. As modified Ltnα and Ltnβ are not involved in the regulation of P_{bac}, we can conclude that there is a correlated effort between fluctuating levels of intracellular LtnR and *ltnA*$_1$*A*$_2$ transcript with P_{imm} and P_{bac} regulation. This results in a steady state of lacticin 3147 immunity, biosynthesis and modification that ultimately lead to export of fully modified Ltnα and Ltnβ from the cell. While the nuances of the contribution of *ltnA*$_1$ need to be finalised, the relative contribution of LtnR and *ltnA*$_2$ have revealed that P_{bac} is a regulated promoter, rather than a constitutive one. With further analysis it may be possible to manipulate this system in order to maximise lacticin 3147 production.

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Tables and Figures

Table 1. Strains used in this study

***L. lactis* MG1363 Strain**	**Relevant characteristics**	**Reference**
MG1363	*L. lactis* devoid of lacticin 3147 operon (Lac^-)	(Gasson, 1983)
pNZ25*gfp+*	Lac^-;P_{CP25} expression of *gfp+*	This study
pNZ32*gfp+*	Lac^-;P_{32} expression of *gfp+*	This study
pNZ44*gfp+*	Lac^-;P_{44} expression of *gfp+*	This study
pNZbac*gfp+*	Lac^-;P_{bac} expression of *gfp+*	This study
pMRC01.pNZ25*gfp+*	Lac^+; P_{CP25} expression of *gfp+*	This study
pMRC01.pNZ32*gfp+*	Lac^+; P_{32} expression of *gfp+*	This study
pMRC01.pNZ44*gfp+*	Lac^+; P_{44} expression of *gfp+*	This study
pMRC01.pNZbac*gfp+*	Lac^+; P_{bac} expression of *gfp+*	This study
pMRC01 ΔA_1A_2	pMRC01 Δ*ltnA*$_1$*A*$_2$	This study
pMRC01 ΔA_1	pMRC01 Δ*ltnA*$_1$	(Cotter *et al.*, 2003)
pMRC01 Δ*R*	pMRC01 Δ*ltnR*	(Deegan, 2007)
pMRC01 ΔM_1	pMRC01 Δ*ltnM*$_1$	(Field *et al.*, unpublished)
pMRC01 ΔM_2	pMRC01 Δ*ltnM*$_2$	(Field *et al.*, unpublished)
pMRC01 Δ*J*	pMRC01 Δ*ltnJ*	(Cotter *et al.*, 2005)
pMRC01 ΔA_1A_2.pNZ44*gfp+*	$Lac^{\Delta ltnA1A2}$; P44 expression of *gfp+*	This study
pMRC01 ΔA_1A_2.pNZbac*gfp+*	$Lac^{\Delta ltnA1A2}$; Pbac expression of *gfp+*	This study
pMRC01 ΔA_1.pNZ44*gfp+*	$Lac^{\Delta ltnA1}$; P44 expression of *gfp+*	This study
pMRC01 ΔA_1.pNZbac*gfp+*	$Lac^{\Delta ltnA1}$; Pbac expression of *gfp+*	This study
pMRC01 Δ*R*.pNZ44*gfp+*	$Lac^{\Delta ltnR}$; P44 expression of *gfp+*	This study
pMRC01 Δ*R*.pNZbac*gfp+*	$Lac^{\Delta ltnR}$; Pbac expression of *gfp+*	This study
pMRC01 ΔM_1.pNZ44*gfp+*	$Lac^{\Delta ltnM1}$; P44 expression of *gfp+*	This study
pMRC01 ΔM_1.pNZbac*gfp+*	$Lac^{\Delta ltnM1}$; Pbac expression of *gfp+*	This study
pMRC01 ΔM_2.pNZ44*gfp+*	$Lac^{\Delta ltnM2}$; P44 expression of *gfp+*	This study
pMRC01 ΔM_2.pNZbac*gfp+*	$Lac^{\Delta ltnM2}$; Pbac expression of *gfp+*	This study
pMRC01 ΔA_1A_2.pNZbac*gfp+*.pNZEm$P_{44}A_2$	pMRC01ΔA_1A_2.pNZbac*gfp+*;P_{44} *ltnA*$_2$ expression	This study
pMRC01ΔA_1A_2.pNZbac*gfp+*.pNZEm$P_{44}A_2^{pp-}$	pMRC01ΔA_1A_2.pNZbac*gfp+*;P_{44} *ltnA*$_2^{PP-}$ expression	This study
pMRC01 ΔA_1A_2.pNZbac*gfp+*.pNZEmP_{44}*R*	pMRC01 ΔA_1A_2.pNZbac*gfp+*;P_{44} *ltnR* expression	This study
Other Strains		
E. coli Top10	Intermediate cloning host	Invitrogen
E. coli EC101	Rep^+ cloning host	(Law *et al.*, 1995)

Table 2. Primers used in this study

Oligo/Primer	Sequence
PCP25 F	gatctCTTTGGCAGTTTATTCTTGACATGTAGTGAGGGGGCTGGTATAATCACATAAGGAGGATATATc
PCP25 R	catggATATATCCTCCTTATGTGATTATACCAGCCCCCTCACTACATGTCAAGAATAAACTGCCAAAGa
P32 F	gatctTTTGTGAGCTTGGACTAGAAAAAAACTTCACAAAATGCTATACTAGGTAGGTAAAAAAATATT CGGAGGAATTTTGAAc
P32 R	catggTTCAAAATTCCTCCGAATATTTTTTTACCTACCTAGTATAGCATTTTGTGAAGTTTTTTTCTAGTC CAAGCTCACAAAa
Pbac F	GAAGATCTTATATACAGAGTTACTAATAGAA
Pbac R	TGCCATGGTTTTTTGTTCATGATA
*ltnA1A2*soeA	ATCTGCAGAATTTAAAAAATCACA
*ltnA1A2*soeB	AGATACTTCTTCCAACCA
*ltnA1A2*soeC	TGGTTGGAAGAAGTATCCTGAGTCTCATGGAGGAA
*ltnA1A2*soeD	ACGAATTCAGGGATATTCCTCAT
ltnR F	CTGCAGCATAGATAGGAGGAAAATGTTTG
ltnR R	GGTCTAGAAAAGGTATTGATATTTTCATTC
ltnA2 F	AACTGCAGTTAATAACAAATTTTTAATTAATTAAG
ltnA2 R	GCTCTAGATTACTCCTAATTAACAAGCACGTG
ltnA2^{pp-} F	GCTTTACGATGTAAGAAAAAAATATGAAAAAGAATGACA
ltnA2^{pp-} R	TGTCATTCTTTTTCATATTTTTTTCTTACATCGTAAAGC
P44 For	GGGCATGCGTTAGTTGAAGAAGGTTTTTATATTAC

Table 3. Plasmids used in this study

Plasmids	Relevant characteristics	Reference
pNZ25	Cm^R; high-copy vector;P_{CP25} promoter	This study
pNZ32	Cm^R; high-copy vector;P_{32} promoter	This study
pNZ44	Cm^R; high-copy vector;P_{44} promoter	(McGrath *et al.*, 2001)
pNZbac	Cm^R; high-copy vector;P_{bac} promoter	This study
pNZEm	Em^R; high-copy vector;P_{nis} promoter	Margolles et al., unpublished
pNZ44*ltnA*$_2$	pNZ44 expression of *ltnA*$_2$	This study
pNZ44*ltnR*	pNZ44 expression of *ltnR*	This study
pNZEmP_{44}*A*$_2$	Em^R; P_{44} expression of *ltnA*$_2$	This study
pNZEmP_{44}*R*	Em^R; P_{44} expression of *ltnR*	This study
pNZEmP_{44}*A2*$^{PP-}$	Em^R; P_{44} expression of untranslated *ltnA*$_2$ pre-pro-peptide	This study
pNZ8150*gfp+*	Source of *gfp+* gene	Field *et al*., unpublished
pVE6007	CmR; temperature-sensitive	(Maguin *et al.*, 1992)
pORI280	$RepA^-$, $LacZ^+$	(Leenhouts *et al.*, 1996)
pORI280*ltnA*$_1$*A*$_2$*soe*	pORI280 containing spliced *ltn*ISOEAD fragment	This study

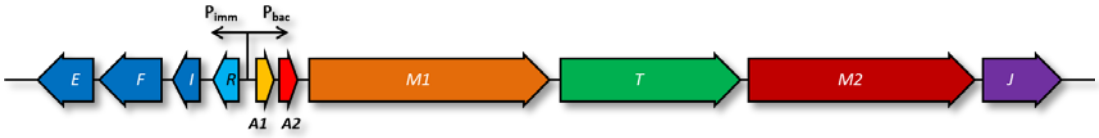

Fig. 1. The 12.6kb lacticin 3147 gene cluster. Two promoters P_{imm} and P_{bac} are located at the interface of the two divergent operons: *ltnRIFE* and *ltn*$A_1A_2M_1TM_2J$, which are responsible for the biosynthesis, immunity and export of the final lantibiotic.

Fig. 2. Overview of the *ltnR-A*$_1$ intragenic region. The -10 and -35 elements of the promoters P_{imm} and P_{bac} are indicated (and underlined). Red triangles indicate the transcriptional start sites. The translational start sites for LtnR and Ltnα pre-pro-peptide are shown indicated with a black arrow and the predicted corresponding ribosome binding sites (RBS). Two sets of three direct repeats are displayed boxed in yellow and blue. An inverted repeat in P_{imm} and a palindrome present in P_{bac} are boxed in red with inverted arrows.

