Each peptide of the two-component lantibiotic lacticin 3147 requires a separate modification enzyme for activity

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The genetic determinants for production and immunity to the two-component lantibiotic lacticin 3147 are encoded by a 12.6 kb region of the plasmid pMRC01. This region contains ten genes arranged in two divergent clusters; these include the structural genes and a number of genes whose products show significant similarity to proteins involved in the biosynthesis of other lantibiotics. Using a strategy of deletion and mutational analysis, the effect of disruption of a number of these genes was investigated. Inactivation of either of the structural genes, *ltnA1* or *ltnA2*, resulted in mutants that were incapable of producing active lacticin 3147; however, the combination of the cell-free supernatant from both mutants resulted in a restoration of bacteriocin activity, confirming that processing and export of the structural peptides can occur independently. An unusual feature of the lacticin 3147 gene cluster is the presence of two *lanM* homologues, whose gene products are proposed to be involved in the dehydration and thioether-forming reactions which result in lanthionine bridge formation. Mutants created in the *ltnM1* and *ltnM2* genes were also incapable of lantibiotic production, confirming an essential role for these enzymes in the lacticin 3147 biosynthetic pathway and supporting the assertion that these proteins are modification enzymes. Interestingly, addition of purified LtnA1, but not purified LtnA2, to the cell-free supernatant of the *ltnM1* mutant restored bacteriocin activity; in contrast, only purified LtnA2 could complement the cell-free supernatant of the *ltnM2* mutant. Creation of a number of double mutants supported these findings, and confirmed that LtnM1 is required to produce mature LtnA1, while LtnM2 is required to produce mature LtnA2.

Keywords: lacticin 3147, lantibiotic production, immunity, modification enzymes

INTRODUCTION

Lacticin 3147 is a two-component bacteriocin produced by *Lactococcus lactis* subsp. *lactis* DPC3147 (Ryan *et al.*, 1996; McAuliffe *et al.*, 1998). Both components are required for the activity of the bacteriocin, which has been shown to result in the formation of ion-specific pores in the membrane of Gram-positive cells (McAuliffe *et al.*, 1998). Lacticin 3147 belongs to the class of antimicrobials known as the lantibiotics (Ryan *et al.*, 1999), a group of post-translationally modified peptides characterized by the presence of a high pro-

portion of unusual amino acids, including the thioether amino acids lanthionine and β -methyllanthionine (Schnell *et al.*, 1988; Sahl *et al.*, 1995). The presence of these residues was first identified in nisin (Berridge *et al.*, 1952) and further analysis has revealed that lanthionine and β -methyllanthionine originate from the sequence-specific dehydration of the hydroxyl amino acids serine (to didehydroalanine) and threonine (to didehydrobutyrine) (Ingram, 1969). These dehydrated amino acids possess electrophilic centres which enable them to react with neighbouring cysteine residues. The thioether lanthionine is formed when the double bond of didehydroalanine is attacked by the thiol group of cysteine; β -methyllanthionine results when the reaction partner is didehydrobutyrine (Ingram, 1969). Some

Abbreviations: ABC, ATP-binding cassette; AU, arbitrary units; CF5, cell-free supernatant.

lantibiotics contain further modified residues, such as S-aminovinylcysteine in mature epidermin (Kupke *et al.*, 1994) and D-alanine, first observed in mature lactocin S (Skaugen *et al.*, 1994), and more recently identified in both lactacin 3147 peptides (Ryan *et al.*, 1999).

