Identification and overexpression of *ltnI*, a novel gene which confers immunity to the two-component lantibiotic lacticin 3147

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Production and immunity of the two-component lantibiotic lacticin 3147 is encoded by the 60.2 kb lactococcal plasmid pMRC01. A 12.6 kb region of this plasmid, containing ten genes in two divergently arranged gene clusters, has been subcloned in *Lactococcus lactis* subsp. *cremoris* MG1363 and has been shown to confer both lacticin 3147 production and immunity. Further subcloning revealed that the smaller of the two clusters (*ltnRIFE*) confers immunity. Although the *ltnF* and *E* genes are homologous to ABC transporters which confer immunity to other lantibiotics, deletion analysis indicates that they do not play a role in the immunity exhibited by this subclone in *L. lactis* subsp. *cremoris* MG1363. Also, a deletion in *ltnR* (which resembles a family of transcriptional repressors) had no effect on immunity. The remaining gene, *ltnI*, encodes a 116 amino acid protein with a predicted membrane location which bears no homology to other bacteriocin immunity proteins. Confirmation of its role in immunity was obtained when it was observed that disruption of *ltnI* resulted in a complete loss of immunity. When *ltnI* was cloned into the expression vector pMG36e, the resulting construct conferred levels of immunity comparable to pMRC01. This confirmed that under the control of a strong promoter, the *ltnI* gene product alone is sufficient to confer lacticin immunity. In addition, heterologous expression of *ltnI* was observed in *Enterococcus faecalis* OG1X. On cloning *ltnI* behind a nisin-inducible promoter, it was observed that the level of immunity was dependent on nisin concentration. Using this construct, the authors have demonstrated a potential role for *ltnI* as food-grade selectable marker. Thus, *LtnI* appears to represent a new class of lantibiotic immunity proteins.

Keywords: lacticin 3147, lantibiotic, immunity, food-grade marker

INTRODUCTION

The term lantibiotics is used to describe a group of post-translationally modified peptides which display activity against Gram-positive bacteria (Klaenhammer, 1993; de Vos *et al.*, 1995; Sahl *et al.*, 1995; Sahl & Bierbaum, 1998). A characteristic of these bacteriocins is the presence of the thioether amino acids lanthionine and/or β -methylanthionine as well as the dehydrated amino acids 2,3-didehydroalanine and 2,3-didehydrobutyrine, which are formed from serine and threonine, respectively. The primary product of the lantibiotic structural gene(s) is a precursor with an N-terminal leader sequence followed by a C-terminal pro-peptide which

undergoes modification. Once modified within the cell, the bacteriocin is secreted by a dedicated transporter and the N-terminal leader is cleaved by a protease (van der Meer *et al.*, 1993). In the case of certain lantibiotics, e.g. lacticin 481 (Rince *et al.*, 1994), activation occurs concomitantly with export as the leader sequence is cleaved by ABC (ATP-binding cassette) transporters which contain a proteolytic domain.

All bacteriocin-producing strains require self-protection mechanisms in order to prevent cell death due to the action of their own bacteriocin(s) (Abee, 1995; Saris *et al.*, 1996). However, despite considerable molecular analysis of the various components involved, the mechanisms of immunity are still poorly understood for most lantibiotic systems. In strains producing nisin, both NisI and NisFEG are suggested to be involved in immunity

Abbreviations: ABC, ATP-binding cassette; AU, arbitrary units.

(Kuipers *et al.*, 1993; Engelke *et al.*, 1994; Siegers & Entian, 1995). NisI is a 245 amino acid protein with a lipoprotein signal sequence which may act to inhibit pore formation by interacting with extracellular nisin (Kuipers *et al.*, 1993). More recently, Qiao *et al.* (1995) have reported a cytoplasmic membrane location for NisI. The *nisFEG* gene cluster encodes a multi-component ABC transporter which is believed to play a role in immunity by transporting the active lantibiotic from the cytoplasmic membrane of immune cells (Siegers & Entian, 1995). The involvement of multi-component ABC transporters in lantibiotic immunity is a common feature which has been linked to subtilin produced by *Bacillus subtilis* (Klein & Entian, 1994), epidermin produced by *Staphylococcus epidermidis* (Peschel & Gotz, 1996) and lacticin 481 produced by *Lactococcus lactis* (Rince *et al.*, 1997). It has recently been demonstrated that EpiFEG acts by active extrusion of peptide from the cytoplasmic membrane of immune cells (Otto *et al.*, 1998). The mechanism of immunity to the *S. epidermidis* bacteriocin Pep5 appears to involve an interaction between the bacteriocin and a 69 amino acid protein, PepI, at the outer surface of the cytoplasmic membrane (Reis & Sahl, 1991; Reis *et al.*, 1994; Pag *et al.*, 1999). A protein with 74.2% similarity to PepI, EciI, has been identified in the epicidin 280 gene cluster (Heidrich *et al.*, 1998). Interestingly, the strain which produces epicidin 280, *S. epidermidis* BN 280, exhibits cross-immunity to Pep5, which suggests a similar molecular mechanism for both of these immunity proteins. In fact, PepI and EciI are more closely related to the immunity proteins of the unmodified peptide (Class II) bacteriocins of Gram-positive bacteria such as lactococcin A (Klaenhammer, 1993; Nissen-Meyer *et al.*, 1993), than to those described for lantibiotics. Recently, a 320 amino acid protein termed CylI has been shown to confer immunity to the two-component haemolysin/bacteriocin, cytolysin (Coburn *et al.*, 1999; Haas & Gilmore, 1999).