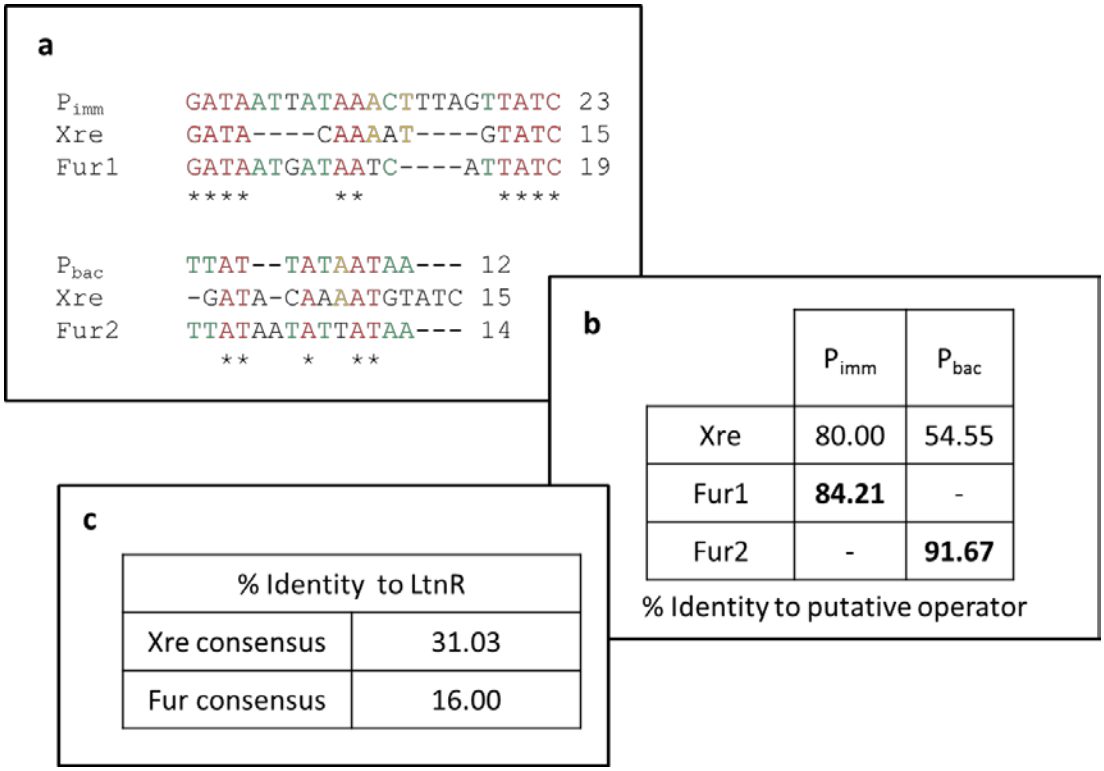

Fig. 3. The putative operator sites identified within P_{imm} and P_{bac} are aligned with consensus sequence homologues (a). These include the Xre binding consensus sequence and two sequences termed here as Fur1 and Fur2, to which a Fur (ferric uptake regulation protein) homologue binds. Nucleotides in red are common across all operators, those in green indicated identity between P_{imm}/P_{bac} and the Fur operators and yellow identifies those bases in common with the Xre consensus binding sequence. The percentage identity of sequences to the P_{imm} and P_{bac} putative operators has also been included (b). The percentage to which LtnR is identical to Xre and Fur protein consensus sequences is also presented (c).

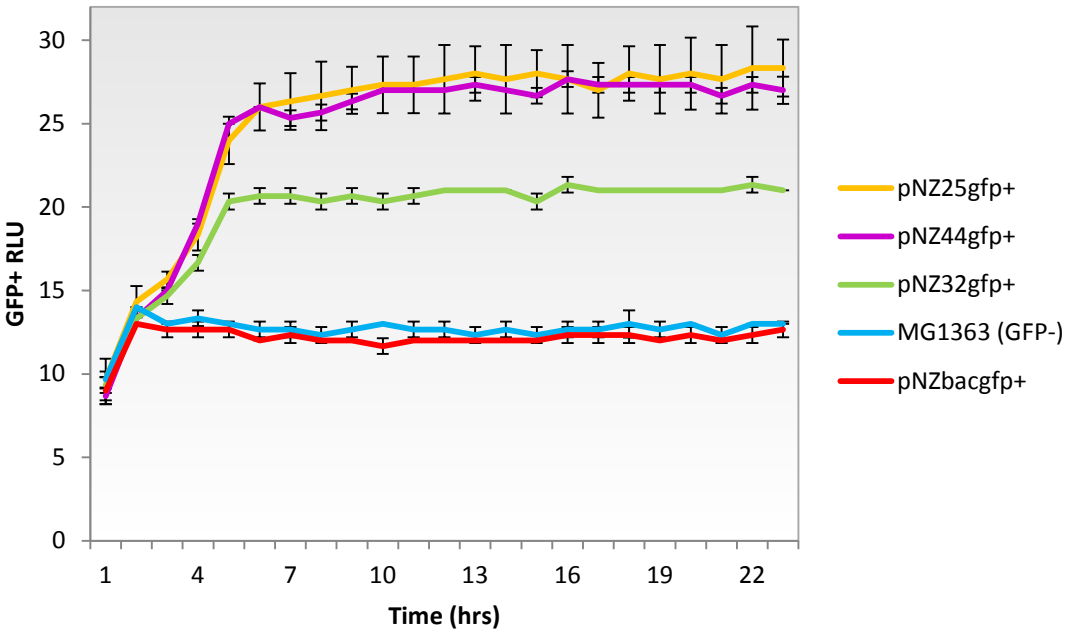

Fig. 4. Expression of *gfp+* in *L. lactis* MG1363 via a variety of known constitutive promoters i.e. P_{25}, P_{32} and P_{44}, present in or replacing P_{44} in the vector pNZ44. A corresponding plasmid pNZbac*gfp+* harbouring the lacticin 3147 associated promoter P_{bac}, is compared to these constitutive promoters and it is revealed that its level of expression is comparable to that of a negative control *L. lactis* MG1363 strain devoid of the *gfp+* gene.

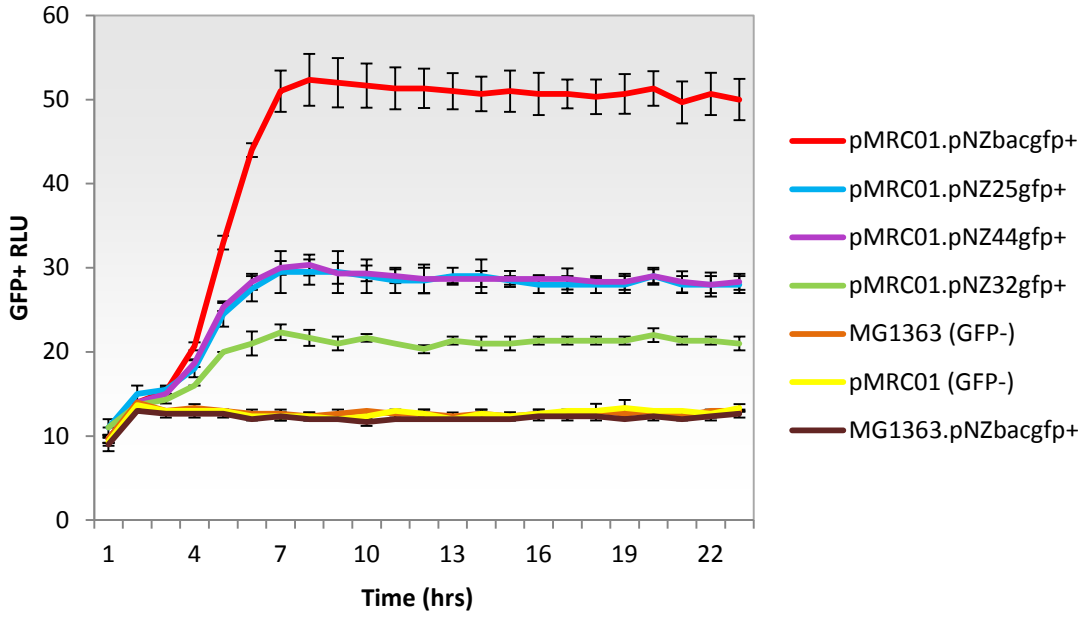

Fig. 5. Expression of *gfp+* via the plasmids pNZ25, pNZ32, pNZ44 and pNZbac in *L. lactis* MG1363.pMRC01. The strain *L. lactis* MG1363.pNZbac*gfp+* was included for comparative purposes, as were two control strain *L. lactis* MG1363 and *L. lactis* MG1363.pMRC01 devoid of the *gfp+* gene.

