Lactococcins: Mode of Action, Immunity and Secretion

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ABSTRACT

Lactococcus lactis subsp. cremoris 9B4 produces three small (around 5kDa), heat-stable, non-lanthionine containing, membrane active bacteriocins. Amino acid uptake experiments and proton motive force measurements have indicated that these peptides most probably form pores in the cytoplasmic membrane of sensitive cells. For bacteriocin activity a membrane protein (receptor) is necessary. The bacteriocin producing strain protects itself against the deleterious action of the bacteriocin by producing an immunity protein expressed from the same operon. The immunity protein works by blocking the receptor, thereby preventing the bacteriocin to form pores.

The producer cell most probably secretes these peptides by a sec-independent transport machinery, homologous to the E. coli haemolysin secretion system. Two membrane-located proteins (LcnC and LcnD) are necessary for extracellular, active bacteriocin. By PhoA-LacZ fusion studies the topology of LcnD has been determined. The protein contains one transmembrane α-helix near its N-terminus. The N-terminus is located inside, the C-terminus is on the outside of the cell. LcnC most probably contains six transmembrane helices. The present model suggests that both membrane proteins are necessary for export of the bacteriocins, forming a dedicated transport-machinery of the ATP-binding cassette family.

INTRODUCTION

The past several years have seen an explosion of research activity on bacteriocins produced by lactic acid bacteria (LAB) (De Vuyst, 1993; Hoover & Steenson, 1993; James et al., 1992; Jung & Sahl, 1991; Klaenhammer, 1988, 1993; Kolter & Moreno, 1992; Ray et al., 1992; Nettles & Barefoot, 1993). As this field continues

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to expand, our knowledge about LAB and their bacteriocins is increasing rapidly, owing to detailed studies of the peptides, their mechanism of action, immunity-, processing and secretion systems, and the genes involved in these processes. Here we focus on lactococcins, peptide bacteriocins produced by *L. lactis*. For data on other (similar) bacteriocins the reader is referred to the excellent reviews that appeared recently (Klaenhammer, 1993; Nettles & Barefoot, 1993; Kok et al., 1993).

Bacteriocins, as defined by Tagg et al. (1976), are proteinaceous compounds that kill closely related bacteria. This definition is true for the majority of bacteriocins investigated, but gradually it has become evident that certain bacteriocins may also elicit bactericidal activity against bacterial species that are not closely related to the bacteriocin producer. Moreover, there are examples of bacteriocinogenic complexes of a protein and another heterogeneous moiety that is required for activity and may include lipid or carbohydrate (Schved et al., 1993; Jimenez-Diaz et al., 1993; Lewus et al., 1992; Upreti & Hinsdill, 1973; Upreti & Hinsdill, 1975).

Several bacteriocins of LAB have been characterized biochemically and genetically and of a number the mode of action has been studied. This allowed the classification of these bacteriocins into four distinct classes [as proposed by Klaenhammer (1993)]:

(i) lantibiotics; small membrane-active peptides that contain the unusual amino acids lanthionine, β-methyl lanthionine, dehydro-alanine and dehydro-butyrine; e.g. lactocin S, carnocin U149, lactacin 481 and nisin (Hurst, 1981; Piard et al., 1993; Stoffels et al., 1992; Mortvedt et al., 1991; Kaletta & Entian, 1989). Nisin is the best studies example and will be dealt with by Oscar Kuipers separately in this issue;

(ii) small (<5 kDa) heat-stable, non-lanthionine containing membrane active peptides characterized by a double glycine processing site in the bacteriocin precursors. This site is not entirely restricted to this class; it is also present in some lantibiotics. Most of the bacteriocins characterized to date, including the lactococcins, belong to this class which may be divided into three subclasses:
   (a) bacteriocins that are active on *Listeria*; the so-called pediocin-like bacteriocins (Nieto-Lozano et al., 1992); e.g. pediocin PA-1, sakacin A and P and curvacin A (Nieto Lozano et al., 1992; Holck et al., 1992; Tichaczek et al., 1992; Marugg et al., 1992);
   (b) complexes that consist of two proteinaceous peptides required for activity; e.g. lactacin F, lactococcin G and lactococcin M (Nissen-Meyer et al., 1992; Allison et al., 1993; Van Belkum et al., 1991a);
   (c) thiol-activated bacteriocins. The only example so far known is lactococcin B (Venema et al., 1993);