Lacticin 3147 is a broad-spectrum bacteriocin produced by *L. lactis* subsp. *lactis* DPC3147 (Ryan *et al.*, 1996). This two-component, membrane-active bacteriocin acts by selectively dissipating the membrane potential of target organisms (McAuliffe *et al.*, 1998). Recently, the presence of lanthionine residues in both lacticin 3147 peptides has been demonstrated, confirming that lacticin 3147 is a lantibiotic (M. P. Ryan, R. P. Ross & C. Hill, unpublished results). The genetic determinants responsible for lacticin 3147 production and immunity are encoded on a 60.2 kb plasmid, pMRC01, the sequence of which has been fully elucidated (Dougherty *et al.*, 1998). A 12.6 kb region of pMRC01 was proposed to encode lacticin 3147 production and immunity based on the homologies of a number of ORFs to genes involved in the production and export of lantibiotics (Klaenhammer, 1993; Jack *et al.*, 1995). This region contains two divergently organized gene clusters, the larger of which was proposed to be responsible for bacteriocin production, modification and export (Fig. 1). It was also noted that the genes which make up the larger cluster

had a similar functional organization to those in the cytolysin operon, which encodes another two-component lantibiotic produced by *Enterococcus faecalis* (Booth *et al.*, 1996; Gilmore *et al.*, 1996). The smaller four-gene cluster was thought to play a role in lacticin 3147 immunity, given that the cluster contains *ltnF* and *ltnE*, two genes related to those encoding multi-component ABC transporters (for reviews see Higgins, 1992; Fath & Kolter, 1993) found in other lantibiotic immunity systems. However, no biological evidence was presented which supported the proposed role of any of the genes. In this communication, we confirm that the 12.6 kb region encodes both lacticin 3147 production and immunity, and that the smaller of the two gene clusters is involved in immunity, as proposed. However, contrary to expectations, *ltnF* and *ltnE* do not appear to be involved in the immunity exhibited by a subclone containing the four-gene cluster in MG1363. Instead, a novel gene, *ltnI*, which encodes a 116 amino acid hydrophobic protein, designated LtnI, is responsible for the level of lacticin 3147 immunity observed. Expression studies using a nisin-controlled inducible promoter system indicate that the degree of immunity conferred upon previously sensitive lactococci depends on the amount of LtnI produced in the cell.

METHODS

Bacterial strains, plasmids and media. Bacterial strains used in this study are listed in Table 1. All lactococci were grown at 30 °C without aeration in M17 broth (Oxoid) supplemented with 0.5% glucose (GM17). *Ent. faecalis* OG1X was maintained in brain heart infusion broth (Difco) and grown at 37 °C without aeration. *Escherichia coli* strains were grown at 37 °C in LB broth (Sambrook *et al.*, 1989) with vigorous agitation. Plasmids used for cloning included pUC19 (Yanisch-Perron *et al.*, 1985), an *E. coli*/*L. lactis* shuttle vector, pCI372 (Hayes *et al.*, 1990), the expression vector pMG36e (van de Guchte *et al.*, 1989) and the nisin-inducible expression vector pNZ8048 (de Ruyter *et al.*, 1996). LB agar supplemented with X-Gal (40 µg ml⁻¹) and IPTG (40 µg ml⁻¹) was used for detection of insertional inactivation of the α -*lacZ* gene in pUC19. Antibiotics were used, where indicated, at the following concentrations: ampicillin, 100 µg ml⁻¹; erythromycin, 100 µg ml⁻¹ for *E. coli* and 5 µg ml⁻¹ for *L. lactis*; chloramphenicol, 20 µg ml⁻¹ for *E. coli* and 5 µg ml⁻¹ for *L. lactis*. The chemical reagents listed were purchased from Sigma.