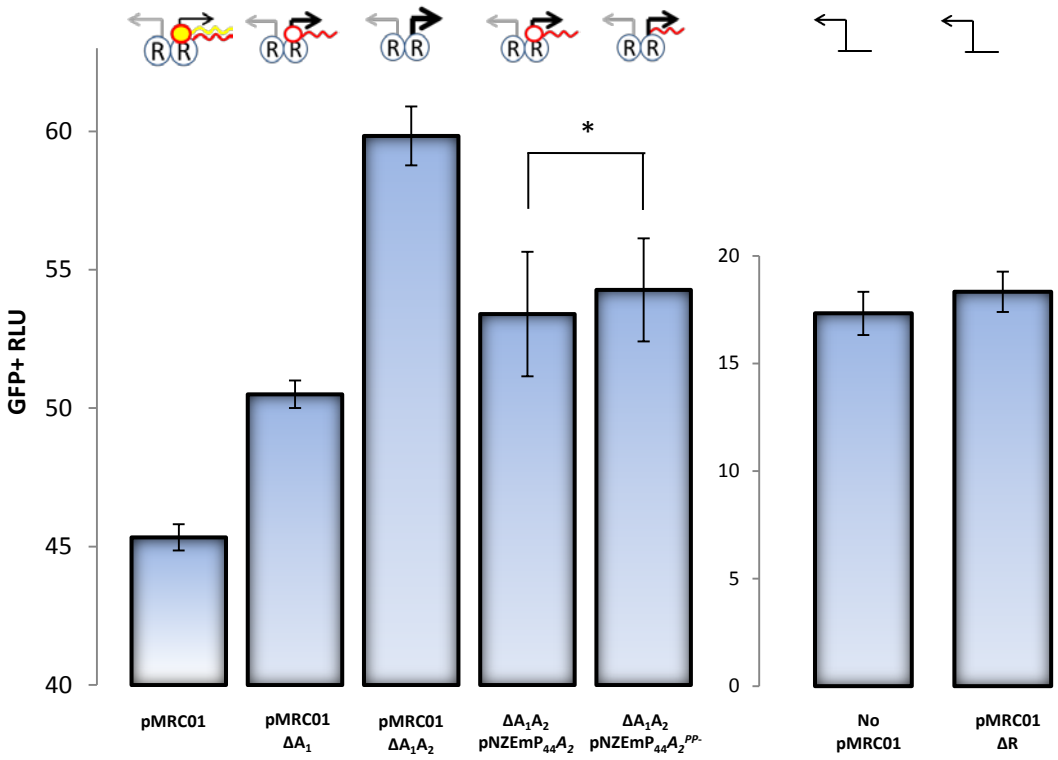

Fig. 6. An overview of GFP+ production by pNZbac*gfp+* in the presence or absence of pMRC01 and derivatives lacking *ltnA*$_1$, *ltnA*$_1$*A*$_2$ or *ltnR* after 18hrs in *L. lactis* MG1363. Complementation of *ltnA*$_2$ in the Δ*ltnA*$_1$*A*$_2$ background gene, using an intact (via pNZEmP$_{44}$*A*$_2$) or mutated *ltnA*$_2$ (via pNZEmP$_{44}$*A*$_2^{PP-}$) was also investigated (*Student's *t*-test $P < 0.4$). The presence or absence of *ltnA*$_1$ and its transcript (indicated by a yellow circle and wavy line), *ltnA*$_2$ and its transcript (indicated by a red circle and wavy line) and *ltnR* (indicated by the circled letter R) are displayed above, along with a representation of the observed P_{bac} strength, where a bold arrow implies increased activity and no arrow indicates lack of expression.

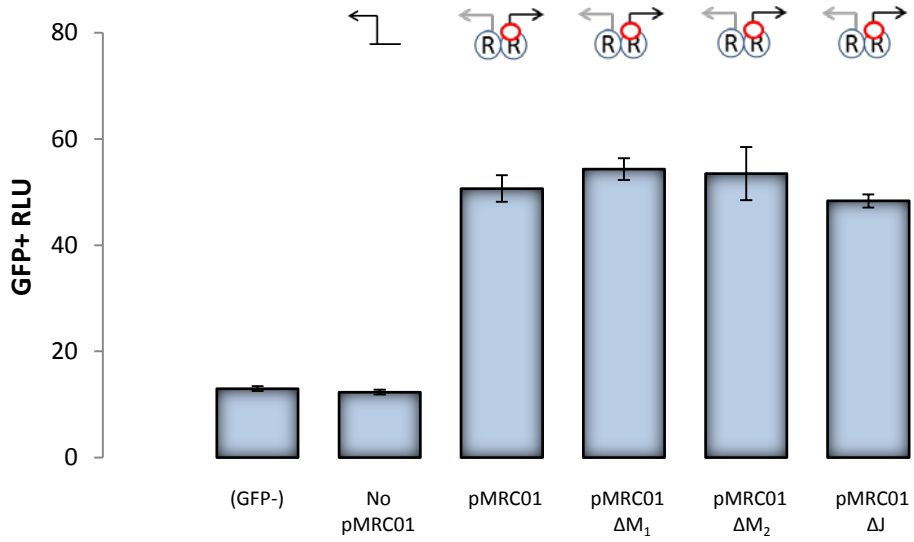

Fig. 7. An overview of GFP+ expression by pNZbac*gfp+* in the presence of pMRC01 and the various deletions therein after 18 hrs in *L. lactis* MG1363. The presence or absence of LtnR (circled R) and *ltnA*$_1$*A*$_2$ (indicated by a red circle) are displayed above along with a representation of the observed P_{bac} activity, where no arrow indicates no expression.

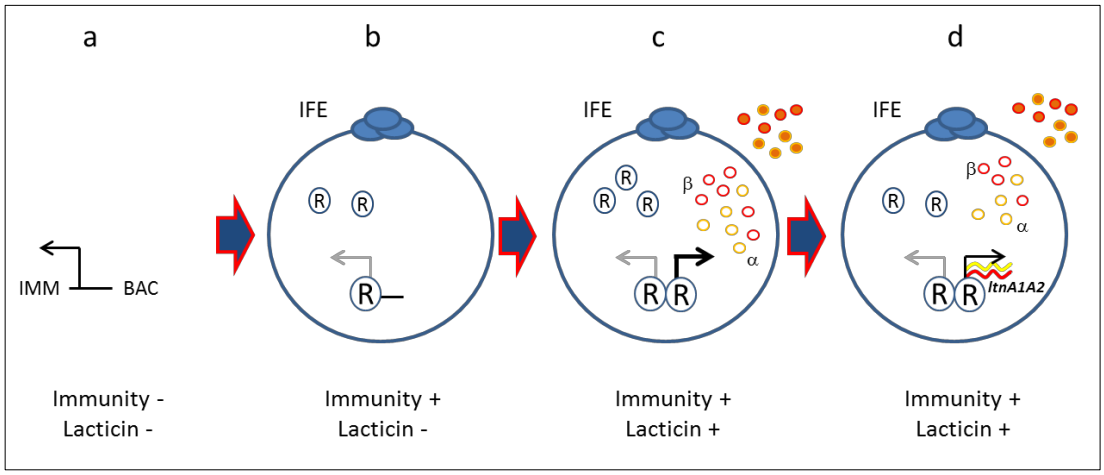

Fig. 8. Hypothesis of internal LtnR and ltnA$_2$ fluctuations and their impact on promoter regulation. In the absence of lacticin 3147 the cell first switches on immunity (a). LtnIFE confer immunity and ltnR suppresses LtnRIFE production (b). LtnR also activates lacticin 3147 production (c). The presence of *ltnA$_2$* and perhaps also ltnA$_1$ (to be confirmed) reduces expression of lacticin 3147 genes to protect the cell against overproduction and allows time for modification of the lantibiotic pre-pro-peptides (d).

Thesis Summary

Lantibiotics are ribosomally synthesised antimicrobial peptides produced by many Gram positive bacteria. They are distinguished by the presence of the unusual amino acids lanthionine and methyl-lanthionine, which are responsible for the characteristic bridges observed in these peptides. Lacticin 3147 and nisin are two well-studied lantibiotics produced by *Lactococcus lactis.* They display activity against a wide spectrum of Gram positive microorganisms at concentrations in the nanomolar range. The genes encoding the two peptide antimicrobial lacticin 3147 are located on two divergent operons on the plasmid pMRC01, whereas the genes responsible for nisin and its natural variants are found on a single operon in several strains of *L. lactis* and *S uberis*. In order to protect themselves from the bactericidal agent they produce, lantibiotics have to employ a resistance or, more accurately, a self-immunity mechanism. In the case of lacticin 3147 and nisin, this is achieved through the action of a dedicated ABC transporter (LanFE(G)) and a separate immunity specific protein (LanI). Given the potency of lacticin 3147 and nisin, it would seem that these antimicrobials represent genuine alternatives to conventional antibiotics. Obstacles that could prevent the commercialisation of lantibiotics include the fact that the potential mechanisms through which resistant strains could emerge have not been extensively investigated as well as the relatively small concentrations of lantibiotic generated by some producers.

Chapter I of this thesis reviews the lantibiotic resistance mechanisms employed by bacteria. Many of these are innate, rather than acquired, mechanisms

of resistance that protect the cell wall and cell membrane of strains under attack from lantibiotics and other antimicrobials with a similar mode of action. Naturally acquired resistance seems quite rare but those that have been identified are also discussed.

Chapter II addresses the possibility of overcoming limitations in producing large quantities of lantibiotic by combining the peptides with other antimicrobials. This is somewhat similar to the hurdle technology employed in food protection in that bacteria are generally more susceptible to the combined effect of more than one antibacterial agent. Ultimately, this study highlights the fact that polymyxin B/polymyxin E (colistin) and lacticin 3147 function synergistically. This was apparent from checkerboard assays that were performed against a number of Gram positive and Gram negative species. The resultant fractional inhibitory concentration (FIC) index values established that, while partial synergy was detected against Gram positive targets, synergy was obvious against Gram negative species, including *Cronobacter* and *E. coli*. Combining lacticin 3147 with low levels of a polymyxin could thus provide a means of broadening the target specificity of the lantibiotic, while reducing the concentrations of the individual antimicrobials. With the potential of lacticin 3147 as an anti-mastitic agent having already been established with respect to Gram positive species, this application highlights a means of also targeting Gram negative species, such as *E. coli,* that are also associated with the disease.