(iii) large (>30 kDa) heat-labile proteins; e.g. helveticin J, helveticin V-1829, lactacins A and B (Joerger & Klaenhammer, 1986; Joerger & Klaenhammer, 1990; Toba et al., 1991; Vaughan et al., 1992);

(iv) complex bacteriocins, composed of protein plus one or more non-proteinaceous moieties (lipid, carbohydrate) required for activity; e.g. leucocin S, lactocin 27 and pediocin SJ-1 (Schved et al., 1993; Jimenez-Diaz et al., 1993; Lewus et al., 1992; Upreti & Hinsdill, 1973, 1975).
Fig. 1. Plasmid p9B4-6 and location of the three operons for lactococcins A, B and M (adapted from Kok et al., 1993). The linear map represents a reconstruction based on nucleotide sequence data and restriction enzyme analyses (Van Belkum et al., 1989, 1992; Holo et al., 1991; Scherwitz-Harmon & McKay, 1987). The baseline represent the DNA sequence and is shown with restriction enzyme cutting sites. Where the line is broken, no nucleotide sequence data is available. The direction of transcription is indicated by arrowheads in the open reading frames. The right/left turn arrows indicate promoters. Lollipops are presumed terminator structures. Below the baseline the size of the ORF's in codons is indicated. C, D, A, iA, B, iB, M, N, iM: lcnC, lcnD, lcnA, lciA, lcnB, lciB, lcnM, lcnN and lciM, respectively. EV, HIII: EcoRV and HindIII, respectively.

GENETICS OF LACTOCOCCIN PRODUCTION

In the most thoroughly characterized examples studied so far, the genes specifying lactococcin production, immunity and secretion are plasmid located (Van Belkum et al., 1989; Van Belkum et al., 1992; Holo et al., 1991; Scherwitz et al., 1983). As an example p9B4-6 from L. lactis subsp. cremoris 9B4 is shown in Fig. 1. From nucleotide sequence and mutational analyses it became clear that this particular plasmid (and probably also pNP2 of L. lactis subsp. lactis biovar. diacetylecoccus WM4 and the 55-kb bacteriocin plasmid of L. lactis subsp. cremoris LMG2130) contains three bacteriocin operons, specifying three different bacteriocins, lactococcin A, B and M, and their corresponding immunity proteins. In addition there is at least one operon present specifying proteins required for lactococcin secretion and processing, namely LcnC and LcnD. In the following we will deal with what is known of the separate genes and gene products.
Van Belkum *et al.* (1991a, 1992) determined the nucleotide sequences of three different bacteriocin determinants from plasmid p9B4-6, specifying low (lactococcin M), high (lactococcin A) and intermediate (lactococcin B) bacteriocin activity. In detailed mutational analyses the lactococcin structural and immunity genes were identified (Fig. 1). Both genes of 69 (\(lcnM\)) and 77 (\(lcnN\)) codons of the lactococcin M determinant were implicated in the production of the bacteriocin (Van Belkum *et al.*, 1991a). Separate expression of \(lcnM\) and \(lcnN\) and mixing of the supernatants of these strains, gave full bacteriocin activity, whereas the separate supernatants were inactive (K. Venema, unpublished results). This indicated that lactococcin M is a member of the IIb class of bacteriocins, in which a complex of two peptides is required for activity. In the case of the high-and intermediate-activity determinants, the ORFs of 75 (\(ZcnA\)) and 68 (\(ZcnN\)) codons were shown to be involved in the production of lactococcin A and B, respectively. This extensive genetic analysis convincingly illustrated that the bactericidal action of a strain may, in fact, reflect the activity of more than one bacteriocin.