Molecular biology techniques. Plasmid DNA was isolated from *E. coli* strains using the alkaline lysis method (Sambrook *et al.*, 1989) or the QIAGEN column purification kit, and from *L. lactis* using the method of Anderson & McKay (1983). pMRC01 DNA (Ryan *et al.*, 1996), which was used as template DNA in PCR, was purified by equilibrium centrifugation in CsCl/ethidium bromide gradients. *L. lactis* and *E. coli* were transformed by electroporation with a Gene-Pulser apparatus (Bio-Rad) as described by Wells *et al.* (1993) and Sambrook *et al.* (1989), respectively. Restriction digestion, Klenow treatment and DNA ligations were done according to standard procedures (Sambrook *et al.*, 1989). Restriction enzymes, the Klenow fragment of the *E. coli* DNA polymerase I and T4 DNA ligase were purchased from New England Biolabs. Restriction-endonuclease-digested DNA was eluted from agarose gels using a GeneClean II kit from Bio101. PCR was

Table 1. Bacterial strains used in this study

Strain	Relevant properties*	Source/reference†
<i>L. lactis</i> subsp. <i>lactis</i>		
DPC3147	Wild-type strain; Ltn ⁺ Imm ⁺	Ryan <i>et al.</i> (1996)
<i>L. lactis</i> subsp. <i>cremoris</i>		
HP	Indicator organism	DPC collection
MG1363	Plasmid-free, indicator organism	Gasson (1983)
NZ9800	NZ9700 derivative; contains Tn5276 <i>ΔnisA</i>	Kuipers <i>et al.</i> (1993)
<i>Ent. faecalis</i>		
OG1X	Indicator organism	Ike <i>et al.</i> (1984)
<i>E. coli</i>		
XL1 Blue	Intermediate cloning host	Stratagene
EC1000	Intermediate cloning host	Leenhouts <i>et al.</i> (1996)

* Ltn, lacticin 3147 production; Imm, immunity to lacticin 3147.

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Table 2. Primers used in plasmid construction

Plasmid*	Primer no.†	Primer sequence (5'–3')‡	Temp (°C)
pOM01	4562 (F)	CGGGATCCCTGGTTGTGTTTCAATTCATT	55
	4567 (R)	CGCGTCGACAAACAGAACCTAATATTCAATA	55
pOM02 (1/2)	3951b (F)	AAAGAGCTCTGCGAATAACATCAAGGGAA	55
	5144 (R)	GATGGATCCATTATTATTACTGTTT	55
(2/2)	5145 (F)	ATGGATCCATCAGTAGGTAGAACTA	60
	5146 (R)	GCTGTCGACTTAAGTATAGGGCAAT	60
pOM05	4562 (R)		
	5356 (F)	CGCGTCGACCATTTTCCTTTTTATATTGATT	55
pOM22	5563 (F)	ATGCATGCAACTATACACCTTCTT	50
	5562 (R)	TATAAGCTTTACCATGTGCTATTGAT	50
pOM23 (1/2)	5534 (F)	AATGAGCTCACTGGTTGTGTTTCA	50
	5533 (R)	AGGATCCCTAATAGAATCTTGAAA	50
(2/2)	5532 (F)	AGGATCCTTTTACCTACGAAC	50
	5531 (R)	TTTGTCGACCCATTGTTGTTT	50
pOM24	5636 (F)	ATTCCATGGAGAATGAAAATAT	50
	5562 (R)		
pOM26	5286 (F)	ATGGATCCATGGTTTTATATTATTATCA	50
	5531 (R)		

* 1/2, 2/2 refers to plasmids which were constructed from two separate PCR fragments.

† F, forward; R, reverse.

‡ Restriction sites in primer sequences are underlined.

performed by following standard procedures using BIOTAQ DNA polymerase (Bioline). The Expand High Fidelity PCR System (Boehringer) was used to generate PCR products for cloning purposes. Thirty cycles were performed with the following conditions: 30 s at 90 °C, 1 min at 50 °C and 1 min at 72 °C. Where longer PCR products were sought, the Expand Long Template PCR System from Boehringer was used according to the manufacturer's instructions. Primers for PCR were synthesized using an Applied Biosystems PCR-MATE DNA synthesizer.

Plasmid construction and mutagenesis. A number of strategies were used to clone and analyse the genes involved in lacticin production and immunity. Plasmid pOM02, containing the entire 12.6 kb coding region, was constructed by cloning the operon in roughly two equal halves (using fragments generated by Long Template PCR and engineered to contain appropriate cloning sites) into the *SacI/SalI* sites in the multiple cloning site of the vector, pCI372 (Hayes *et al.*, 1990). Plasmid pOM01 contains the smaller four-gene cluster cloned in pCI372 and was constructed using a single 2.9 kb

Fig. 1. Schematic representation of the organization of various constructs and the phenotypes associated with these mutated derivatives. Arrows indicate ORFs and the direction of transcription; lines represent parts of the 12.6 kb fragment remaining in the various subclones. The 'X' indicates a frameshift mutation at this point; P32 and *nisA* represent the P32 lactococcal promoter and the nisin-inducible *nisA* promoter, respectively. Bac and Imm indicate the phenotype for bacteriocin production and immunity of the different constructs, respectively (Imm +/– indicates that the immunity phenotype was dependent on the level of nisin used to induce the *nisA* promoter).