Chapter III describes an in-depth investigation of the lacticin 3147 immunity peptide LtnI. Characterisation of structure-function relationships in LtnI was

achieved through the creation of a series of fusion peptides and random and site directed mutagenesis of *ltnI*. The results are consistent with *in silico* predictions that LtnI is a transmembrane protein and reveals the presence of regions, such as those between amino acid 20-27 and 76-83, which are essential for LtnI function. Finally, as a consequence of the screening of a bank of 28,000 strains producing LtnI variants, we identified one (LtnI I81V) that provides enhanced protection. To our knowledge, this is the first report of a lantibiotic immunity protein with enhanced functionality. This enhanced knowledge may be of benefit when overcoming hurdles relating to the discovery of derivatives with enhanced activity, or increasing levels of lantibiotic production, which might otherwise lead to the growth of the producing strain being inhibited.

Chapter IV describes the presence of nisin U immunity gene homologues in *Streptococcus infantarius* subsp*. infantarius* BAA-102. Nisin U is a member of the extended nisin family of lantibiotics. Heterologous expression of the BAA-102 *spiFEG* genes (homologues of the nisin U immunity specific ABC transporter *nsuFEG*) in *L. lactis* subsp*. cremoris* HP conferred protection to nisin U and other members of the nisin family. This is an example of immune mimicry, a phenomenon in which non-lantibiotic producing strains express functional homologues of lantibiotic immunity systems. This study established that this mechanism of resistance, only recently identified for lacticin 3147, also occurs with respect to nisin. Immune mimicry has the potential to pose a real problem if lantibiotics are to be employed in a clinical setting, and so a greater understanding of this

phenomenon may allow the identification of strategies to prevent such resistance from developing.

Chapter V investigates the regulation of lacticin 3147 production. It was formerly believed that expression of the lacticin 3147 biosynthetic genes was constitutive, even though most lantibiotic systems are regulated. We demonstrated that, in fact, the regulatory protein LtnR, previously associated with repression of itself and the immunity genes, also acts as an activator of the P_{bac} promoter and plays a role in the expression of the biosynthetic, modification and transport genes. Different levels of expression are observed depending on the absence or presence of specific genes of the lacticin 3147 gene cluster. While *ltnR* is required to obtain the intermediate/homeostasis levels of expression associated with the un-mutated pMRC01 plasmid, the absence of *ltnA*$_1$ and *ltnA*$_2$ results in a greatly heightened level of expression. As the modification genes *ltnM*$_1$ and *ltnM*$_2$ are not involved in this enhanced expression, it would seem that either the unmodified pre-propeptides or gene transcripts act as the regulatory elements. The expression of different forms of *ltnA*$_2$ in pMRC01Δ*A*$_1$*A*$_2$, resulting in translated and untranslated versions of the *ltnA*$_2$ gene, revealed that the transcribed but untranslated gene also complemented this phenomenon. While the contribution of *ltnA*$_1$ has yet to be defined, these results do suggest a role for *ltnA*$_2$ and *ltnR* in repression and activation of P_{bac}, respectively. It would seem that these mechanisms are employed such that a balance between production and immunity can be maintained, whereby the expression, processing and export of the *ltnA*$_1$ and *ltnA*$_2$ gene products are regulated to ensure that the cell is not overloaded. Investigating the processes

involved in regulating the P_{bac} promoter may facilitate the production of greater levels of lacticin 3147 and, in such a situation, the improved immunity provided by the enhanced version of LtnI discovered here may be useful.

Overall, this thesis makes several contributions to our knowledge of lantibiotics, particularly in the area of potential applications in clinical settings. The major limitations in developing clinical applications for lantibiotics include production levels, efficacy against selected pathogens and the issue of resistance development. All three areas are addressed in this thesis. A thorough understanding of regulatory controls (Chapter V) and associated immunity mechanisms (Chapter III) may be useful in addressing production levels. Alternatively, the need for enhanced production could be avoided through the use of antimicrobial combinations (Chapter II). In addition, an awareness of the threats associated with the emergence of resistance through immune mimicry can allow researchers to develop strategies to prevent this phenomenon from leading to the dissemination of lantibiotic resistance (Chapter IV). Given the dearth of novel antibiotics and the continuing threat posed by antibiotic resistance development, lantibiotics (and other bacteriocins) present a realistic alternative, worthy of further investigation. This thesis represents a significant advance in our knowledge, and brings the likely deployment of lantibiotics in the clinic a little bit closer.

Appendix

Saturation mutagenesis of selected residues of the α-peptide of the lantibiotic lacticin 3147 yields a derivative with enhanced antimicrobial activity.

Des Field, Evelyn M. Molloy, Catalin Iancu, **Lorraine A. Draper**, Paula M. O' Connor, Paul D. Cotter, Colin Hill and R. Paul Ross.

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During my Ph.D. I collaborated with the authors of the following study and performed all of the minimum inhibitory concentration assays published within.

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Saturation mutagenesis of selected residues of the α-peptide of the lantibiotic lacticin 3147 yields a derivative with enhanced antimicrobial activity

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Summary

The lantibiotic lacticin 3147 consists of two ribosomally synthesized and post-translationally modified antimicrobial peptides, Ltnα and Ltnβ, which act synergistically against a wide range of Gram-positive microorganisms. We performed saturation mutagenesis of specific residues of Ltnα to determine their functional importance. The results establish that Ltnα is more tolerant to change than previously suggested by alanine scanning mutagenesis. One substitution, LtnαH23S, was identified which improved the specific activity of lacticin 3147 against one pathogenic strain, *Staphylococcus aureus* NCDO1499. This represents the first occasion upon which the activity of a two peptide lantibiotic has been enhanced through bioengineering.

Introduction

Lantibiotics [lanthionine-containing antibiotics (Schnell *et al*., 1988)] are a member of the family of antimicrobial peptides termed bacteriocins (Willey and van der Donk, 2007; Bierbaum and Sahl, 2009). In lantibiotics, dehydroalanine (Dha) and dehydrobutyrine (Dhb) residues are formed through the dehydration of serine and threonine respectively. The eponymous lanthionine (Lan) and β-methyllanthionine (MeLan) residues are enzymatically introduced when a covalent (thio-ether) bridge forms between a neighbouring cysteine and one of these unsaturated amino acids. These post-translational modifications confer structure and function to the previously inactive precursor peptide. Lantibiotics have been the subject of intensive studies as a result of their broad target range, potent activity and their potential as safe, natural food additives or as chemotherapeutic agents (Cotter *et al*., 2005b; Galvez *et al*., 2007; Piper *et al*., 2009a).

Lacticin 3147 is a lantibiotic produced by the food-grade bacterium *Lactococcus lactis* spp. *lactis* DPC3147. It is active against a variety of clinically significant Gram-positive organisms, including methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus* strains (VRE) and penicillin-resistant *Pneumococcus*, in addition to foodborne pathogens such as *Listeria monocytogenes* and *Bacillus cereus* (Lawton *et al*., 2007b; Piper *et al*., 2009b; Carroll *et al*., 2010). Lacticin 3147 is a two peptide lantibiotic and thus both peptides, Ltnα and Ltnβ, are required for full antimicrobial activity (Wiedemann *et al*., 2006). Lacticin 3147 is active at single nanomolar concentrations through a dual mechanism in which Ltnα first interacts with the cell wall precursor lipid II to inhibit peptidoglycan synthesis. It is proposed that the resulting Ltnα : lipid II complex then interacts with Ltnβ to facilitate pore-formation (Wiedemann *et al*., 2006). An unusual feature of lacticin 3147 is the presence of three D-alanines (D-Ala) that are enzymatically derived from ribosomally introduced L-serines (Cotter *et al*., 2005c). Lacticin 3147 is one of only two examples of prokaryotic gene-encoded peptides in which such modified residues have been identified (Skaugen *et al*., 1994).

Two peptide lantibiotics remain relatively uncommon. To date only seven other lacticin 3147-like antimicrobials have been characterized; staphylococcin C55 (Navaratna *et al*., 1999), plantaricin W (Holo *et al*., 2001), Smb (Yonezawa and Kuramitsu, 2005), BHT-A (Hyink *et al*., 2005), haloduracin (McClerren *et al*., 2006; Lawton *et al*.,

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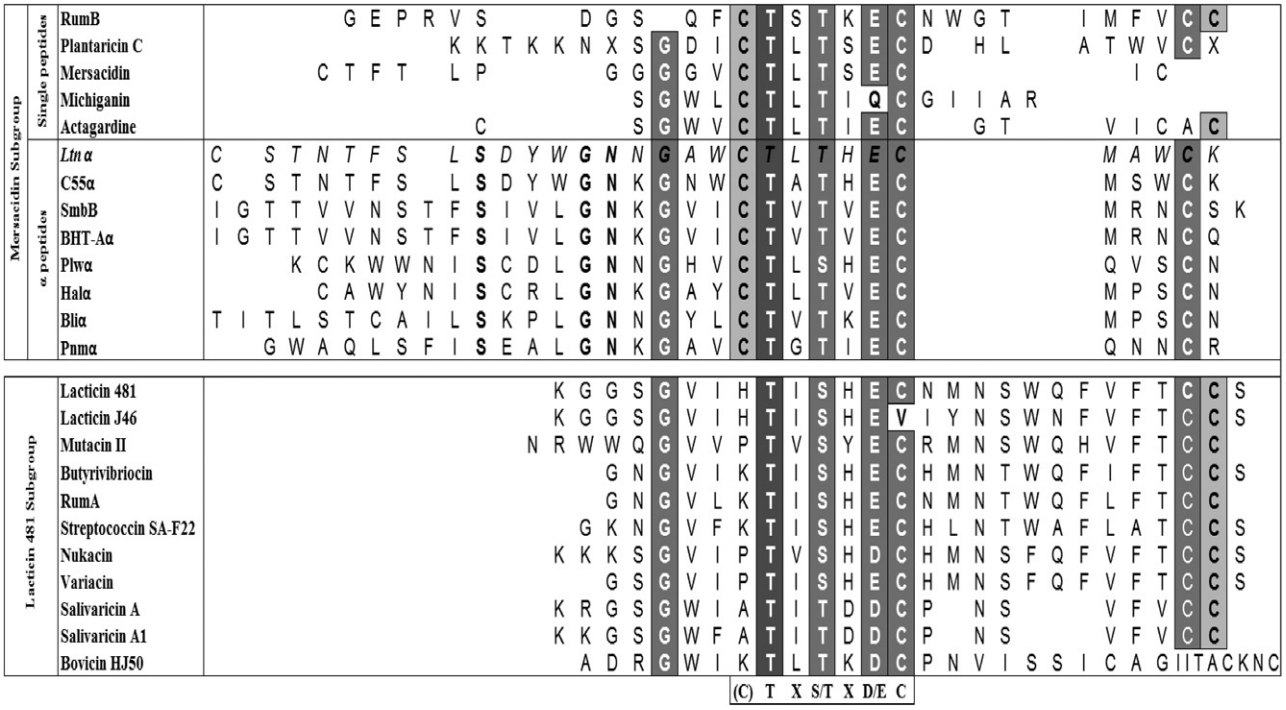

