Insights into lantibiotic immunity provided by bioengineering of LtnI.

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Abstract

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. 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 20-27 and 76-83, which are essential for LtnI function. Finally, as a consequence of the screening of a bank of 28,000 strains producing 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 (5, 7, 18, 35, 43). As a consequence, lantibiotics have been the subject of much investigation with respect to clinical and/or food applications (3, 4, 7, 15, 30, 55). 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 (50). The lacticin 3147 producer employs two systems to provide immunity (12, 13, 34). 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) (17, 41, 44, 48, 49). 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) (27, 31, 34, 40, 42). 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 (6, 19, 21, 47).

Relatively little is known regarding the mechanism by which LtnI provides protection to lacticin 3147. Although this 116 amino acid protein is predicted to be membrane associated on the basis of hydrophobicity profiling (34), 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 (51).

Similar investigations have been carried out to identify essential domains within PepI, a LanI protein associated with Pep5 immunity (37), and its homologue, EcI, which is responsible for epicidin 280 immunity and cross-immunity to Pep5 (19). 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 (21). To investigate the importance of the C-terminal domain of PepI, a truncated protein, PepI 1-65, was created that lacked the four C-terminally located charged amino acids. The immunity provided by this truncated version was greatly reduced (42). A further study focused on three other C-terminally truncated versions of PepI; PepI1-63, 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 truncated versions of PepI (21).

Finally, the structure and function of the LanH protein associated with immunity to the type II lantibiotic nukacin ISK-1, NukH (92 amino acids), has been extensively investigated (1). NukH, although distinct from LanI proteins in that it functions as an accessory protein to the ABC-transporter immunity system NukFEG, has a transmembrane location. Through the creation of truncated versions of NukH fused to an alkaline phosphatase (PhoA) reporter and by evaluating their sensitivity to proteinase K, it was established that NukH contains 3 transmembrane domains. The PhoA fusion sites of NukH(1-33)-PhoA and 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 (40). To identify functional domains within NukH, amino acid substitutions, deletions and truncated versions were created. Deletion of either the N-terminus (position 1-6) or the C-terminus (position 89-92) 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 to 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 (40).

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

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 K2HPO4 (36 mM), KH2PO4 (13.2 mM), sodium citrate (1.7 mM), MgSO4 (0.4 mM), (NH4)2SO4 (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 broth (LB broth; (45)) 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 manufacturers guidelines for transformation. *L. lactis* strains were made electrocompetent following the procedure described by Holo and Nes (23). 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-Aldrich) 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* (12) using the Maxi-prep 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 one base pair change in the 488 bp cloned fragment, amplification was performed in a 50 μl reaction 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 pre-heated at 96°C for 1 min, and 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 re-amplified with KOD DNA polymerase before being digested with *KpnI* and *XbaI*, ligated with similarly digested and SAP-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 the Genetix QPIX II-XT colony-picking robot and 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 LtnIxbF (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. LacZ reverse primers (LtnI21B-LtnI114B) all contain a *Bam*HI restriction site to facilitate in-frame fusion with the *lacZ* gene of pRMCD70 (11), whereas reverse primers for *phoA* fusions (LtnI21H to LtnI114H) contain a *Hind*III site to facilitate in-frame fusion with *phoA* of pRMCD28 (11). All constructs were electroporated into *E. coli* CC118, transformants were selected on LB plates containing ampicillin and initially checked using a pho 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 P44 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 (9) using pNZ44*ltnI* as template and primers as listed in Table 2, i.e. the primers LtnIL1AF/R and LtnIL2AF/R were used for constructing pNZ*ltnIL*87A and pNZ*ltnIL*94A, respectively and pNZ44*ltnID*57A, pNZ44*ltnIR*59A and a double mutant pNZ44*ltnID*57A-R59A were created using the primers LtnID57AF/R, LtnIR59AF/R respectively for the single mutants. For the double mutant one pair of primers (LtnID57/R59F/R) where used, 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\(^{-1}\) (7680 AU ml\(^{-1}\)) lacticin 3147 powder. Sensitivity to lacticin 3147 was also assessed using a gradient agar sensitivity test (21). 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° 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 1.1µM lacticin 3147 and monitoring optical density at 600 nm (OD\(_{\text{600}}\)) with a Spectromax 340 spectrophotometer (Molecular Devices, Sunnyvale, California) over a 16-h period. Minimum inhibitory concentration (MIC) assays were performed as described previously (12).
β-galactosidase and alkaline phosphatase assays.