The events leading to the production of a mature lantibiotic begin with the synthesis of a gene-encoded precursor peptide composed of a leader peptide and a propeptide region. This latter region is extensively modified prior to processing of the leader peptide and translocation across the membrane, as first proposed for epidermin by Schnell *et al.* (1988). The genes encoding the biosynthetic machinery of the lantibiotics characterized to date are arranged in either plasmid- or chromosomally-encoded gene clusters which encode a range of enzymes, including specific proteases (involved in removal of the leader sequence), regulatory proteins, immunity proteins and transport proteins [members of the ATP-binding cassette (ABC)-superfamily] (Jack *et al.*, 1995; Sahl *et al.*, 1995; Siezen *et al.*, 1996). In addition, a number of genes have been identified whose products do not share sequence similarity with any known proteins. These include *lanB* and *lanC* in the nisin-like lantibiotics (Engelke *et al.*, 1992; Gutowski-Eckel *et al.*, 1994; Meyer *et al.*, 1995), and *lanM* in the lantibiotic subclass containing lactacin 481 (Rince *et al.*, 1994), lactocin S (Skaugen *et al.*, 1997) and the two-component lantibiotics, cytolysin (Gilmore *et al.*, 1994) and staphylococcin C55 (Navaratna *et al.*, 1999). Disruption of these genes in various lantibiotic systems has revealed their essential role in biosynthesis, as production of active bacteriocin is abolished in their absence (Augustin *et al.*, 1992; Klein *et al.*, 1992; Gilmore *et al.*, 1994; Siegers *et al.*, 1996). It has been suggested, therefore, that these proteins are strong candidates as catalysts for the novel reactions responsible for the dehydration of hydroxyamino acids and subsequent thioether ring formation, although the molecular mechanisms involved remain to be elucidated. For example, it has been demonstrated that inactivation of PepC in a Pep 5-producing strain resulted in the secretion of completely dehydrated Pep 5 peptides; however, the majority of these peptides did not contain thioether amino acids (Meyer *et al.*, 1995). More recently, Karakas Sen *et al.* (1999) reported the obvious importance of NisB in the dehydration of serine 33 to didehydroalanine 33 in mature nisin. Therefore, it is proposed that the dehydration of serine and threonine is catalysed by LanB, while LanC catalyses lanthionine ring formation. It is likely that the *lanM* gene products combine the functions of dehydration and ring formation, although this has yet to be proven.

The genes for lactacin 3147 production and immunity are encoded on a 60.2 kb conjugative plasmid, pMRC01; the complete sequence of pMRC01 has been reported previously (GenBank accession no. AE001272; Dougherty *et al.*, 1998). It was proposed that up to ten ORFs identified in pMRC01 were involved in lactacin 3147 biosynthesis and immunity on the basis of sequence similarity to proteins from other lantibiotic biosynthetic

pathways, including transport and modification proteins (Dougherty *et al.*, 1998). An unusual feature is the presence of two *lanM* homologues within the gene cluster. Only one such gene is found in the cytolysin gene cluster (Gilmore *et al.*, 1994). In the staphylococcin C55 system, one *lanM* homologue, *sacM1*, has been completely sequenced (Navaratna *et al.*, 1999), while an incomplete ORF shows significant sequence similarity to *ltnM2* (GenBank accession no. AF147744). The role of the lactacin 3147 gene clusters was confirmed on cloning a 12.6 kb region of pMRC01, containing the ten ORFs implicated in production and immunity, in *L. lactis* MG1363 (McAuliffe *et al.*, 2000). The resultant strain, MG1363 (pOM02), exhibited wild-type immunity and produced similar levels of lactacin 3147 as did MG1363 harbouring the parental plasmid, pMRC01. A role in immunity has also been confirmed for the gene product of *ltnI* (McAuliffe *et al.*, 2000). Here, the functional analysis of *ltnM1* and *ltnM2*, genes whose products are proposed to be involved in the dehydration and thioether-forming reactions in mature lactacin 3147, is presented. We report the essential role of these putative modification enzymes in the production of active lactacin 3147, and demonstrate that each of the prepeptides of lactacin 3147 requires a separate modification enzyme.