Fig. 1. Alignment of the unmodified sequence of structural peptides of the mersacidin-like lantibiotic subgroup (Mersacidin Subgroup), which includes single-peptide members and the closely related α peptides of two peptide lantibiotics, and the lacticin 481-like lantibiotic subgroup (Lacticin 481 Subgroup). The sequence of Ltnα is italicized. Dark grey boxes indicate residues that are fully conserved across both subgroups, medium grey indicates residues that are highly conserved across both subgroups, while light grey indicates partially conserved residues. The putative lipid II binding motif conserved among the mersacidin and lacticin 481 lantibiotic subgroups (with the exception of (C), which is not found in the lacticin 481 subgroup) is given at the bottom of the alignment.

2007a), lichenicidin (Begley *et al*., 2009; Dischinger *et al*., 2009) and a predicted pneumococcin (Majchrzykiewicz *et al*., 2010). The α peptides of the two peptide lantibiotics cluster with the mersacidin-like peptides (Cotter *et al*., 2005a), with the exception of the enterococcal cytolysin (Cox *et al*., 2005). Despite their different overall structures, alignment of the mersacidin-like peptides with those from the more distantly related lacticin 481 subgroup (Fig. 1) reveals the presence of a conserved stretch of amino acids, (C)TXS/TXD/EC, a motif encompassing a conserved ring that has been suggested to comprise the core site for lipid II binding (Breukink and de Kruijff, 2006; Cotter *et al*., 2006). Significantly, this region represents the first point of contact between mersacidin and the bacterial cell and is thus believed to contain core amino acids that are essential with regard to the functionality of these peptides (Hsu *et al*., 2003).

Lantibiotics represent one possible solution to combat the emergence of multi-drug resistant strains (Boucher *et al*., 2009). The fact that lantibiotics are gene-encoded and ribosomally synthesized has facilitated the development of versatile expression systems capable of producing novel derivatives. This approach has generated information about the structure-function relationships of lantibiotics, as well as allowing one to screen for the enhancement of chemical and antimicrobial properties (Field *et al*., 2010a). A description of the role of each individual amino acid or domain will be required for a rational approach to design new, improved lantibiotics. To this end, an attempt has been made to predict the identity of essential and variable residues across both peptides of lacticin 3147 using alanine scanning mutagenesis (Cotter *et al*., 2006). However, the consequences of changing a residue to alanine do not always reflect the overall tolerance/intolerance of a specific residue to change. To address this issue, saturation mutagenesis of the lipid II-binding component of lacticin 3147, i.e. Ltnα, was performed. We focused on the residues of Ltnα that are not involved in bridge formation and while this approach frequently confirmed the relevance of the alanine scanning approach with regard to the importance of specific amino acids, a limited number of substitutions were found to be tolerated at positions previously designated as being intolerant of change. We also noted that amino acids highly conserved across related lantibiotics are not necessarily inviolate. More significantly, we identified the first derivative of a two peptide lantibiotic with increased potency against a strain of clinical significance.

Results and discussion

Saturation mutagenesis was performed using a PCR-based approach and a two-plasmid expression system was subsequently applied in generation of banks of Ltnα mutants (Field *et al*., 2007), with a particular focus on mutants that retained at least some bioactivity against the sensitive indicator strain *L. lactis* HP (Table 1). As the aim of the current study was to confirm whether individual residues are tolerant or intolerant of change, no attempt was made to distinguish between mutations that impact on production and those that impact on specific activity.

Targeting of 'essential' residues in Ltnα for site-saturation mutagenesis

The conversion of a number of Ltnα residues to alanine resulted in the abolition of bioactivity (Cotter *et al*., 2006); as such, these residues were designated as being 'essential' for bioactivity of lacticin 3147. These include residues proposed to be involved in the interaction with Ltnβ (F6, S7, W12, N14), putative lipid II binding residues (L21, E24) and two tryptophans (W18 and W28). Despite not being conserved (Fig. 1), the replacement of F6 and W12 with alanine was previously found to eliminate bioactivity (Cotter *et al*., 2006). Here saturation mutagenesis established that conservative substitutions are tolerated at position 12 (Fig. 2), with bioactivity decreasing relative to the size of the newly incorporated residue (Trp > Tyr > Phe; Table 1). However, a critical role was confirmed for F6 with respect to bioactivity (Cotter *et al*., 2006) (Table 1). Based on previous observations (Jing *et al*., 2003; Sanderson and Whelan, 2004), there is a likelihood that aromatic amino acids in membrane-acting peptides such as these are likely to be situated at the lipid-water interface and promote hydrophobic interaction with the cytoplasmic membrane. Thus, replacing the native residue with any amino acid other than another aromatic residue could be expected to have a detrimental effect on antimicrobial activity (Cotter *et al*., 2006).

Position S7 is subject to a two-step post-translational modification to form D-alanine (Cotter *et al*., 2005c). Despite the natural presence of an alanine at the corresponding location in Bliα and the fact that only Ltnα, and potentially Sacα (Suda *et al*., 2011), possess a D-alanine at this location (Fig. 1), an S7A mutant was previously found to be inactive (Cotter *et al*., 2005c; Cotter *et al*., 2006). Here we confirm the negative consequences of such a S7A change and note that many other substitutions also result in the elimination of bioactivity. However, in line with previous investigations, S7T (dehydrated to Dhb, data not shown) and S7G substitutions (Cotter *et al*., 2005c) were both found to result in active mutants (Table 1), confirming a limited tolerance to change (Fig. 2).

The previously generated N14A mutant lacked bioactivity (Cotter *et al*., 2006), in accordance with the complete conservation of N14 among lantibiotic α peptides (Fig. 1). This suggested a pivotal role for this residue in the synergistic interaction between both component peptides (Cotter *et al*., 2006). However, it is now apparent that some substitutions are tolerated to some degree, including replacements with positively charged residues arginine or lysine (Table 1).

Despite the variability of L21 across the mersacidin and lacticin 481 subgroups and the presence of alanine at the corresponding location in the closely related C55α (Fig. 1), a LtnαL21A mutant was previously found to be inactive (Cotter *et al*., 2006; O'Connor *et al*., 2007). Thus, this position was previously designated as being essential. However, site-saturation of L21 found that two

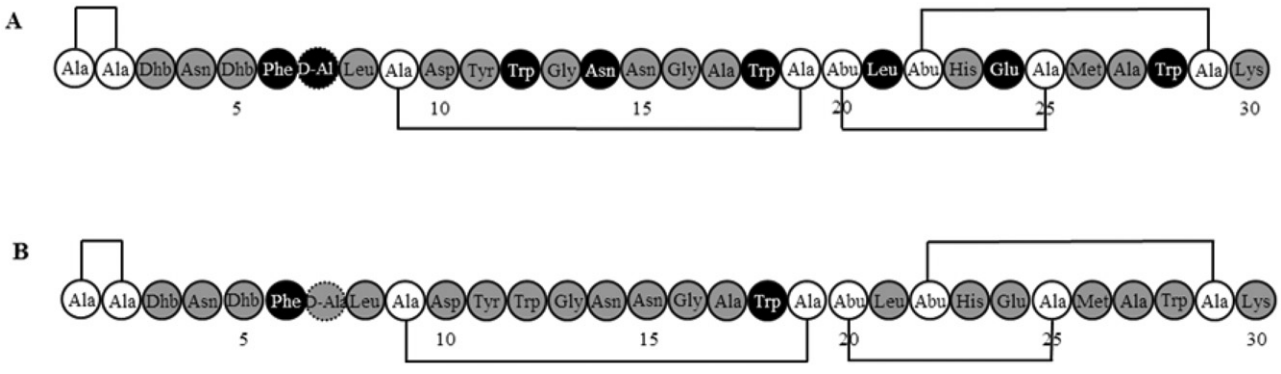

Fig. 2. Tolerance of residues of Ltnα to change as determined by (A) alanine scanning, with the assumption that lack of bioactivity on substitution with alanine (or glycine in the case of a native alanine) indicates an immutable residue, while retention of bioactivity suggests that other residues could be tolerated at the specific position, and (B) saturation mutagenesis, with those that retain bioactivity only when the native residue is present designated as immutable residues, and those retaining bioactivity on substitution with one or more other residues classified as tolerant to change. Grey circles indicate tolerant positions while black circles indicate immutable positions. White circles represent residues involved in bridge formation, which were not targeted in this study.