Conclusive evidence that \(ZcnA\) was indeed the structural gene of lactococcin A came from Ho10 *et al.* (1991). These authors cloned and sequenced a DNA fragment from the 55-kb bacteriocin plasmid from strain LMG2130 that reacted with a DNA probe designed from the N-terminal amino acid sequence of purified lactococcin A. The nucleotide sequence appeared to be identical to that of the high-activity determinant of p9B4-6. By comparing the amino acid sequence determined from the purified bacteriocin with that deduced from the structural gene, it was evident that lactococcin A was synthesized as a 75-amino-acid-precursor with an N-terminal extension of 21 amino acids. Processing of this precursor takes place behind a glycine doublet (Fig. 2). The N-terminal extension, hereafter called leader peptide, and the processing site do not follow the rules of Von Heijne (1983) for genuine signal peptides required for sec-dependent protein export, which suggests that the extension in pre-lactococcin A is not a signal sequence in its classical sense (Ho10 *et al.*, 1991). \(LcnB\), M and N all contain N-terminal sequences with homology to the lactococcin A leader peptide (Fig. 2). Although the latter three bacteriocins have not been purified, it is tempting to assume that they are also processed behind the glycine doublet (or glycine–serine

![Alignment of LcnA, B, M and N. (*) identical and conserved residue, respectively. The arrowhead indicates the processing site for LcnA and the presumed processing site for the other peptides. The sequence that could possibly form a membrane-spanning \(a\)-helix is underlined in LcnA.](image-url)
Lactococcins, mode of action, immunity, secretion

The glycine processing site is found in numerous class II peptide bacteriocins of LAB, and even in some lantibiotics and colicin V, an *E. coli* bacteriocin (Hastings et al., 1991; Marugg et al., 1992; Fremaux et al., 1993; Quadri et al., 1994; Tichaczek et al., 1992; Havarstein et al., 1994, Hynes et al., 1993).

Van Belkum et al. (1991a, 1992) made deletion or frameshift mutations in either of the three larger genes, expected to be the immunity genes [the genes of 154, 98 and 91 codons for the lactococcin M, A and B determinants, respectively, (Fig. 1)] without disturbing the corresponding bacteriocin structural genes. Unexpectedly, *L. lactis* transformants carrying the various constructs proved viable, although the colonies were initially small and grew poorly. After serial transfers in fresh medium the cells gradually started to grow more rapidly. If lactococcin [50% (vol/vol) of a supernatant of a lactococcin producing strain] was added to the plates on which the initial transformants were to be selected, no transformants were obtained. Also, when the presumed immunity genes were cloned separately from the bacteriocin genes under the control of a promoter, they provided immunity to the transformed lactococcal strain. These results indicated that the presumed immunity genes indeed specify bacteriocin immunity, and also that cells can overcome the lethal action of lactococcin at a stage when little bacteriocin is produced. Several possibilities leading to tolerance towards lactococcin can be envisaged at this point, one of which, in the case of lactococcin A, is the loss or mutational alteration of its receptor (see below). The number of transformants obtained with a plasmid carrying *lcnA* and a mutated immunity gene is the same as that obtained with a plasmid carrying functional *lcnA* and *lciA* (K. Venema, unpublished results). Apparently, the absence of a functional immunity gene does not interfer with the viability of these transformants. It is unlikely that each of the viable transformants would carry a mutation in the lactococcin receptor. A more likely explanation is that the viability of these transformants is due to a non-genetic change in membrane-lipid composition, such that the lactococcin cannot form pores in the altered membranes. Because of the frequent occurrence of the acquisition of tolerance towards lactococcin, we envisage this change in lipid composition to be an reversible adaptation rather than a mutation. Here we will restrict the term immunity to lactococcin-insensitivity caused by the *lci* product. We propose to denote the reversible acquisition of insensitivity to bacteriocins as tolerance, whereas the term resistance should be reserved for permanent acquisition of insensitivity to bacteriocins.