METHODS

Bacterial strains, plasmids and media. The bacterial strains and plasmids used in this study are listed in Table 1. Lactococci were grown at 30 °C without aeration in M17 broth (Oxoid) supplemented with 0.5% glucose (GM17). *Escherichia coli* strains were grown in LB broth (Sambrook *et al.*, 1989) at 37 °C with vigorous shaking. Agar media were prepared by adding 1.5% granulated agar (Difco) to broth, whereas overlay agar was prepared with 0.7% agar. Antibiotics used in the selection media were added at the following concentrations: ampicillin, 100 µg ml⁻¹; and chloramphenicol, 20 µg ml⁻¹ (*E. coli*) and 5 µg ml⁻¹ (*L. lactis*). LB agar supplemented with 40 µg X-gal ml⁻¹ and 40 µg IPTG ml⁻¹ were used to screen for insertional inactivation of the α -lacZ gene in pUC19. Chemical reagents listed were obtained from Sigma.

Bacteriocin preparation. Concentrated lactacin 3147 was prepared as described previously (McAuliffe *et al.*, 1999) from supernatants of *L. lactis* subsp. *lactis* DPC3147 cultured in MRS broth (Oxoid). The activity of the concentrate was determined by critical dilution (Parente & Hill, 1992) using a well-diffusion assay. Basically, molten agar was cooled to 48 °C and seeded with the strain of interest (approx. 2×10^7 fresh overnight-grown cells). The inoculated medium was dispensed into sterile Petri plates, allowed to solidify and dried. Wells (approx. 4.6 mm in 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 30 °C. The arbitrary units per millilitre (AU ml⁻¹) were calculated as the reciprocal of the last dilution that gave a distinct zone of inhibition (1/dilution $\times$ 20).

DNA preparation and transformation. Plasmid DNA was isolated from *E. coli* strains using the Qiagen column purification kit, and from *L. lactis* using the method of Anderson & McKay (1983). pMRC01 DNA (Ryan *et al.*,

Table 1. Bacterial strains and plasmids

Strain/plasmid	Relevant properties*	Source/reference†
Strains		
<i>L. lactis</i>		
DPC3147	Lacticin 3147 producer	Ryan <i>et al.</i> (1996)
HP	Indicator organism	DPC collection
AM2	Indicator organism	DPC collection
MG1363	Plasmid-free cloning host	Gasson (1983)
<i>E. coli</i>		
XL1-Blue	Intermediate cloning host	Stratagene
EC1000	Intermediate cloning host	Leenhouts <i>et al.</i> (1996)
Plasmids		
pCI372	Cm ^R ; cloning vector	Hayes <i>et al.</i> (1990)
pUC19	Amp ^R ; cloning vector	Yanisch-Perron <i>et al.</i> (1985)
pMRC01	Ltn ⁺ Imm ⁺	Ryan <i>et al.</i> (1996)
pOM02	Lacticin gene cluster in pCI372	McAuliffe <i>et al.</i> (2000)
pOM09	<i>ltnEFIRA1A2M1</i> in pUC19	This study
pOM10	<i>ltnTM2D</i> in pUC19	This study
pOM13	pOM09 Δ <i>ltnM1</i>	This study
pOM18	pOM02 Δ <i>ltnM1</i>	This study
pOM30	pOM10 Δ <i>ltnM2</i>	This study
pOM31	pOM02 Δ <i>ltnA2</i>	This study
pOM32	pOM02 Δ <i>ltnM2</i>	This study
pOM33	pOM02 Δ <i>ltnA2M2</i>	This study
pOM39	pOM02 Δ <i>ltnA1</i>	This study
pOM47	pOM02 Δ <i>ltnA1M1</i>	This study

*Ltn, lacticin 3147 production; Imm, immunity to lacticin 3147; Cm^R, chloramphenicol resistance; Amp^R, ampicillin resistance.

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1996), which was used as template DNA in PCR, was purified by equilibrium centrifugation in CsCl/ethidium bromide gradients (Sambrook *et al.*, 1989). *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.