Table 1. Bioactivity of *L. lactis* MG1363 pOM44 pDF02 derivatives producing mutant Ltnα peptides as determined by triplicate deferred antagonism assays against the sensitive indicator strain *Lactococcus lactis* spp. *cremoris* HP.

		Ltnα position																										
	Amino acid	T3 (Dhb)	N4	T5 (Dhb)	F6	57 (D-Ala)	L8	D10 (–ve	Y11	W12	G13	N14	N15	G16	A17	W18	L21	H23 (+ve)	E24 (–ve)	M26	A27	W28	K30 (+ve)
Hydrophobic	A	0	+	+	0	0	0	0	+	0	++	0	+	+	++	0	0	+	0	0	++	×	+
	G	0	+	×	0	+	0	+	0	×	++	0	×	++	+	×	×	+	0	0	+	0	+
	C	×	×	×	0	×	×	×	0	×	×	×	×	0	×	×	×	+	×	×	×	0	×
	V	0	+	+	×	0	×	0	+	×	×	×	+	×	++	×	++	+	×	+	+	×	+
	P	0	0	×	0	0	×	0	0	×	0	0	0	×	0	0	×	0	0	0	0	×	+
	L	×	×	+	×	×	++	0	×	0	×	+	0	0	×	0	++	+	×	+	+	0	+
	I	0	×	×	×	×	0	0	×	×	×	×	0	×	×	×	×	×	×	+	×	×	×
	M	+	×	×	×	×	0	0	++	×	+	×	+	×	×	×	++	×	0	++	+	×	×
	F	×	0	0	++	×	×	×	0	+	×	×	×	0	×	×	0	×	×	×	+	×	×
	W	×	×	×	×	0	×	×	×	++	×	×	0	×	×	++	×	+	0	+	++	0	++	×
Hydrophilic: neutral	N	+	++	×	0	0	×	×	0	0	++	++	++	×	×	×	0	×	0	++	++	×	++	+
	Q	×	×	0	×	0	×	×	×	0	+	×	×	0	+	0	0	0	0	×	×	×	+
	S	×	×	0	×	++	0	++	+	×	+	+	++	+	+	0	0	++	0	0	++	×	×
	T	++	0	++	0	+	0	+	×	×	0	×	++	×	0	0	0	++	×	×	++	×	×
	Y	++	0	0	×	×	×	×	++	+	×	×	0	×	×	×	+	×	×	×	++	+	×	+
Hydrophilic: charged	R +ve	0	0	0	×	0	×	0	+	×	+	+	+	0	+	×	×	+	0	0	+	×	+	++
	K +ve	×	0	×	×	0	×	×	×	×	×	+	++	×	×	×	×	×	×	++	+	×	×	
	H +ve	×	0	0	0	×	0	+	×	×	+	0	+	×	0	×	0	++	0	+	×	0	×	
	–ve E	×	×	×	×	0	+	++	×	×	×	×	×	0	0	×	×	×	++	×	++	×	×	0
	–ve D	+	0	×	×	×	×	++	0	×	×	×	×	×	+	×	×	×	+	+	++	×	×	×
	Active derivatives identified	4	3	3	0	2	1	5	5	2	7	4	8	2	6	0	3	8	1	8	12	1	8

Bioactivity was estimated by zone of inhibition (diameter of zone minus diameter of bacterial growth) relative to that of the wild-type lacticin 3147 producer represented by +++ (approximately 15 mm) and marked in grey. 0 = absence of inhibition, × = mutants not identified. The bioactivities of those mutant Ltnα peptides selected for purification are boxed.

conservative substitutions are tolerated, L21M and L21V, and perhaps surprisingly, a L21Y mutant retained a small amount of bioactivity (Table 1). The retention of bioactivity by the L21V mutant is notable in that it renders Ltnα more similar to other α peptides (SmbB, BHT-Aα and Bliα; Fig. 1). The results of saturation mutagenesis at positions N14 and L21 highlight the risk of relying solely on alanine scanning as an indication of tolerance to change (Fig. 2).

E24 is highly conserved among the mersacidin and lacticin 481 subgroups (Fig. 1) and LtnαE24A showed no bioactivity (Cotter *et al*., 2006). When the corresponding residues in mersacidin (Szekat *et al*., 2003), actagardine (Boakes *et al*., 2009) and Halα of haloduracin (Cooper *et al*., 2008) were substituted with Ala (and also to Gln in the case of HalαE22), all antimicrobial activity was also lost. HalαE22Q was also deficient with regard to the inhibition of peptidoglycan formation by the enzyme PBP1b, which uses lipid II as a substrate for glycan polymerization (Oman *et al*., 2011). Unsurprisingly, on saturation mutagenesis of LtnαE24, the only mutant to retain activity, albeit low levels, is one in which the negative charge at this position is maintained (E24D; Table 1). This is consistent with previous findings (Deegan *et al*., 2010) and renders the peptide more similar to many members of the lacticin 481 subgroup (Fig. 1). This observation contradicts a previous investigation that designated E24 as intolerant of change (Cotter *et al*., 2006) (Fig. 2). However, while a preference for the native glutamate is apparent, it can be said that a negatively charged residue at this position of Ltnα is of critical importance.

Despite their variable nature across the mersacidin-like peptides (Fig. 1), bioactivity was abolished when W18 and W28 were converted to alanine (Cotter *et al*., 2006). It was noted that on saturation mutagenesis of position W18, none of the Ltnα mutants identified retained detectable bioactivity (Table 1). In contrast, a W28Y mutant retained bioactivity, albeit at a reduced level compared to the wild-type, in disagreement with the anticipated intolerance to change at this location (Cotter *et al*., 2006) (Table 1; Fig. 2).

Targeting of 'variable' residues in Ltnα for site-saturation mutagenesis

Many Ltnα residues were classified as variable in that they could be altered to alanine (or glycine in the case of native alanines) without resulting in complete loss of bioactivity (Cotter *et al*., 2006). These include the N-terminal variable residues (T3, N4, L8, T5), ring B variable residues (D10, Y11, G13, N15, G16, A17) and C-terminal variable residues (H23, M26, A27, K30). It should be noted that some changes which were previously found to be tolerated (Cotter *et al*., 2006) did not yield bioactive strains on this occasion, presumably as a consequence of the reduced activity associated with the expression system used in this study.

Residues T3 and T5 of Ltnα are both dehydrated to Dhb in mature lacticin 3147. Given the degree to which the previously generated T3A mutant retained bioactivity (Cotter *et al*., 2006), coupled with the fact that a threonine at this position is not conserved across the α peptide group (Fig. 1), it is perhaps not surprising that four bioactive mutants, T3M, N, Y and D, were identified on saturation mutagenesis of this residue (Table 1). Interestingly, none of these substitutions were residues present at the corresponding locations in other members of the group (Fig. 1). The retention of low levels of bioactivity by T5A, which renders the peptide more similar to Bhaα, was replicated (Cotter *et al*., 2006), and two additional bioactive mutants were identified (T5V, L; Table 1). Significantly, T5V rendered Ltnα more similar to the related α peptides SmbB and BHT-Aα (Fig. 1).

Because some activity was retained upon conversion of N4 to alanine (Cotter *et al*., 2006) and the residue at this position varies across the α peptide group (Fig. 1), N4 was previously categorized as being non-essential with respect to the bioactivity of lacticin 3147. Three active mutants were identified (Table 1), two of which involved substitutions which rendered the peptides more similar to other α peptides (A, Pnmα; V, SmbB and BHT-Aα) (Fig. 1). Similarly, L8 is variable across the group and a L8A mutant previously displayed bioactivity (Cotter *et al*., 2006). In accordance with this, an additional bioactive mutant was detected (L8E; Table 1).

Previous mutagenesis of the residues within ring B of Ltnα suggested that this region is tolerant of change in that six of the nine corresponding mutants retain bioactivity when altered to alanine (Cotter *et al*., 2006). The first of these residues, the negatively charged residue D10, is not conserved across the α peptide group (Fig. 1) and five active mutants were identified (Table 1). In particular, the retention of bioactivity following substitution with another negatively charged residue, D10E, was anticipated in light of the natural presence of a glutamate at the corresponding position of Pnmα (Fig. 1). Despite the non-production of a D10K mutant previously (Deegan *et al*., 2010), it seems that the presence of a negative charge here is not essential, given that a D10H mutant still retained some bioactivity.

In the case of four of the five aromatic residues in Ltnα (F6, W12, W18 and W28), conversion to alanine completely eliminated bioactivity (Cotter *et al*., 2006). The exception, Y11A, displayed greatly reduced bioactivity. This aromatic residue also varies across the α peptide group (Fig. 1). Indeed, five mutants with reduced bioactivities were identified (Table 1), including substitutions that rendered Ltnα more similar to other members of the

group: Y11A (Pnmα), Y11V (SmbB, bhtA-alpha) and Y11R (Bhaα) (Fig. 1).

G13A was previously found to retain a considerable level of bioactivity (Cotter *et al*., 2006), even though glycine is conserved in all α peptides (Fig. 1). It was suggested that alanine alone, because of its similarity to the native glycine, could be tolerated (Cotter *et al*., 2006). However, saturation mutagenesis established the tolerance of the G13 residue to a variety of substitutions, ranging from other hydrophobic residues (G13A), to non-conservative hydrophilic neutral (G13N and G13Q) and charged residues (G13H and G13R) (Table 1). It should be noted that the bioactivity level of all G13 mutants was much reduced when compared to wild-type.