**LcnC and LcnD are essential for lactococcin production**

Nucleotide sequence analysis of a 5.2-kb DNA fragment derived from plasmid pNP2 of *L. lactis* subsp. *lactis* biovar. *diacetylactis* WM4 revealed that this plasmid also specifies lactococcin A (Stoddard et al., 1992). Moreover, it was shown that two additional genes were required for bacteriocin activity. These genes, designated *lcnC* and *lcnD*, are located in an operon immediately upstream of the lactococcin A structural and immunity genes (Fig. 1). Another copy of these genes is probably also present upstream of the lactococcin M operon (Kok et al., 1993). Tn5 insertions in either *lcnC* or *lcnD* eliminated lactococcin production without
negating immunity (Stoddard et al., 1992). A promoter immediately upstream of lcnA drives expression of both lactococcin A activity and immunity (Van Belkum et al., 1991a). Therefore, loss of lcnC or lcnD functions were reasoned to impact the maturation and/or secretion processes (Stoddard et al., 1992). Comparisons of LcnC and LcnD with protein database sequences revealed that these proteins share significant similarities to Gram-negative proteins implicated in signal sequence-independent secretion pathways. Stoddard et al. (1992) showed that LcnC is a member of the HlyB-like family of ATP-binding cassette (ABC) transporters (Higgins, 1993; Fath & Kolter, 1993). The similarity is most pronounced in the C-terminal stretch of 200 amino acids of LcnC, which contains the ATP-binding cassette. Protein structure-predicting computer programs indicate six hydrophobic regions in the N-terminus of LcnC which could promote binding at the cytoplasmic membrane, a situation similar to HlyB (Gentschev & Goebel, 1992). Hydrophobicity analysis of LcnD indicated that it is largely hydrophilic, with the exception of the N-terminal 43 amino acids. This region in LcnD might be a transmembrane domain, as was shown for HlyD (see also below) (Schulein et al., 1992). LcnD does not show similarity with HlyD, however. These results strongly suggested that LcnC and LcnD are required for secretion of the lactococcins via a system dedicated to bacteriocin export (Stoddard et al., 1992; Kok et al., 1993). Similar dedicated secretion systems have now been proposed for other LAB bacteriocins (Marugg et al., 1992; Kuipers et al., 1993).

It appears that L. lactis strains IL1403 carries genes for LcnC and LcnD on its chromosome, explaining why plasmids carrying only the bacteriocin structural and immunity genes still produced active bacteriocin (Van Belkum et al., 1991a, 1992; Holo et al., 1991). Using a DNA probe encompassing lcnC and lcnD, in Southern hybridizations, a signal with the chromosome of IL1403 was obtained (Kok et al., 1993). When probed with the various bacteriocin structural and immunity genes, no chromosomal signal was observed (K. Venema, unpublished data). Also, PCR products encompassing almost the entire operon were obtained using primers designed from the plasmid encoded lcnC an lcnD genes (K. Venema, unpublished results), indicating that the chromosomally located lcnC and lcnD of IL1403 are highly homologous to their plasmid encoded counterparts. Part of the chromosomally encoded lcnC was cloned and sequenced. Several nucleotide substitutions were found compared to the plasmid encoded lcnC (K. Venema, unpublished data), but none of these led to amino acid changes in the translation product. The reduced production of lactococcin in IL1403 carrying a plasmid with only the bacteriocin operon, as compared to the wild type strain carrying all of the essential genes on a plasmid, can be explained by assuming that the lower copy number of the chromosomally located genes (most probably one) results in a lower secretion efficiency. This conclusion was corroborated by introducing lcnC and lcnD, originating from p9B4, on a plasmid in IL1403. This led to a drastically increased production of lactococcin A.

**TOPOLOGY OF LcnD IN THE LACTOCOCCAL CYTOPLASMIC MEMBRANE**

The results presented so far indicate that LcnC and LcnD might form a dedicated secretion machinery for lactococccins. However, no evidence has been presented to
Lactococcins, mode of action, immunity, secretion

justify such a conclusion. To characterize the putative secretion machinery, Franke et al. (1995) used fusions with the E. coli reporter proteins LacZ and PhoA to determine the topology of LcnC and LcnD in the cytoplasmic membrane. Since the studies with LcnC are still in progress, only the data for LcnD will be discussed here.

Fusions of LacZ, a cytoplasmic protein, to sequences that are normally located in the cytoplasm result in an active β-galactosidase. Fusions between LacZ and parts of a protein that are normally extracellular will result in highly reduced β-galactosidase activities. For PhoA, a periplasmic enzyme, the reverse is true (Manoil, 1990; Muller et al., 1993; Boyd et al., 1993).