General molecular techniques. PCR was performed by following standard procedures using BIOTAQ DNA polymerase (Bioline) in a GeneAmp PCR Systems 2400 DNA thermal cycler (Perkin-Elmer). The Expand High Fidelity PCR System (Roche Biochemicals) 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 Roche Biochemicals was used according to the manufacturer's instructions. Oligonucleotide primers for PCR were synthesized using an Applied Biosystems PCR-MATE DNA synthesizer. Restriction digestion, Klenow treatment and DNA ligations were performed according to standard procedures (Sambrook *et al.*, 1989). Restriction enzymes, the Klenow fragment of *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.

Plasmid construction and mutagenesis. The following strategies were used to analyse the genes in the lacticin 3147

gene cluster. Mutagenesis of *ltnM1* was achieved by sub-cloning a 6.6 kb fragment from pOM02, representing approximately the first half of the bacteriocin-coding region (*ltnEFIRA1A2M1*), into pUC19. The resultant plasmid, pOM09, was digested with *Cla*I, the recessed 3' ends were filled in with the Klenow fragment from DNA polymerase I and the plasmid was religated to create pOM13. The 6.6 kb insert from pOM13, now with a frameshift in *ltnM1*, was subcloned into pCI372 containing a 6.0 kb fragment, representing the remainder of the bacteriocin-coding region, creating plasmid pOM18. A similar strategy was used to create pOM32; a 6.0 kb fragment from pOM02, containing the genes *ltnTM2D*, was cloned into pUC19, creating pOM10. pOM10 was subsequently digested with *Cla*I, filled-in and religated, creating the plasmid pOM30 with a frameshift in *ltnM2*. The mutated 6.0 kb fragment was used to replace the original fragment in pOM02, resulting in the plasmid pOM32. Plasmids pOM31 and pOM39 were generated as follows: in each case, primers with built-in restriction sites were designed to amplify the first half of the bacteriocin-coding region in two separate fragments, which resulted in deletion of part of the genes of interest, i.e. *ltnA1* and *ltnA2*. These primers are listed in Table 2. The relevant fragments were amplified by High Fidelity PCR, ligated and used to replace the original 6.0 kb fragment in pOM02, creating pOM31 (Δ *ltnA2*) and pOM39 (Δ *ltnA1*). The double mutants, pOM33 and pOM47, were created by mutagenesis of *ltnM2* in pOM31 and *ltnM1* in pOM18, respectively, as described above for pOM32. Rel-

Table 2. Primers used in plasmid construction

Plasmid*	Primer no.†	Primer sequence (5′–3′)‡	Temp (°C)	Position in gene cluster	
pOM31 (1/2)	3951b (F)	AAAAGAGCTCTGCGAATAACATCAAGGGA	55	1–18	
	5434 (R)	ATGGTACCCAGCTAATTCAATCATATC	55	3183–3200	
	(2/2)	5431 (F)	ATGGTACCAATACTTGTCCAACAACA	55	3276–3293
	5144 (R)	GATGGATCCATTATTATTACTGTT	50	6992–6607	
pOM39 (1/2)	3951b (F)				
	5866 (R)	AAGGTACCCTGGTTGTGTTTCAA	55	2915–2928	
	(2/2)	5865 (F)	AAGGTACCTGTACACTCACTCAT	55	3040–3054
	5144 (R)				

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

† F, forward; R, reverse.

‡ Restriction sites in primer sequences are underlined.

evant plasmids are graphically represented in Fig. 1. Whenever PCR was used in plasmid construction, a number of independent clones were tested to confirm the associated phenotype.

RESULTS AND DISCUSSION

The type-A group of lantibiotics have previously been classified into two subgroups based on the sequence of their leader peptides and the composition of their biosynthetic gene clusters (Jung, 1991; de Vos *et al.*, 1995). Analysis of the products of the lacticin 3147 gene cluster (Dougherty *et al.*, 1998) revealed prepeptides with 'double-glycine'-type leader sequences, in addition to two LtnM proteins and LtnT, an ABC transporter with an N-terminal proteolytic domain. Consequently, the presence of such gene products clearly identifies lacticin 3147 as a member of the type-A(II) group, which also includes lacticin 481 (Rince *et al.*, 1994) and another two-component lantibiotic, the haemolysin/bacteriocin cytolysin (Gilmore *et al.*, 1994).