Residue G16 is even more highly conserved than G13, being fully conserved across both the mersacidin-like peptides (except RumB) and lacticin 481-like peptides (Fig. 1). This hyper-conserved nature, and the reduced and absent bioactivity of G16A (Cotter *et al*., 2006) and G16E (Field *et al*., 2007), respectively, suggested that G16 is less tolerant of change than its G13 counterpart. This was indeed the case as only one additional substitution retained detectable bioactivity (G16S, in which the Ser residue remains unmodified; data not shown) (Table 1). Similarly, on saturation mutagenesis of the corresponding glycine in mersacidin (G9), only three bioactive mutants were identified, including G9A and G9S (Appleyard *et al*., 2009). The corresponding position in nukacin ISK-1 (G5) has been shown by saturation mutagenesis to be essential to bioactivity (Islam *et al*., 2009).

Residues N15 and the previously discussed N14 are noteworthy due to the contrasting consequences on conversion to alanine, with N15A retaining significant bioactivity (Cotter *et al*., 2006). Saturation mutagenesis of N15 revealed eight mutants that retained bioactivity (Table 1), including the previously described N15A. In line with previous studies (O'Connor *et al*., 2007; Deegan *et al*., 2010), an N15K mutant, which more closely resembles the related C55α, SmbB, BHT-Aα, Halα and Pnmα peptides (Fig. 1), exhibited relatively high levels of bioactivity (Table 1). A N15S substitution that alters Ltnα to more closely resemble rumB, plantaricin C, michiganin and actagardine and many of the lacticin 481 peptides (Fig. 1) was also tolerated (Table 1).

Residue A17 is expected to be amenable to substitution based on its variation among related peptides (Fig. 1) and the fact that high activity was observed on substitution with glycine (Cotter *et al*., 2006). In keeping with this hypothesis, site-saturation at this position yielded a number of active mutants (Table 1). Surprisingly, no residues found at the corresponding locations in other group members were identified. We did not detect a previously described mutation, A17N, that makes Ltnα more closely resemble Sacα and which has little impact on bioactivity (O'Connor *et al*., 2007).

Although M26 is conserved in six out of eight α peptides (Fig. 1), the retention of some activity on substitution with alanine led to its classification as a residue that is amenable to change (Cotter *et al*., 2006). Indeed, following saturation mutagenesis, many active substitutions were identified (Table 1), including M26L that more closely resembles some members of the lacticin 481 subgroup. While an M26I mutant was only slightly active, mutation of the corresponding residue (V22) in nukacin ISK-1 to isoleucine resulted in a variant with increased potency (Islam *et al*., 2009).

In keeping with its designation as a variable residue (Cotter *et al*., 2006), and its non-conserved nature (Fig. 1), position A27 was found to be very tolerant of change when subjected to site-saturation mutagenesis (Table 1) (Cotter *et al*., 2006). In fact, site-saturation mutagenesis of LtnαA27 yielded the greatest number of bioactive mutants. A change to arginine, which is found in the equivalent position in SmbBα and BHT-Aα, resulted in a mutant that retained much of its bioactivity. A change to valine, which renders the peptide more similar to Plwα, had a more damaging impact. The identification of an A27S variant was interesting given that previous attempts to construct this mutant in order to generate a derivative of Ltnα that more closely resembled C55α were unsuccessful (O'Connor *et al*., 2007). We established that this mutant retained close to wild-type levels of bioactivity (Table 1), and like A27T, remained in an unmodified form (data not shown). The A27S variant also displayed levels of bioactivity comparable to those of the wild-type against *S. thermophilus* NCDO2525 and *L. lactis* AM2 (data not shown). A27S was purified in order to determine its specific activity (see below).

Ltnα has a net neutral charge (two positive residues, H23 and K30; and two negative; D10 and E24). While alanine substitution of the negatively charged residues had a relatively major impact, changing the positively charged amino acids had a lesser effect (Cotter *et al*., 2006). Indeed, a previously described derivative substituting alanine for both positive residues still retained considerable bioactivity (Deegan *et al*., 2010). The H23 location also merited attention by virtue of being the only position in the region of Ltnα within the predicted lipid II binding domain (residues 19–25), which retained bioactivity on conversion to alanine. Furthermore, both H23 and K30 are variable across the mersacidin-like peptides (Fig. 1). Saturation mutagenesis indicated many permissible substitutions for these positively charged residues (Table 1). In two instances they were replaced by other positively charged amino acids (K30R and H23R). Mutants where the substitution mimics a natural variation between Ltnα and the other α peptides (Fig. 1) were

identified, namely H23V (SmbB, BHT-Aα and Halα), K30N (Plwα, Halα and Bliα) and K30Q (BHT-Aα). Although it has previously been established that both H23D- and K30D-producing strains are bioactive (Deegan *et al*., 2010), these mutants were not identified in the current study, suggesting that they were not created or were not among those tested.

It was apparent that Ltnα H23S- and H23T-producing mutants retained close to wild-type levels of bioactivity against *S. aureus* NCDO1499, a clinical isolate involved in bovine mastitis, *S. thermophilus* NCDO2525 and *L. lactis* AM2 (data not shown). As a consequence of this bioactivity, coupled with our inability to detect these peptides by CMS, it was postulated that production of these peptides may be reduced and thus that specific activity may be relatively high. On that basis, the H23T and H23S peptides were selected for purification and further analysis. Following purification, masses of 3269 and 3255 Da were ascertained for H23T and H23S, respectively, indicating that both hydroxyl residues remained in an unmodified form. Significantly, a serine residue is naturally present at the corresponding positions in both mersacidin and plantaricin C (Fig. 1), which in the case of mersacidin is known to be modified to Dha.

Peptide purification and specific activity studies

Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) of H23S, H23T and A27S confirmed peptides of the expected mass, with the exception of an additional peak corresponding to 3285 Da for H23T, which was indicative of oxidation. This occurred despite the use of a variety of strategies designed to minimize this phenomenon. As a result its specific activity could not be accurately assessed. Accordingly, only purified H23S and A27S were utilized for specific activity studies.

The MICs of Ltnα A27S against *L. lactis* AM2 and *S. thermophilus* NCDO2525, both alone and when combined with Ltnβ, are higher than those of Ltnα and the wild-type Ltnα–Ltnβ combination (Table 2). H23S-Ltnβ had a MIC of 0.0313 μM against *S. thermophilus* NCDO2525, similar to the wild-type Ltnα–Ltnβ combination (Table 2). The MICs of the two Ltnα peptides were also identical when determined in isolation against *S. thermophilus* NCDO2525. It was noteworthy that the H23S variant alone is twofold more active than its wild-type counterpart against *L. lactis* AM2 but, when combined with Ltnβ, had a MIC the same as that of wild-type Ltnα–Ltnβ (Table 2). This is only the second example where one of the peptides of a two peptide lantibiotic exhibits increased solo specific activity relative to the parental molecule. The first such peptide, LtnβR27A, displayed a twofold increased specific activity against *L. lactis* HP when compared to Ltnβ alone (Deegan *et al*.,

Table 2. Minimum inhibitory concentration (MIC) of purified Ltnα, Ltnα–H23S and Ltnα–A27S alone, and in combination with equimolar concentrations of purified Ltnβ, against various Gram-positive organisms.

Peptide	*L. lactis* AM2	*S. aureus* NCDO1499[a]	*S. thermophilus* NCDO2525	*S. aureus* Newman	*S. aureus* Farm1	*E. casseliflavus* 5053	*E. faecium* 5119	*L. lactis* HP
Ltnα + Ltnβ	0.03125	0.500	0.03125	2.5	0.156	0.250	0.250	0.0156
LtnαH23S + Ltnβ	0.03125	**0.250**	0.03125	5.0	0.312	0.500	0.500	0.0313
LtnαA27S + Ltnβ	0.062	ND	0.0625	ND	ND	ND	ND	ND
Ltnα	0.937	1.875	0.937	> 10	10	> 3.75	3.75	0.937
LtnαH23S	**0.468**	1.875	0.937	> 10	> 10	> 3.75	> 3.75	1.875
LtnαA27S	1.874	ND	3.784	ND	ND	ND	ND	ND

a. Clinical mastitis isolate.
Values given are identical results from three independent determinations (μM). Those values in bold represent MICs that are improved relative to that of the wild-type against the relevant strain.
ND, not determined.

2010). However, in that case an eightfold decreased specific activity was observed when LtnβR27A was combined with its sister peptide Ltnα against HP. Most notably, further MIC-based investigations revealed that when LtnαH23S is combined with Ltnβ, their combined specific activity (0.25 μM) was twofold greater than the natural lacticin 3147 (0.50 μM) against *S. aureus* NCDO1499 (Table 2), thus making it the first example of the application of bioengineering to successfully enhance the activity of lacticin 3147, or indeed any two peptide lantibiotic. Prompted by this finding, further MIC-based analysis of LtnαH23S combined with Ltnβ against a wider selection of indicator strains including other staphylococcal isolates (*S. aureus* Newman, *S. aureus* Farm 1), enterococci (*E. casseliflavus* 5053, *E. faecium* 5119) and *L. lactis* HP revealed that in each case, a twofold decrease in specific activity compared to wild-type Ltnα–Ltnβ was apparent (Table 2). LtnαH23S alone did not show enhanced solo activity against any of the targets. Thus, although LtnαH23S exhibits enhanced specific activity, both alone and in combination with Ltnβ, this enhanced activity is very much a strain specific phenomenon.