Hydrophobicity analysis of LcnD indicates that the stretch of amino acids from residue 22 to 38 might form a transmembrane α-helix. The rest of the protein is rather hydrophilic. Taking this computer prediction as a guide, Franke et al. (1995) constructed four LacZ and four PhoA fusions in front of, in, and behind the predicted transmembrane helix and at the complete C-terminal end of LcnD. The analysis showed that, indeed, the N-terminus of LcnD was in the cytoplasm, the stretch of amino acids between residue 22 to 38 crossed the membrane, and the remainder of the protein was located at the outside of the cell.

PROCESSING OF BACTERIOCINS; INVOLVEMENT OF THE ABC-TRANSPORTERS IN PROCESSING

As discussed above, class II bacteriocins are synthesized as prebacteriocins, containing a leader peptide that is cleaved behind a glycine doublet before or during transport. Until recently, it was unclear whether the presumed leader peptidase activity was also encoded by the bacteriocin operon, or whether it was a more general peptidase present elsewhere on the chromosome. The first indication that a protein encoded by the bacteriocin operon was involved in this process came from studies by Venema et al. (1995, submitted) on the pediocin operon.

As for lactococcins, production of active pediocin requires, in addition to the structural gene, pedA, two additional genes (pedC and pedD) that show homology to the proteins involved in set-independent secretion of polypeptides (Marugg et al., 1992). PedC has only structural homology to LcnD, whereas PedD has considerable amino acid sequence homology to LcnC (49% identity with an overall homology of 67%). The homology of the latter two proteins is not restricted to the ABC-box, but extends over the entire polypeptide. This is also the case with several other ABC-transporters involved in secretion of bacteriocins that contain the double glycine motif in the leader peptide. Notably, a region in the N-terminal 190 amino acids is conserved in these proteins (Venema et al., 1995).

E. coli cells containing the complete ped operon (pedABCD) produce active, secreted pediocin (Fig. 3A). Apparently, all the information for both secretion and processing is present in this operon. Mutations in the structural gene (pedA), pedC or pedD abolish production of extracellular pediocin activity by E. coli. The primary translation product of pedA contains 62 amino acids encompassing the 18-amino-acid-leader peptide and the 44 amino acids that constitute mature pediocin (Marugg et al., 1992). pedA was expressed in E. coli behind the strong T7 RNA polymerase promoter. When lysates of this strain were run on an SDS–PAA gel and overlaid with pediocin sensitive cells, a faint halo of activity was


Fig. 3. The effect of pedC and pedD on pediocin secretion and processing. Ext. ped, int. prep, int. ped: extracellular pediocin, intracellular prepediaocin and intracellular pediocin activity, respectively. (+), (-): activity present and absent, respectively. The presence of the various genes (pedA, pedC, pedD) in the E. coli clones (A, B, C, D) is indicated by the arrows. For details, see text.

observed, corresponding to a peptide with a lower electrophoretic mobility than that of mature pediocin produced by E. coli containing the complete ped operon. Apparently, the primary translation product of pedA is biologically active albeit at a very reduced level. No extracellular activity was found in the strain carrying pedA alone (Fig. 3B). To determine which of the genes in the operon was responsible for pediocin secretion and/or processing, pedC and pedD were expressed separately or together in the strain producing prepediaocin from the T7 promoter. When both pedC and pedD were present, active extracellular pediocin was produced. However, when only pedC (Fig. 3C) or pedD (Fig. 3D) were present, no extracellular activity was found. Lysates of the strain carrying pedA and pedC contained prepediaocin activity only. However, lysates of the strain carrying pedA and pedD showed, in addition to prepediaocin activity, an activity on the gel that was indistinguishable from that of mature pediocin (Fig. 3D). These results conclusively show that both PedC and PedD are involved in secretion of pediocin. In addition, we demonstrate that PedD is capable of processing the pediocin precursor. Since mature pediocin was found intracellularly in the strain containing only pedA and pedD, we postulate that the conserved 190-amino-acid-domain in the N-terminus of the protein, which is shown to be intracellular by topology studies (E. Emond, pers. commun.), is involved in processing of the leader peptide. The results also show that bacteriocin processing can be uncoupled from secretion.

We predict that, similar to PedD, LcnC will be the processing enzyme for the lactococcins. Since cloning of lcnC in E. coli is lethal we have not yet been able to verify this prediction.