PCR fragment. Deletion of the *ltnF* gene in pOM01, to create pOM04, was accomplished by digestion with *Xba*I at three sites internal to this gene and subsequent religation. Similarly, pOM14, which contains intact *ltnR* and *ltnI*, was also created by *Xba*I digestion and religation. Mutagenesis of *ltnI* was achieved by cloning the 2.9 kb insert from pOM01 into pUC19. The resultant plasmid, pOM08, was digested with *Afl*III, the recessed 3' ends were filled in with the Klenow fragment from DNA polymerase I, and the plasmid was religated to create pOM20. The 2.9 kb insert from this plasmid, now with a frameshift mutation in *ltnI*, was subcloned into pCI372 to create pOM15. Plasmids pOM22, pOM23, pOM24 and pOM26 were generated by High Fidelity PCR and cloning. Primers used in plasmid construction are listed in Table 2 and all plasmids created in this study are graphically represented in Fig. 1.

Induction of strains with *nisA* promoter-containing plasmids.

L. lactis NZ9800 was transformed by electroporation with the plasmids pNZ8048 and pOM24. The transformation mixes were plated onto GM17 containing antibiotic only (no nisin was included in the plates at this stage). Transformants were initially grown in GM17 broth containing chloramphenicol (10 µg ml⁻¹); on subculturing, strains were induced with levels of purified nisin ranging from 0.5 to 50 ng ml⁻¹ (purified nisin was a kind gift from Michiel Kleerebezem, NIZO, Ede, The Netherlands). Induced overnight cultures were used in the agar-well-diffusion assay to assess the sensitivity of these strains. The same levels of nisin as in liquid media were used in solid media for these assays.

Bacteriocin preparation and assay. Concentrated lacticin 3147 was prepared as described previously (McAuliffe *et al.*, 1999)

from supernatant of *L. lactis* subsp. *lactis* DPC3147, and the activity determined by critical-dilution assay (Ryan *et al.*, 1996) using a well-diffusion assay. Basically, molten agar was cooled to 48 °C and seeded with the strain of interest (approx. 2 × 10⁷ fresh overnight-grown cells). The inoculated medium was dispensed into sterile Petri plates, allowed to solidify and dried. Wells (approx. 4.6 mm diameter) were made in the seeded agar plates. Aliquots (50 µl) of a twofold serial dilution of the bacteriocin preparation were dispensed into the wells, and the plates incubated overnight at the appropriate temperature for the strain in question. The arbitrary units (AU) ml⁻¹ were determined as described by Ryan *et al.* (1996).

Use of lacticin 3147 as a selective agent. *L. lactis* NZ9800 cells which had been electroporated with pOM24 (*LtnI*⁺) and pNZ8048 (*LtnI*⁻) were resuspended in 1 ml GM17 broth, or GM17 broth containing 10 ng purified nisin ml⁻¹. After incubation for 2 h at 30 °C, transformants were plated on GM17 plates containing chloramphenicol (10 µg ml⁻¹) and lacticin 3147 (250 AU ml⁻¹). An ammonium sulphate precipitation was used as a source of concentrated lacticin 3147.

RESULTS

Cloning and expression of a region encoding lacticin 3147 production and immunity

The nucleotide sequence of pMRC01, the plasmid encoding lacticin 3147 production and immunity, has been previously reported (GenBank accession no. AE001272; Dougherty *et al.*, 1998). The bacteriocin-coding region was inferred on the basis of homology to

Fig. 2. Inhibitory action of a concentrated lactacin 3147 preparation (left well) and a twofold serial dilution of the preparation (concentration decreasing from left to right) against various clones exhibiting different levels of immunity. (a) *L. lactis* MG1363 containing the various plasmids indicated; (b) *L. lactis* NZ9800 containing pOM24 induced with the concentrations of purified nisin indicated.

be located within a 12.6 kb region which contains 10 ORFs arranged in two divergent gene clusters (Fig. 1). This region was PCR amplified and cloned into the *E. coli*/*L. lactis* shuttle vector pCI372 (Hayes *et al.*, 1990), using *E. coli* as an intermediate host. The resultant plasmid, designated pOM02, was introduced into plasmid-free *L. lactis* subsp. *cremoris* MG1363 by electroporation, and a number of transformants were examined for their ability to produce lactacin 3147. In a number of independent clones, the plasmid conferred the ability to produce lactacin 3147 on MG1363, and overnight cultures of the transformants produced similar levels of bacteriocin (320 AU ml⁻¹) as MG1363 transconjugants containing the parental plasmid, pMRC01. pOM02 also conferred a significant level of immunity on MG1363 (Fig. 2), a strain normally quite sensitive to the bacteriocin. This level of immunity was comparable to that conferred by pMRC01 (data not shown). Consequently, it was concluded that this cloned region contained sufficient information to encode both lactacin 3147 production and immunity; this confirmed the region formerly proposed only on the basis of homology comparisons.