Cloning and expression of a 12.6 kb region which contains the lacticin 3147 ten-gene cluster to create the plasmid pOM02 has previously been described (McAuliffe *et al.*, 2000). To gain an insight into the biosynthetic pathway of lacticin 3147, a strategy of deletion and/or mutational analysis was adopted to functionally analyse some of the genes in the lacticin 3147 gene cluster. A number of derivatives of pOM02 were constructed as described in the Methods section and are shown in Fig. 1. Each construct was isolated from *E. coli* XL1-Blue and electroporated into the non-producing, lacticin-sensitive strain *L. lactis* MG1363. Resultant transformants were confirmed by plasmid isolation and restriction analysis. Phenotypic analysis of the mutants involved determination of the levels of lacticin 3147 production and immunity, in addition to complementation between the products of strains harbouring different constructs.

Inactivation of either *ltnA1* and *ltnA2* results in loss of lacticin 3147 production

Previously, it has been shown that lacticin 3147 is a two-component bacteriocin composed of two small (3–4 kDa) peptides (McAuliffe *et al.*, 1998). The genes *ltnA1* and *ltnA2* (originally referred to as *ltnA* and *ltnB*) were originally proposed as candidates for the lacticin 3147 structural genes (Dougherty *et al.*, 1998) on the basis that they encode prepropeptides that are rich in serine, threonine and cysteine residues that could be acted on to give rise to dehydrated amino acids, and subsequently, lanthionine rings (Dougherty *et al.*, 1998). In addition, *ltnA1* and *ltnA2* are located directly upstream of genes whose products are homologous to proteins involved in lantibiotic maturation, a context similar to the structural genes of some other lantibiotic gene clusters. Recent chemical analysis, including a combination of MS, amino acid analyses and N-terminal sequencing has confirmed *ltnA1* and *ltnA2* as the lacticin 3147 structural genes, and also confirmed the presence of lanthionine residues in each peptide (Ryan *et al.*, 1999).

Plasmids pOM39 ($\Delta ltnA1$) and pOM31 ($\Delta ltnA2$) (Fig. 1) were created as described in the Methods section, using *E. coli* XL1-Blue as the intermediate host. Subsequent transformation of these plasmids into *L. lactis* MG1363 resulted in transformants that exhibited wild-type levels of immunity to exogenously applied lacticin 3147 (using a lacticin 3147 stock solution of 160 000 AU ml⁻¹; data not shown). It has been previously reported by Ryan *et al.* (1999) that LtnA1 has independent antimicrobial activity. However, this activity was only observed following a purification protocol which gave rise to high concentrations of LtnA1, concentrations which would not be reached in the culture supernatant. Therefore given that, at low concentrations, both peptide products are required for activity, deletion of either of the lacticin 3147 structural genes would be expected to result in a loss of the bacteriocin-producing phenotype. Pre-

Fig. 1. Schematic representation of the organization of various constructs and the phenotype associated with these mutated derivatives. Arrows indicate ORFs and the proposed direction of transcription. Black arrows represent the ORFs remaining in the various subclones; grey arrows indicate those ORFs which have been deleted/mutated. 'X' indicates a frameshift mutation at this point; square brackets indicate where an ORF has been deleted. Important restriction sites are marked; *SacI* and *SalI* are engineered sites. Bac, bacteriocin production; + + +, wild-type levels; -, no activity detected. *, Bacteriocin activity was restored by the addition of purified LtnA1 or LtnA2 as indicated.

dictably, neither mutant was able to generate active lactacin 3147, in that the transformants failed to inhibit a number of test strains including the hypersensitive indicator *L. lactis* subsp. *cremoris* AM2 in either direct or deferred antagonism assays (data not shown). However, it proved possible to restore lactacin 3147 activity to the cell-free supernatant (CFS) of the $\Delta ltnA1$ mutant by the simple addition of purified LtnA1, or by supplying purified LtnA2 to the $\Delta ltnA2$ CFS (data not shown). Furthermore, combining the CFS of both mutants also restored activity (data not shown). These results confirm that each mutant is capable of producing one modified and active peptide, and that processing and export of LtnA1 and LtnA2 can be carried out independently.