β-galactosidase and alkaline phosphatase assays were carried out as described by Miller (38) and Manoil (32), respectively. Briefly, for LacZ constructs, *E. coli* were grown in 10 ml LB broth until the OD$_{600\text{nm}}$ 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 (24). Subsequently, 4 mg ml$^{-1}$ 2-Nitrophenyl β-D-galactopyranoside (ONPG) was mixed with the permeabilised cells, 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 ($522 \times \text{OD}_{420\text{nm}}$ of reaction mixture/OD$_{600\text{nm}}$ of culture $\times$ volume per ml of culture used $\times$ 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, permeabilised 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; ($1000 \times \text{OD}_{420\text{nm}}$ of reaction mixture/OD$_{600\text{nm}}$ $\times$ volume of culture $\times$ time of reaction).

Results

In silico analysis of LtnI.

It was reported previously 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 (34). We can now report a more extensive bioinformatic analysis, using the TMHMM (29), HMMTOP (52), SPLIT 4 (26), SOUSI (20) and TMpred (22)
servers, which also strongly predict that these hydrophobic regions correspond to three trans-
membrane regions (TMR). All predictions suggest that the N-terminus of LtnI is located outside
of the cell, that TMR1 and TMR3 have an outside to inside orientation, that TMR2 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 4.0 (10) and thus the structures predicted
by TMHMM2 and SPLIT 4.0 were used as 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 genome sequenced microorganisms (Fig 2). This
includes a bliI that has been previously shown to provide protection against lacticin 3147 when
expressed heterologously (12). 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 (34), the identification of leucine zippers can be easily assigned incorrectly
when the correct orientation of leucines alone is employed. Notably, when reassessed using 2ZIP
(2), 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 (33) 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 (32). 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 TMRs (Fig 1 (C)), were 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/lacZ genes lacking the first eight codons. These were under the control of a lacI promoter. Fourteen hybrids were generated in which 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+ PhoA- phenotype indicating that, as predicted, K55 is located in the cytoplasm (Fig 3). Similarly, the G85 fusions behaved as expected (LacZ- PhoA+), indicating that this region is located outside the cytoplasmic membrane. A21 fusions have a negative LacZ and PhoA 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- 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 1-13 of LtnI, while a second mutant that lacks this region as well as residues 14-19, and a third lacking residues 1-27, were also generated (Fig 2). The construct pNZ44*ltnIΔNT(1-13) was cloned into MG1363 and was initially tested by well diffusion assay for an immunity phenotype. The level of immunity provided by MG1363/pNZ44*ltnIΔNT1-13 was comparable with that of MG1363/pNZ44*ltnI, as was MG1363/pNZ44*ltnIΔNT1-19, a fact that was confirmed by MIC studies (Table 3). In contrast, MG1363/pNZ44*ltnIΔNT1-27 was as sensitive as the control host strain MG1363, thus demonstrating that the region between W20 and N27 inclusive of the N-terminal of LtnI is essential for its functionality. In contrast, it was apparent that the absence of the C-terminal region of LtnI pNZ44*ltnIΔCT110-116 did not alter the immunity phenotype, with an MIC of 1.25μM (identical to that of MG1363/pNZ44*ltnI). While the removal of larger regions of the C-terminus (facilitated by the creation of pNZ44*ltnIΔCT90-116 and pNZ44*ltnIΔCT83-116) 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\textit{ltnI} and expression in MG1363. A number of conserved residues were also investigated, corresponding to residues D57, E58, R59 and T60 in LtnI. One positively charged (R59) and one negatively charged (D57) amino acid 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\textit{ltnI} 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 \textit{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\textit{ltnI} 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 \textit{ltn}I amplicons. Ligation of these amplicons into pNZ44 and heterologous expression in \textit{L. lactis} MG1363 led to the creation of a bank of 28,000 strains expressing randomly mutated forms of \textit{ltnI}. Spotting of the bank onto GM17 infused with 3 mg ml\textsuperscript{-1} (1920 AU ml\textsuperscript{-1}) lacticin 3147-skim milk powder revealed more than 200 strains that were unable to grow despite PCR confirmation that a copy of the \textit{ltnI} gene was present.
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 with respect to 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 membrane. Two different changes occur at phenylalanine
36, conversion to an isoleucine and to a leucine. Within the second transmembrane domain, two
mutations resulted in the elimination of activity, D79N and F82S. 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.

31 strains expressing mutated forms of *ltnI* were sensitive as a consequence of frameshift mutations (Table 4, Fig 4). These represented 17 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 with the effects of the 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 2-4 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
A single mutant was identified with an ability to survive in the presence of increased levels of lacticin 3147. To ensure that this enhanced protection was as a consequence of the \textit{ltnI}-associated change, rather than a spontaneous change within the host’s genome, the associated pNZ44\textit{ltnI} plasmid was isolated, re-introduced into a fresh MG1363 background and found to again provide enhanced protection. The corresponding \textit{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\textit{ltnI} and MG1363/pNZ44\textit{ltnI}(I81V) were grown in the presence and absence of 1.1\textmu M lacticin 3147. This confirms the enhanced resistance of LtnI(I81V) to the lantibiotic (Fig 5).