ltnI confers immunity on previously sensitive strains

The theory that the smaller four gene cluster (*ltnRIFE*) encoded lactacin 3147 immunity was largely based on the fact that two of the four genes, *ltnF* and *ltnE*, encode proteins with significant sequence similarities to bacterial multi-component ABC transporters involved in bacteriocin immunity (Peschel & Gotz, 1996; Rince *et*

al., 1997). To test this hypothesis, a 2.9 kb region containing all four genes and including the region between *ltnR* and the first structural gene, *ltnA1*, was amplified by PCR and cloned into pCI372 to create pOM01 (Fig. 1). MG1363(pOM01) became significantly more immune to lactacin 3147 than the plasmid-free strain (Fig. 2), although the immunity levels exhibited by MG1363(pOM02) were not observed. In order to verify a role for these genes in lactacin immunity, a number of deletion derivatives were constructed (Fig. 1), and transformed into MG1363. The effect of each deletion was assessed by means of the agar-well-diffusion assay using concentrated lactacin 3147. Unexpectedly, those constructs in which *ltnF* and *ltnE* were inactivated, both individually (pOM04, pOM05) and in combination (pOM14), conferred the same level of immunity on *L. lactis* MG1363 as pOM01 (data not shown). This confirmed that *ltnF* and *ltnE* are not involved in the levels of lactacin 3147 immunity exhibited by MG1363(pOM01). In addition, introducing the plasmid pOM26 (a construct with *ltnIRA1A2*; Fig. 1) into MG1363 resulted in transformants exhibiting the same level of immunity as strains harbouring pOM14 (*ltnRI*). To determine if this immunity phenotype was specific to this strain, pOM14, which contains *ltnRI*, was transformed into the heterologous host *Ent. faecalis* OG1X, which was then tested for immunity to lactacin 3147. Similar results were obtained in the heterologous host, in that pOM14 also conferred immunity on strain OG1X (data not shown).

Unlike the other putative proteins encoded by the four-gene cluster, *LtnI* does not display significant similarity to any known protein sequences. In contrast, *LtnR*, a 79 amino acid protein, is homologous to a number of transcriptional regulators of the repressor type (Fig. 3). Significantly, when plasmid pOM15, constructed from pOM01 by creating a frameshift mutation in *ltnI*, was introduced into MG1363, the immunity phenotype was lost and all transformants were as sensitive to concentrated lactacin 3147 as the plasmid-free strain. This indicated that the product of *ltnI* was involved in providing immunity against lactacin 3147. To confirm this observation, *ltnI* was cloned downstream of the strong lactococcal promoter in the expression plasmid pMG36e, creating pOM22. This plasmid conferred a significant level of immunity on MG1363, comparable to that seen with pOM02. These experiments confirm that *ltnI* alone, under the control of a strong promoter, is capable of conferring complete immunity on a sensitive strain.

Although there is significant similarity between the mechanisms of immunity described for a number of lantibiotics, to our knowledge there is only one reported case of cross-immunity between lantibiotic producers, i.e. Pep5 and epicidin 280 (Heidrich *et al.*, 1998). The interactions resulting in lactacin 3147 immunity appear to be highly specific, since the various *LtnI*⁺ constructs created in this study did not confer immunity to a number of other lantibiotics tested, including nisin, lactacin 481 and cytolysin (data not shown).

LtnR	M K K K F Q D S I S N S V Y K Y R V L S R	21
AF1793	M R T R I K E - - - - - F R A K F N	13
MTH1328	M R T L I R E - - - - - H R K E L G	13
MJ0272	M K N K L K Y - - - - - Y R A L H N	13

LtnR	L S Q Q D L A D K V G V S K Q T F V M	41
AF1793	M T Q E E L A K R V G V R R E T V F L	33
MTH1328	L T Q E E L A E R V G V T R Q T I A L	33
MJ0272	L T Q E D L A K K L G V S R Q T I A I	33

LtnR	E K G N Y T P S L L L A L R A N F F	60
AF1793	E K G K Y N P S L K L A Y K I A R V F	52
MTH1328	E K G R Y S P S L I L A H R I A R A L	52
MJ0272	E K G K Y D P S L K L A F K I A K F F	52

LtnR	D - V E T T K F T Y E P G G H K N E E	79
AF1793	N - A K I E D F I F D E E L W E K R	71
MTH1328	G R E H I E D F I L D E D G - - A K	69
MJ0272	G - V K I E D F I Y E D D E	66