MODE OF ACTION OF LACTOCOCCIN A AND B

Lactococcins A and B specifically inhibit the growth of lactococci. Both are small cationic, hydrophobic peptides that structurally resemble several peptide anti-
Lactococcins, mode of action, immunity, secretion

Biotics permeabilizing membranes (Gao et al., 1991; Galvez et al., 1991; Kordel & Sahl, 1986; Kordel et al., 1988; Schaller et al., 1989). A possible target for their action could thus be the cytoplasmic membrane of sensitive cells. Van Belkum et al. (1991b) investigated this possibility in detail for lactococcin A. The mode of action of purified lactococcin A was studied on whole cells and membrane vesicles of sensitive and immune lactococcal strains, and on liposomes obtained from lactococcal phospholipids. Venema et al. (1993) performed similar studies with partially purified lactococcin B on whole cells. At lactococcin concentrations that did not affect immune cells, both bacteriocins rapidly dissipated the membrane potential (and in the case of lactococcin B also the pH-gradient across the membrane) of glucose-energized sensitive cells. A non-metabolizable alanine analogue (AIB, 2-α-amino isobutyric acid) that is taken up in symport with a proton [and of which the uptake is thus driven by the proton motive force (pmf)] (Konings et al., 1989) was rapidly lost from sensitive whole cells treated with lactococcin (Van Belkum et al. 1991b; Venema et al., 1993). To show that efflux was a direct consequence of permeability changes in the cytoplasmic membrane, and not caused by the absence of a pmf, uptake of L-glutamate was studied. Glutamate uptake by L. lactis is driven by a phosphate bond-linked unidirectional process (Poolman et al., 1987) and dissipation of the pmf should, therefore, not lead to efflux of accumulated glutamate. However, addition of either lactococcin to sensitive cells that had accumulated glutamate resulted in the immediate efflux of the amino acid, even when the pmf in the cells was dissipated prior to the addition of the bacteriocin (Van Belkum et al., 1991b; Venema et al. 1993). These results indicated that both lactococcins are able to form pores in the membrane in a voltage-independent manner.

Lactococcin A inhibited leucine uptake in cytoplasmic membrane vesicles from sensitive lactococcal cells, but not from vesicles derived from Bacillus subtilis, Clostridium acetobutylicum or E. coli membranes (Van Belkum et al., 1991b). Liposomes derived from lactococcal phospholipids were not affected by lactococcin A. From these data, and the observation that lactococcin A specifically inhibits lactococcal strains, Van Belkum et al. (1991b) concluded that lactococcin A forms pores in the cytoplasmic membrane of sensitive cells using a Lactococcus-specific receptor protein (Van Belkum et al., 1991b).

A 21-amino acid sequence between residues Ala30 and Phe50 in lactococcin A (Fig. 2) could possibly form a membrane-spanning α-helix (Kok et al., 1993). Lactococcin A could be anchored to the cytoplasmic membrane of sensitive cells by this hypothetical transmembrane helical segment. A large number of pore-forming toxins are known to create channels through a "barrel stave" mechanism (Ojcius & Young, 1991; Sansom et al., 1991). Pore formation requires the molecules to aggregate like barrel staves surrounding a central water-filled pore. It is assumed that lactococcin A and lactococcin B form pores by this mechanism, although in the case of lactococcin B the method of Kyte & Doolittle (1982) does not predict a (clearcut) membrane spanning helix. The size of the pore would be dictated by the number of molecules involved in pore formation. An indication that different pore sizes can exist was derived from the observation that low concentrations of lactococcin B allow leakage of protons and ions, whereas for leakage of glutamate 150 times more bacteriocin was needed (Venema et al., 1993). Apparently, at low concentrations a multi-peptide complex of lactococcin B molecules forms a pore which is too narrow...
Fig. 4. Effect of LcnB on the membrane potential of glucose-energized lactococcal cells (adapted from Venema et al., 1993). Δ: no addition, +: addition of non-DTT-treated LcnB, O: addition of DTT-treated LcnB, □: addition of HgCl₂-treated LcnB, ●: addition of HgCl₂-treated LcnB, subsequently reduced with DTT.

for the passage of amino acids, but allows the passage of protons and other ions.