\section*{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 Pepl are anchored to the cytoplasmic membrane, and unusually, NisI has also been found as a lipid free form, presumably scavenging for exogenous nisin (21, 28, 48, 49). In contrast, it was previously recognized that LtnI was very likely to traverse the membrane (34) in a manner similar to that predicted for CylI (6) and established for NukH (40). 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 4 and TMHMM algorithms, which have previously
been established to be 85% and 83% accurate, respectively, in predicting the location of residues
(10). 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 (46, 53, 54). Based on 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 (39). 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 is highly reflective of the situation in lactic acid bacteria (25). Therefore, it
is likely that the topology of LtnI predicted is a true reflection of the situation in a lacticin 3147-
immune L. lactis.

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 Pepl, where loss of a charged residues at the C-terminus reduces immunity function (21),
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 1st or 2nd 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 (40), 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 but does not eliminate immunity, this is a phenomenon also observed for NukH, whereby the TMD3 is only essential for a full immunity level (40). Interestingly, the last 7 amino acids of LtnI would not appear to make any contribution to protection as pNZ44\(\text{ltnI}\Delta\text{CT110-116}\) provided the same level of immunity as pNZ44\(\text{ltnI}\). 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\(\text{ltnI}\Delta\text{NT1-27}\) is not immune but MG1363/pNZ44\(\text{ltnI}\Delta\text{NT1-20}\) 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 NT1-13 region have a greater impact than the entire
deletion of this region. The first of two absolutely essential domains identified in this study is therefore within the N-terminus, and contains a region between W20–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\(^{-1}\) (1920 AU ml\(^{-1}\)) lacticin 3147 in agar was effective in only identifying 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-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, another region of significance is the external loop (40), here we find that 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 (14). 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.

Acknowledgements

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functional analysis of the subtilin immunity genes spaFEG in the subtilin-sensitive host Bacillus

nisl immunity genes nisI and nisFEG after coordinated expression in the surrogate host Bacillus

Figure Legends and Tables

Fig 1. LtnI topology predictions
Membrane Topology models of LtnI using Trans-Membrane (TM) prediction sites. TMHMM, (red) indicates amino acids predicted to be in membrane, (blue) amino acids inside and (pink) amino acids outside (A). Split 4.0 hatched boxes indicate amino acids in membrane (B). Based on study by Cuthbertson et al., (10) algorithms were compared to indicate LtnI transmembrane regions TMHMM, SPLIT 4, Sousi and TMpred (C). Protein sequence of LtnI also indicates residues fused to reporter enzymes LacZ and PhoA (residues numbered and with asterixes).

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 black) 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/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, as is I81V, the amino acid change resulting in a functionally enhanced
LtnI peptide. 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.
### Table 1. Strains and plasmids

<table>
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<th>Strains and Plasmids</th>
<th>Description</th>
<th>Source/reference</th>
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<tr>
<td>E. coli CC118</td>
<td>ΔphoA20</td>
<td>(32)</td>
</tr>
<tr>
<td>E. coli Top10</td>
<td>Intermediate cloning host</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>L. lactis MG1363</td>
<td>Plasmid free, lacticin 3147 sensitive</td>
<td>(16)</td>
</tr>
<tr>
<td>L. lactis MG1363/pMRC01</td>
<td>MG1363 with lacticin 3147 producing plasmid</td>
<td>(8)</td>
</tr>
<tr>
<td>pRMCD28</td>
<td>E. coli phoA in pWSK29; Amp&lt;sup&gt;+&lt;/sup&gt;</td>
<td>(11)</td>
</tr>
<tr>
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<td>E. coli lacZ in pWSK29; Amp&lt;sup&gt;+&lt;/sup&gt;</td>
<td>(11)</td>
</tr>
<tr>
<td>pNZ44</td>
<td>L. lactis P44 promoter in pNZ8048; Cm&lt;sup&gt;+&lt;/sup&gt;</td>
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Table 2. Primers used for truncation and site-directed mutagenesis of LtnI (top), and also those used to construct LacZ and PhoA fusions (bottom).

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<td>Cterm109</td>
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<td>Pho/lac check</td>
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Underline represents restriction sites.
Table 3. MIC of N- and C-terminally truncated derivatives of LtnI.

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

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<th>Mutant</th>
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<th>Mutant</th>
<th>Change</th>
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<th>Change</th>
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