Fig. 3. Alignment and sequence comparison of the predicted amino acid sequence of LtnR with a number of putative transcriptional regulators of the PBSX (Xre) family. AF1793, produced by *Archaeoglobus fulgidus*; MTH1328, produced by *Methanobacterium thermoautotrophicum*; MJ0272, produced by *Methanococcus jannaschii*. Identical residues in all four proteins are indicated by shading; boxes indicate where residues are identical in three of four proteins. Amino acid residues are numbered; residues 16–35 of AF1793, MTH1328 and MJ0272 contain a helix–turn–helix motif, indicative of DNA-binding sites.

It is possible, given the lower levels of immunity conferred by pOM01 (*ltnRIFE*) in comparison to pOM02 (containing the complete 12.6 kb insert), that immunity is a regulated phenomenon, and that LtnR, which is homologous to the PBSX (Xre) family of transcriptional repressors (Wood *et al.*, 1990), may play a role in this regulation. To investigate this possibility, a deletion was made in plasmid pOM14 (*ltnRI*) to eliminate *ltnR*. An MG1363 transformant harbouring the resulting plasmid, pOM23 (Fig. 1), exhibited the same levels of immunity to lacticin 3147 as MG1363-(pOM14), and preliminary mRNA analysis suggests that the level of *ltnI* transcription is not affected by deletion of *ltnR* (data not shown).

The level of immunity depends on *ltnI* transcription

A fragment encoding *ltnI* was amplified and cloned into the expression plasmid pNZ8048 under the control of the nisin-inducible *nisA* promoter (de Ruyter *et al.*, 1996). The resulting plasmid, pOM24 (Fig. 1), was transformed into the expression host *L. lactis* subsp. *cremoris* NZ9800 (Kuipers *et al.*, 1993), a strain which contains the nisin operon in the chromosome. This strain does not produce nisin due to a 4 bp deletion in *nisA*, the nisin structural gene. Increasing concentrations of purified nisin can be supplied exogenously to induce the *nisA* promoter, resulting in increasing levels of gene

Fig. 4. (a) Predicted amino acid sequence of LtnI; the box indicates the leucine-zipper motif. (b) Hydrophobicity profile of LtnI, determined according to the method of Kyte & Doolittle (1982). Bars indicate hydrophobic segments proposed to be membrane spanning.

expression. Nisin concentrations ranging from 0.5 to 50 ng ml⁻¹ were used to induce expression of *ltnI* and the strains were assayed as before. As can be seen from Fig. 2, increasing the level of inducer resulted in a concomitant increase in the level of immunity of NZ9800, and the promoter strength could be adjusted to a point where a level of immunity similar to that seen in strains containing pMRC01 was observed.

Characterization of LtnI, a novel lantibiotic immunity protein

Having confirmed a role for LtnI in lacticin immunity, we further examined the predicted amino acid sequence to establish the nature of the protein. As previously mentioned, LtnI did not show any significant sequence similarity to any known proteins. The presence of a leucine zipper motif (Leu-6-Leu-6-Leu-6-Leu; Fig. 4a) in the C-terminus of the 116 amino acid protein suggests that this protein forms homodimers in order to create an active complex. Leucine zipper motifs are usually associated with DNA-binding proteins such as transcription factors (Abel & Maniatis, 1989); the leucine residues are critical for the dimerization of these proteins. Kyte & Doolittle (1982) hydrophobicity plots revealed the presence of three hydrophobic domains (aa 30–49, 68–81, 91–112; Fig. 4b), suggesting a transmembrane location for this protein. An immunity protein of this type has not been found previously in other lantibiotic systems.

Table 3. Comparison of the efficiency of *ltnI* with that of an antibiotic-resistance gene as a selectable marker in transformation experiments

Plasmid	No.* of transformants of <i>L. lactis</i> NZ9800 with the following selection marker†			
	Cm	Cm/Ltn	Cm‡	Ltn‡
pNZ8048	243	0	248	0
pOM24	283	0	250	194
pOM24§	> 300	0	290	238

* $10^{-2} \times$ No. of colonies per μg DNA.

† Cm, 10 μg chloramphenicol ml^{-1} ; Ltn, 250 AU lacticin ml^{-1} .