The gene products of *ltnM1* and *ltnM2* are also essential for the production of active lactacin 3147

Two large genes, *ltnM1* and *ltnM2*, are present downstream of the structural genes, separated by an ORF encoding the putative bacteriocin translocator, *ltnT* (Fig. 1). These genes encode the proteins LtnM1 (980 amino acids) and LtnM2 (814 amino acids), respectively (Dougherty *et al.*, 1998). Both LtnM1 and LtnM2 share sequence similarity with CylM, an enzyme proposed to be involved in the post-translational modification of the haemolysin/bacteriocin, cytolysin (Gilmore *et al.*, 1994), and a protein implicated in lactacin 481 modification, LctM (Rince *et al.*, 1994).

The role of the products of the *ltnM1* and *ltnM2* genes in the biosynthesis of lactacin 3147 was investigated by creation of frameshift mutations within each of these genes in pOM02, creating the plasmids pOM18 ($\Delta ltnM1$) and pOM32 ($\Delta ltnM2$). This was achieved by filling-in *ClaI* sites in each of the genes, as described in the Methods section. The resultant plasmids were then introduced into *L. lactis* MG1363 by electroporation, and the phenotypic properties of a number of transformants examined. In all cases, MG1363 harbouring either pOM18 ($\Delta ltnM1$) or pOM32 ($\Delta ltnM2$) was immune to exogenously applied lactacin 3147 (160 000 AU ml⁻¹; Fig. 2a). In addition, neither mutant was capable of producing active lactacin 3147. This con-

firmed that while both LtnM1 and LtnM2 play an important role in lactacin 3147 production, they do not contribute to immunity. This supports the initial presumption that these proteins are indeed modification enzymes.

LtnA1 and LtnA2 require separate LtnM enzymes for activity

Although there was no activity associated with the CFS of the *ltnM1* or *ltnM2* mutants (Fig. 2b), it was possible to restore bacteriocin activity by combining the CFS of both strains in a single well (Fig. 2b). If the CFS from each mutant was placed in closely adjacent wells, a zone of inhibition was observed between the wells (Fig. 2b). This suggested that each mutant was producing a single active peptide. Confirmation of this, and an indication of which peptide is being produced, was provided when CFS of both the *ltnM1* and *ltnM2* mutants were combined with purified FPLC fractions corresponding to the lactacin 3147 components LtnA1 and LtnA2. On combining CFS from the *ltnM1* mutant with fractions corresponding to the A1 peptide, bacteriocin activity was restored against *L. lactis* subsp. *cremoris* HP (Fig. 2c), but not on combination with the A2 peptide (data not shown). In contrast, inhibition was observed only when the A2 peptide was added to the CFS from the *ltnM2* mutant (data not shown).

To further substantiate the proposition that LtnM1 acts to create active LtnA1, while LtnM2 acts on LtnA2, we were able to show that addition of the CFS from the *ltnA2* mutant restored activity to the CFS of the *ltnM1* mutant, but not to the *ltnM2* mutant (data not shown). As expected, combining the CFS from the *ltnA1* mutant with that of the *ltnM2* mutant, but not the *ltnM1* mutant, also resulted in a restoration of lactacin 3147 activity.