Conclusion

Only a small number of bioengineered lantibiotics had been created prior to 2005, including derivatives with enhanced antimicrobial activity (Liu and Hansen, 1992; Kuipers *et al*., 1996; Wiedemann *et al*., 2001; Yuan *et al*., 2004; Rink *et al*., 2007), derivatives with enhanced properties including improved solubility and stability (Liu and Hansen, 1992; Rollema *et al*., 1995; Yuan *et al*., 2004), or ones which enabled researchers to gain an appreciation of structure/function relationships (Chan *et al*., 1996; van Kraaij *et al*., 1997; 2000; Chen *et al*., 1998; Wiedemann *et al*., 2001; Szekat *et al*., 2003). These pioneering studies suggested that lantibiotic peptides are quite adaptable and it was evident that further bioengineering-based approaches could be rewarding. Some recent examples have been successful with regard to the generation and identification of lantibiotic derivatives with improved antimicrobial and/or physicochemical properties (Rink *et al*., 2007; Field *et al*., 2008; 2012; Appleyard *et al*., 2009; Islam *et al*., 2009; Field *et al*., 2010b; Rouse *et al*., 2012).

The two peptide lantibiotics have been the subject of much interest as they offer many possibilities with respect to the design of new, and possibly more potent, antimicrobials. To facilitate the rational design of such peptides, we performed saturation mutagenesis on one of the two lacticin 3147 peptides. There are already encouraging signs that Ltnα would make an excellent candidate for bioengineering considering the significant number of residues (16/30) that retained bioactivity following alanine scanning mutagenesis (Cotter *et al*., 2006), and the fact that it can function in combination with the β peptide from another two peptide lantibiotic (O'Connor *et al*., 2007). This flexibility is coupled with the fact that the involvement of two peptides facilitates the examination of distinct functional domains in isolation (Morgan *et al*., 2005). While both Ltnα and Ltnβ each possess solo activity, Ltnα is significantly more active than Ltnβ. Thus, Ltnα derivatives can be more easily assessed in isolation, as well as in combination with Ltnβ. It has been speculated that once the basis of the mutual interaction between the α and β peptides is revealed, theoretically the α peptide could be directed to other more strain-specific targets than lipid II (Breukink and de Kruijff, 2006), while continuing to interact with the β peptide to facilitate pore formation.

To this end, site-saturation mutagenesis was performed on all residues of Ltnα other than those involved in bridge formation, facilitating a more comprehensive determination of the tolerance of Ltnα to change than that provided by alanine scanning (Fig. 2). It was apparent that a number of positions in particular were more amenable to change (N14, L21) than was previously predicted (Cotter *et al*., 2006). Furthermore, a limited number of mostly conservative changes were tolerated at positions previously designated as intolerant (S7, W12, E24 and W28) (Cotter *et al*., 2006). Significantly, despite the conserved nature of positions G13, G16 and M26, it was found that within lantibiotics a high degree of conservation does not necessarily mean that change at this location is not tolerated.

Additionally, during this process, a H23S substitution was found to improve the specific activity of lacticin 3147 against a strain of *S. aureus* responsible for bovine mastitis, and that of the Ltnα peptide alone against *L. lactis* AM2. While the bioengineering of lantibiotics has produced some successes and the activity of a number of one peptide lantibiotics has been enhanced, this is the first description of a bioengineered two-peptide lantibiotic with an improved specific activity. The fact that such enhanced combinations have not been described previously most likely stems from the requirement for two peptides to act synergistically for full activity. This imposes a greater structural constraint on each peptide, and thus alterations made to enhance the interaction of the α peptide with its cell target for instance may have a negative impact on its ability to function synergistically with the β peptide. One might have predicted that the H23S alteration could enhance lipid II binding as a consequence of the peptide more closely resembling mersacidin, whose activity is solely based on lipid II binding without pore formation. However, the fact that the solo activity of Ltnα H23S against *S. aureus* is not improved confirms that the enhanced activity is dependent on the presence of Ltnβ. We speculate that this change must either improve the Ltnα–Ltnβ interaction at the target site or that an enhanced Ltnα–lipid II interaction may require a Ltnβ-

induced conformational change. Future work will focus on the elucidation of the mechanistic basis for the strain-specific enhanced activity of lacticin 3147 H23S relative to lacticin 3147.

In summary, through the study of > 200 mutants, this systematic mutagenesis has provided significant information on the key residues that contribute to the bioactivity of lacticin 3147, which should prove valuable for the rational design of novel lantibiotics with improved properties. Furthermore, while the vast majority of mutants were less potent, the high number of derivatives that were produced in this study can also be interpreted as a test of the *in vivo* promiscuity of the enzymatic machinery, showing that the biosynthetic pathway of lacticin 3147 has a relatively relaxed specificity when it comes to mutants of Ltnα. Perhaps most importantly, a Ltnα–H23S change was found to improve the specific activity of lacticin 3147 against a strain of *S. aureus*, representing the first instance in which an enhanced bioengineered derivative of a two peptide lantibiotic has been identified.

Experimental procedures

Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in Table S1. *L. lactis* and *Enterococcus* strains were grown in M17 broth (Oxoid) supplemented with 0.5% glucose (GM17) or GM17 agar at 30°C and 37°C respectively. *Escherichia coli* was grown in Luria–Bertani broth with vigorous shaking or agar at 37°C. *S. aureus* strains were grown in Mueller-Hinton broth (Oxoid) at 37°C. *S. thermophilus* NCDO2525 was grown in Litmus Milk (Difco BD, USA) before routine subculturing in M17 broth supplemented with 0.5% lactose (LM17) at 37°C. Chloramphenicol and tetracycline were used at 5 and 10 μg ml^{-1}, respectively, for *L. lactis* (unless otherwise stated) where required and at 20 and 10 μg ml^{-1}, respectively, for *E. coli.* Xgal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was used at a concentration of 40 μg ml^{-1}.

Site-saturation mutagenesis

Oligonucleotide pairs (Table S2) were designed to replace each target *ltnA1* codon with the NNK triplet, which should result in the substitution of the relevant residue with all 19 possible alternatives (Cwirla *et al.*, 1990; Scott and Smith, 1990). Plasmid pDF01 was used as template DNA for saturation mutagenesis and PCR amplification was performed as previously described (Field *et al.*, 2008). Following plasmid amplification and introduction into the intermediate *E. coli* MC1000 host, plasmid DNA from a pooled bank of pDF01 derivatives (each corresponding to a targeted amino acid) was isolated using a Roche High Pure Plasmid Isolation Kit. DNA sequence analysis with pCI372FOR (MWG Biotech, Germany) confirmed randomization at the relevant codon. PbacA1A2 (containing bioengineered *ltnA1* genes, the partner *ltnA2* gene and the associated promoter region Pbac) was re-amplified using the primers pPTPLA1A2FOR and pPTPLA1A2REV and template DNA isolated from the individual mutagenized pDF01 pools. Amplified products were purified as before, digested with BglII and XbaI (Roche), ligated with similarly digested and shrimp alkaline phosphatase (Fermentas)-treated pPTPL and introduced by electroporation into *E. coli* MC1000. Transformants were pooled and stored in 80% glycerol at –20°C. Plasmid DNA isolated from each mutant bank was introduced by electroporation into the strain *L. lactis* MG1363 pOM44 to facilitate expression of the bioengineered Ltnα peptide (in the presence of unaltered Ltnβ) for further analysis. A total of 144 transformants were chosen at random and inoculated into 96-well plates containing GM17 chloramphenicol and tetracycline (5 μg ml^{-1} each), incubated overnight and stored at –20°C after addition of 80% glycerol. Mutants were identified by MS analysis and, in instances where the nature of the change remained ambiguous after MS or a peptide could not be detected, sequencing with TETK P1. All bioactive derivatives in each bank were identified. Ten representative inactive derivatives were chosen from each bank for further analysis, with loss of activity attributed to the particular substitution, an insertion, numerous mutations or the introduction of a stop codon. Varying levels of success were observed in the identification of unique inactive derivatives. Steps were taken to ensure that the companion peptide was unmutated (by MS and/or sequencing) in all cases.

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS)

Colony mass spectrometry (CMS) was performed with an Axima TOF^2 MALDI-TOF mass spectrometer (Shimadzu Biotech, Manchester, UK) as previously described (Field *et al.*, 2010b). For purified peptide, a small amount of lyophilized peptide resuspended in 70% IPA 0.1% TFA was used for analysis.

Bioassays for antimicrobial activity

Deferred antagonism assays were performed as previously described (Field *et al.*, 2007). For high throughput screening of the Ltnα site-saturation banks against *L. lactis* HP, deferred antagonism assays were performed by spotting strains using a 96-pin replicator (Boekel) on GM17 agar plates. Zone size was measured with callipers and calculated as the diameter of the zone of clearing minus the diameter of bacterial growth.

Minimum inhibitory concentration determinations were performed as described previously (Wiedemann *et al.*, 2006), with incubation for 16 h at 30°C (*L. lactis*) or 37°C (*S. aureus*, *S. thermophilus* and *Enterococci*). The MIC was read as the lowest peptide concentration causing inhibition of visible growth.

RP-HPLC purification of lacticin 3147 and Ltnα derivatives

Reverse phase-high performance liquid chromatography (RP-HPLC) was used to obtain pure lacticin 3147 and Ltnα derivatives as previously described (Suda *et al.*, 2011).

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Conflict of interest

None declared.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Strains and plasmids used in this study. UCC, University College Cork; NCDO, National Collection of Dairy Organisms.
Table S2. Oligonucleotides utilised in this study. Pho indicates 5′ phosphate. Boldface represents randomized nucleotides (N = A + C + G + T, K = G + T, M = A + C). Underlined sequences represent restriction sites.