‡ Nisin added to selection plates at an inducing concentration of 10 ng ml^{-1}

§ No nisin added to outgrowth medium.

ltnI can be used as a selectable marker

To evaluate the feasibility of using lacticin 3147 to select for *LtnI*⁺ transformants in *Lactococcus*, NZ9800 cells transformed with pOM24 and pNZ8048 (*LtnI*[−] control) were plated onto agar containing lacticin 3147, and the number of transformants compared to that obtained on the antibiotic plates. Purified nisin was used, in the outgrowth medium and in the selection plates, to induce the *nisA* promoter for expression of *LtnI*. The results from a representative experiment are presented in Table 3; a number of experiments established that these results were reproducible. Similar numbers of colonies were obtained on both the antibiotic and lacticin plates when the cells were induced with nisin, whereas NZ9800 transformed with pNZ8048 did not yield any colonies on lacticin plates, as expected. Inclusion of nisin in the outgrowth medium only did not allow expression of *ltnI*, presumably because the induction time was too short. All transformants tested from the lacticin selection plates were capable of growth on chloramphenicol and the presence of pOM24 was confirmed in a number of cases, demonstrating the efficiency of lacticin 3147 as a food-grade selective agent.

DISCUSSION

To date, at least two different mechanisms of lantibiotic immunity have been identified. The presence of putative immunity proteins located at the outer surface of the cytoplasmic membrane, as in the nisin, subtilin and Pep5 systems (Kuipers *et al.*, 1993; Klein & Entian, 1994; Pag *et al.*, 1999) may result in an interaction with the bacteriocin molecules to prevent pore formation. In addition, ABC transport systems have been shown to play a role in immunity, possibly by active transport of the bacteriocin from the membrane (Siegers & Entian, 1995; Peschel & Gotz, 1996; Rince *et al.*, 1997). In this study, we provide evidence that a novel protein, *LtnI*, is responsible for immunity to the two-component lanti-

biotic lacticin 3147 and describe how this protein is capable of conferring wild-type levels of immunity on a previously sensitive strain. This novel protein may represent a new class of immunity mechanism.

Cloning and expression of a 12.6 kb fragment of pMRC01 in *L. lactis* MG1363 confirmed the involvement of genes already identified (Dougherty *et al.*, 1998) in lacticin 3147 production and immunity. It seems unlikely that genes outside of this region are involved in bacteriocin production, as the level of bacteriocin produced by cells harbouring pOM02 was similar to that produced by transconjugants containing pMRC01. The operon-like arrangement of the genes for processing, transport and immunity is a common feature of lantibiotic gene clusters; however, Northern analysis of the lacticin-coding region is required to ascertain if the genes in this region are transcribed as operons. Future work will involve mutational analysis of the various genes in this region, as well as site-directed mutagenesis of the structural genes, in order to gain an insight into the biosynthetic pathway of lacticin 3147.

Further subcloning and deletion analysis confirmed that the immunity region is associated with *ltnRIFE*; however, surprisingly, *ltnFE* did not appear to be involved, as *ltnRI* conferred the same level of immunity as the four-gene cluster. However, the levels of immunity exhibited by strains harbouring constructs containing these genes under the control of their own promoter were reduced compared to that of the wild-type strain, and also compared to MG1363(pOM02). Similar observations have been reported in other lantibiotic systems. In the nisin system, it was initially observed that expression of the structural gene and the level of immunity were correlated. Kuipers *et al.* (1993) demonstrated that mutations in the structural gene, *nisA*, resulted in strains which were more sensitive to exogenously applied nisin, and clones expressing the nisin immunity gene, *nisl*, in the absence of *nisA* showed quite a low level of immunity. This was shown later to be a result of nisin autoregulation; mature active nisin is essential to activate transcription of the nisin gene cluster by signal transduction at both the *nisA* and *nisFEG* promoters, which control the expression of the *nisl* and *nisFEG* genes respectively, via the two-component regulator NisRK (Kuipers *et al.*, 1995; Dodd *et al.*, 1996). With Pep5, it was initially observed that mutants harbouring the immunity gene, *pepI*, without the structural gene, *pepA*, or with a deleted *pepA* were not able to express the Pep5 immunity phenotype and did not produce the *pepI* gene product (Reis & Sahl, 1991; Reis *et al.*, 1994). However, recently Pag *et al.* (1999) have shown that *pepI* is sufficient for immunity, and that the apparent coupling of immunity to Pep5 production occurs at the level of transcription by the presence of a transcriptional terminator located downstream of *pepA* which acts to stabilize *pepI*-containing transcripts. In the lacticin 3147 system, strains containing constructs with *ltnI* and the lacticin structural genes, *ltnA1* and *ltnA2*, were found not to exhibit the levels of immunity seen in the parent strain. It is possible

that the level of immunity exhibited by the parent is in some way influenced by production of active lactacin 3147, and not solely by the presence of the structural genes. In fact, 'wild-type' immunity levels were not observed in any construct containing the native promoter. This may be due to regulatory factors on the parent plasmid which are absent from these constructs, as seen in the Pep5 system, although there is no evidence of a similar transcriptional terminator following the structural genes in the lactacin 3147 coding region. It should, however, be considered that perhaps other genes in the 12.6 kb bacteriocin region have a role in the levels of immunity exhibited by the producer. Recently, Ra *et al.* (1999) reported that while NisI has an important function in nisin immunity, cooperation from NisFEG is necessary for full nisin immunity. Similar cooperative interactions may occur in the lactacin 3147 system; this remains to be investigated.