If the modification of each prepeptide is truly independent then it should be expected that double *ltnA1M1* and *ltnA2M2* mutants should each produce one active peptide, modified A1 and A2, respectively. To confirm this theory, two such double mutants were created. Firstly, plasmid pOM33 ($\Delta ltnA2M2$, Fig. 1) was created

Fig. 2. (a) Inhibitory activity of a twofold serial dilution of concentrated lactacin 3147 ($160\,000\text{ AU ml}^{-1}$) against *L. lactis* MG1363(pOM18) ($\Delta ltnM1$; upper panel), and the plasmid-free *L. lactis* MG1363 (lower panel). (b) Activity of the cell-free supernatant of *L. lactis* MG1363(pOM18) (well $\Delta M1$) and *L. lactis* MG1363(pOM32) (well $\Delta M2$) against the sensitive indicator *L. lactis* subsp. *cremoris* HP. Heat-treated supernatant (25 μ l) was added to each well. $\Delta M1 + \Delta M2$, 25 μ l supernatant from each culture was added to this well. (c) Activity of (1) 25 μ l FPLC-derived fraction corresponding to purified LtnA2; (2) 25 μ l cell-free supernatant of *L. lactis* MG1363(pOM33) ($\Delta ltnA2M2$); (3) combination of 25 μ l each of both (1) and (2), assayed against *L. lactis* subsp. *cremoris* HP.

to eliminate any possibility that LtnM1 plays a role in the production of A2. As expected, the complete absence of the A2 peptide combined with a non-functional LtnM2 resulted in an MG1363 derivative which did not produce active lactacin 3147. However, providing the A2 peptide restored activity to this construct, as did the CFS from either the *ltnM1* and *ltnA1* mutants. The opposite was also true in that CFS from transformants of MG1363 harbouring pOM47 ($\Delta ltnA1M1$) could be complemented by purified LtnA1 as well as CFS from the *ltnM2* and *ltnA2* mutants. These data confirm that LtnM1 acts solely on LtnA1, and LtnM2 solely on LtnA2.

The LanM proteins represent a novel group of enzymes which have not been identified outside a lantibiotic context, and therefore, it has been proposed that these enzymes catalyse the novel reactions involved in thioether ring formation. Disruption of the *lanM* gene in other systems resulted in a Bac^- phenotype, establishing a crucial role for the LanM protein in lantibiotic biosynthesis (Gilmore *et al.*, 1994; Rince *et al.*, 1994; Skaugen *et al.*, 1997); however, their function is as yet speculative. Similarly, inactivation of *ltnM1* or *ltnM2* by introducing a frameshift abolished lactacin 3147 production. A feature of the lactacin 3147 gene cluster is the presence of two *ltnM* genes. It could be envisaged that one LtnM protein is responsible for dehydration of the hydroxy amino acids, while the other catalyses thioether ring formation, much like the LanB and LanC enzymes in the type-A(I) group (for a review see Sahl *et al.*, 1995). However, the absence of a second modification enzyme in the lactacin 481, lactocin S and cytolysin systems makes this unlikely. A more probable scenario is that each lactacin 3147 prepeptide requires a separate modification enzyme. There is no significant sequence homology between the lactacin 3147 and cytolysin peptides, whereas protein sequence alignments show that LtnA1 and LtnA2 are very closely related to

the staphylococcin C55 components Sac α A and Sac β A, with LtnA1 and Sac α A sharing 83% identity and LtnA2 and Sac β A sharing 44% identity (Navaratna *et al.*, 1999). Furthermore, in the cytolysin system, both peptide components are much more related to each other than is the case for lactacin 3147 or staphylococcin C55. This may explain why cytolysin can rely on only one LanM modification enzyme, while two modification enzymes may be necessary in both the lactacin 3147 and staphylococcin C55 systems.

The presence of two modification genes and two structural genes within the lactacin gene cluster presented a number of possible scenarios. First, both prepeptides could be modified sequentially by both M proteins; secondly, either one of the M proteins may have modified both prepeptides (with the second M protein redundant); or thirdly, each prepeptide may simply have been modified by the first M protein encountered within the cell. However, on the basis of the various knockout experiments presented here, we conclude that the only possibility which is supported by the evidence is that each prepeptide requires a dedicated modification enzyme. Given the low level of identity between LtnA1 and A2 peptides this also provides the most logical explanation of the requirement for two modification enzymes. Further analysis of the peptide products from the various knockout mutants will shed further light on the precise interactions between prepeptides and modification proteins.

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