A prerequisite for lactococcin B activity is that its only cysteine residue is in the reduced state (Cys24, Venema et al., 1993). Partially purified lactococcin B was almost inactive on whole cells (Fig. 4, curve +). Only after addition of small amounts of dithiothreitol (DTT) was lactococcin B capable of dissipating the membrane potential (Fig. 4, O). Reduction of Cys24 by DTT was counteracted by HgCl₂ (Fig. 4, □), while HgCl₂-oxidized lactococcin B could be reactivated by DTT (Fig. 4, curve ●).

Cys24 of lactococcin B was replaced via site directed mutagenesis by all other possible 19 amino acids. Surprisingly, 16 of the 19 mutants were active, and even more so than the wild type bacteriocin. The inactive mutants were the ones in which the cysteine had been replaced by a positively charged amino acid (Cys24His, Cys24Lys and Cys24Arg; K. Venema, unpublished data). Apparently, Cys24 is not essential for activity of lactococcin B. When Cys24 is present, oxidation leads to inactivation of lactococcin B. In this regard lactococcin B resembles a group of thiol-activated toxins in which the reduced state of a cysteine residue appears to be essential for the generation of functional lesions in toxin-treated membranes (Boulnois et al., 1991). Also in these molecules the cysteine can be replaced without loss of activity. The reason for inactivation of lactococcin B by oxidation of Cys24 is unclear, but since all mutants carrying a positively charged amino acid at position 24 are inactive, for the
bacteriocin to be active a negative or no charge at this specific site is apparently essential.

**MODE OF ACTION OF THE IMMUNITY PROTEIN LCI A**

Since lactococcin A is active on membrane vesicles derived from sensitive cells, but not on those derived from immune cells, Van Belkum *et al.* (1991b) concluded that the immunity protein, LciA, was present in the membrane. To study the mode of action of the immunity protein, we raised monoclonal antibodies against the protein (Venema *et al.*, 1994). To facilitate the purification of LciA, for which no assay was available, LciA was fused to the C-terminus of the maltose binding protein (MBP) of *E. coli* (Fig. 5). The fusion protein was used to raise monoclonal antibodies. With one of the antibodies directed against the LciA moiety of the fusion, LciA was localized in the membrane fraction of fractionated cells of *L. lactis* (Venema *et al.*, 1994). However, similar amounts of the protein were also present in the cytosolic and membrane-associated fractions. LciA was not detectable in the supernatant or soluble cell wall fraction. Four deletion variants of the MBP-LciA fusion were constructed that contained the N-terminal 80, 60, 40 and 20 amino acids of LciA fused to MBP (Fig. 5). On Western-bLOTS only the full length fusion and the fusion protein containing the 80 N-terminal amino acids of LciA reacted with the monoclonal antibody, indicating that its epitope in LciA is located in the C-terminus between amino acids 60 and 80. By performing ELISA (enzyme-linked-immunosorbent assay) using the monoclonal antibody on right-side-out and inside-out vesicles it was shown that the C-terminus of LciA is

![Fig. 5. MBP-LciA fusions and its deletion derivatives (adapted from Venema *et al.*, 1994). MBP, LciA: maltose binding protein and lactococcin A immunity protein, respectively. 1, 98, 80, 60, 40, 20: amino acid residue number in LciA. The fusions are not drawn to scale.](image-url)
located at the outside of the cytoplasmic membrane of an LciA-producing strain (Venema et al., 1994). Right-side-out membrane vesicles derived from a strain producing both lactococcin A and the immunity protein did not react with the antibody. The same was observed when lactococcin A was added externally to right-side-out vesicles derived from a strain producing only LciA. Apparently, the bacteriocin shielded the epitope in LciA from reacting with the monoclonal antibody. Right-side-out membrane vesicles of immune as well as sensitive cells were treated with proteinase K and subsequently used in leucine uptake experiments. Although LciA is cleaved by proteinase K (Venema et al., 1994) immune vesicles incubated with the proteinase were not affected by the bacteriocin. However, also proteinase K-treated sensitive vesicles became insensitive to the bacteriocin. These data suggested that, in addition to digesting LciA, proteinase K had also digested the bacteriocin receptor, previously inferred by van Belkum et al. (1991b) on the basis of the lactococcin A mode of action studies, rendering the membrane vesicles insensitive to the action of lactococcin A.