Given that *ltnR* shows significant homology to a family of transcriptional regulators, it seemed likely that *ltnI* may represent the actual immunity gene. This was confirmed when it was shown that immunity levels similar to those conferred by pMRC01 were exhibited by strains harbouring *ltnI* cloned under the control of the strong lactococcal promoter in pMG36e. Also, *ltnI* cloned downstream of the *nisA* promoter in the nisin-inducible system was used to demonstrate that the level of immunity is dependent on the level of transcription of *ltnI*, since increasing the concentration of nisin resulted in an increase in the level of immunity. It could be conceived then that the promoter controlling the transcription of *ltnI* in the wild-type strain is somehow regulated or induced. Homology comparisons revealed that the gene product of *ltnR* is similar to the PBSX (Xre) family of transcriptional regulators (Wood *et al.*, 1990; McDonnell *et al.*, 1994; McDonnell & McConnell, 1994). It has been proposed that the Xre protein regulates transcription of its own gene and divergently transcribed genes by binding to a series of operator sites in the region between these genes. The system has been compared to that of the immunity regions of λ -type phages, where an autoregulated repressor is divergently transcribed from genes which are also controlled by the repressor (Ptashne, 1992). It is possible that LtnR may act similarly and serve to coordinate expression of the transcripts from the divergent gene clusters in the lactacin 3147 coding region, ensuring that active lactacin 3147 is produced only when immunity has been established. A possible operator site for this putative regulator is the intergenic region between *ltnR* and *ltnA1*. This region contains a number of direct and palindromic repeats including an 8 bp inverted repeat in the vicinity of a number of sequences consistent with consensus promoter sequences. The plasmid pOM23, created by deletion of *ltnR* from pOM14, conferred the same level of immunity on MG1363 as pOM14. This result is in agreement with preliminary mRNA analysis, but not necessarily with the role for LtnR proposed here, as the level of immunity might be expected to increase. It is, however, important

to note that other regulatory elements may be involved in the wild-type, which are not present on pOM23, and also that other genes, besides *ltnI*, may be involved in immunity. Nevertheless, the presence of *ltnR* appears to provide the only evidence of regulation in the bacteriocin-coding region. A transcriptional regulator has also been identified in the epidermin gene cluster, and has been shown to positively activate gene expression. Peschel *et al.* (1993) demonstrated that EpiQ, a 205 amino acid protein, binds to palindromic sequences in the *epiA* promoter region and activates the immunity gene cluster of *epiFEG*. Genes homologous to the *lanR* and *lanK* genes encoding two-component regulatory systems, identified in the operons of other characterized lantibiotics (Engelke *et al.*, 1994; Klein *et al.*, 1993) have not been found in the lactacin 3147 operon, suggesting that a quorum-sensing mechanism (Kleerebezem *et al.*, 1997) does not regulate gene expression in this system.

A role for lactacin 3147 as a food-grade selectable marker has also been demonstrated. It has previously been reported that the parent plasmid, pMRC01, can be electroporated into *L. lactis* MG1614 using lactacin 3147 as a selection (Coakley *et al.*, 1997). The results presented here show that *ltnI* alone, at high expression levels, confers a level of immunity which can be used to select transformants in the absence of antibiotic selection. A study performed employing *lafl*, the lactacin F immunity gene, as a selectable marker yielded similar results (Allison & Klaenhammer, 1996); in that case, it was concluded that *lafl* confers immunity specifically to lactobacilli, and therefore has certain limitations for use as a food-grade marker. In contrast, we have shown that the immunity phenotype conferred by *ltnI* can be expressed in a heterologous host, namely *Ent. faecalis*. This may be an important factor in considering *ltnI* for use in the construction of a food-grade cloning vector.

That this protein is most likely localized at the cytoplasmic membrane is strongly suggested by the presence of three putative transmembrane domains, each consisting of approximately 20 amino acids. While the mode of action of LtnI remains to be determined, the absence of a typical signal sequence implies that LtnI is not a secreted protein, and therefore may be an integral membrane protein where it may serve to hinder the insertion of the bacteriocin molecules in the membrane, or it may interact directly with and inactivate the bacteriocin. There is little sequence similarity between LtnI and CylI (Coburn *et al.*, 1999), the protein which confers immunity to the two-component cytolysin; it will be interesting to determine whether a similar immunity gene is present in the gene system of the two-component lantibiotic staphylococcal C55 (Navaratna *et al.*, 1998), especially given the high degree of similarity between this system and lactacin 3147.

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