Thus far, the results indicate that LciA is present in the cytoplasmic membrane and prevents the action of lactococcin A. It could do so by binding and, thus, neutralizing the bacteriocin, or by interacting with and blocking the bacteriocin receptor. To distinguish between these two possibilities, right-side-out vesicles isolated from immune and sensitive cells were fused and the ability of the fused membranes to accumulate leucine was examined (Venema et al. 1994). In case LciA would bind and neutralize lactococcin A, the fused vesicles would still be immune (Fig. 6A). If LciA would block the bacteriocin receptor, the fused vesicles would be sensitive because the unblocked receptor molecules contributed by the sensitive vesicles would still be able to interact with lactococcin A (Fig. 6B). As leucine uptake in the fused vesicles was completely blocked in the presence of lactococcin A, we concluded that the immunity protein can not protect the fusion vesicles against lactococcin A and, therefore, does not titrate away lactococcin A. These data conclusively show that LciA directly interacts with the lactococcin A receptor.

Nissen-Meyer et al. (1993) have purified the LciA protein by using the physicochemical characteristics of its deduced amino acid sequence. As judged from its amino acid composition and from amino acid sequencing, the immunity protein is not post-translationally modified. Protein structure computer programs predict that the stretch of amino acids from residue 29 to 47 in LciA could form an α-amphiphilic helix with a strong hydrophobic moment of 0.52 (Fig. 7). All the results presented above can be united in a model for LciA topology, in which the C-terminus, from residue 48 to 98 and containing the epitope, is at the outside of the cell. Residues 29 to 47 might span the cytoplasmic membrane as an α-amphiphilic helix by virtue of its interaction with another transmembrane protein, possibly the lactococcin A receptor. The N-terminus of LciA resides on the cytoplasmic face of the membrane. A model visualizing the immunity mechanism is presented in Fig. 8. This mechanism also provides an explanation for the observation that only part of the synthesized immunity protein molecules is present in the cytoplasmic membrane. Apparently, this portion is enough to interact with all receptors. We envisage the cytoplasmic and membrane-associated fractions to constitute a continuously available reservoir from which molecules can be drawn when new receptors are synthesized and inserted into the membrane, thus resulting in immediate blocking of the receptor (Venema et al., 1994).
CONCLUSIONS AND PERSPECTIVES

From this overview, it is clear that, over the past few years, very significant progress has been made in the field of lactococcin production and immunity against lactococcins by L. lactis. Apart from the cloning and sequencing of the
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Fig. 7. Helical wheel representation of residues 29 to 47 in LciA that could form an amphipathic helix. Shaded part of the helix: hydrophobic side, white part of the helix: hydrophilic side.

Fig. 8. Model for the complex of the lactococcin A immunity protein with the bacteriocin receptor (adapted from Venema et al., 1994). In this model, the bacteriocin receptor (R) is depicted as a transmembrane protein. The amphipathic helix in LciA (I) is located in the cytoplasmic membrane (CM), such that the hydrophilic side (light shaded area) is tightly associated with a hydrophilic area in the receptor (dark shaded area). The bacteriocin (L) is still capable of binding to the receptor, thereby shielding the epitope in the C-terminus of LciA from interacting with the LciA-specific antibody. The presence of LciA prevents the bacteriocin from successful insertion into the cytoplasmic membrane to form pores. N, N-terminus.

structural and immunity genes and genes encoding the secretion machinery, we now begin to understand the mode of action of lactococcin A and lactococcin B, and the way the lactococcin A immunity protein LciA might work. Although the gene specifying the receptor necessary for activity of lactococcin A has, as yet, not
been identified, the presumed receptor is likely to be the site of action of the immunity protein. In analogy to the system for pediocin secretion, it is likely that LcnC and LcnD are involved in processing and secretion of the lactococcins. In the coming years we expect that the receptor will be identified and that molecular details concerning the mode of action combined with structure-function studies of the lactococcins will allow the construction of bacteriocins with enhanced or altered activities and broader specificities.

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