Bacteriocins from lactic acid bacteria
Venema, Konraad

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CHAPTER V

Mode of action of LciA, the lactococcin A immunity protein.

Koen Venema, Roel E. Haverkort, Tjakko Abee, Alfred J. Haandrikman, Kees J. Leenhouts, Lou de Leij, Gerard Venema and Jan Kok.

Chapter V

Mode of action of LciA, the lactococcin A immunity protein.

SUMMARY

Monoclonal antibodies were raised against a fusion between the *Escherichia coli* maltose-binding protein and LciA, the immunity protein that protects *Lactococcus lactis* against the effects of the bacteriocin lactococcin A. One of the antibodies directed against the LciA moiety of the fusion protein was used to locate the immunity protein in the *L. lactis* producer cell. LciA was present in the cytosolic, the membrane-associated, and the membrane fractions in roughly equal amounts, irrespective of the production by the cells of lactococcin A.

The monoclonal antibody specifically reacted with right-side-out vesicles obtained from a strain producing the immunity protein. It did not react with inside-out vesicles of the same strain, or with right-side-out vesicles obtained from a strain producing both LciA and lactococcin A. Also, externally added lactococcin A blocked the interaction between the antibody and right-side-out vesicles obtained from a strain producing only LciA.

The epitope in LciA was localized between amino acid residues 60 and 80. As the epitope could be removed from right-side-out vesicles by proteinase K, it is located at the outside of the cell.

The immunity protein contains a putative α-amphiphilic helix from residue 29 to 47. A model is proposed in which this helix is thought to traverse the membrane in such a way that the C-terminal part of the protein, containing the epitope, is on the outside of the cell.

Vesicle-fusion studies together with leucine-uptake experiments suggest that the immunity protein interacts with the putative receptor for lactococcin A, thus preventing pore formation by the bacteriocin.

INTRODUCTION

All species of lactic acid bacteria have been shown to produce bacteriocins (Klaenhammer, 1993), i.e. proteinaceous molecules that usually inhibit a narrow range of closely related species. Lactococcin A (LenA) is a bacteriocin produced by *Lactococcus lactis* (Van Belkum et al., 1989; 1991a; Holo et al., 1991). Treatment of sensitive cells of *L. lactis* with lactococcin A permeabilizes the cytoplasmic membrane, which results in leakage of ions and amino acids from the cells (Van Belkum et al., 1991b). By comparing amino acid uptake in cytoplasmic membrane vesicles and liposomes prepared from phospholipids of *L. lactis*, Van Belkum et al. (1991b) concluded that a membrane-associated protein component is involved in lactococcin A activity. Genetic studies have identified a gene (licI) that specifies immunity against lactococcin A: disruption of *licI* led to sensitivity of the cell to lactococcin A (Van Belkum et al., 1991b). Uptake and efflux experiments have shown that cells producing the immunity protein (LciA) do not leak ions or amino acids upon addition of lactococcin A. Furthermore, membrane vesicles obtained from an immune strain showed no inhibition of uptake or efflux of amino acids by lactococcin A, in contrast to membrane vesicles obtained from a sensitive strain (Van Belkum et al., 1991b).

Although the mode of action of several bacteriocins from lactic acid bacteria has been investigated, and all are thought to form pores in the membrane of susceptible cells (Van Belkum et al., 1991b; Venema et al., 1992; Chikindas et al., 1993, for a review see Kok et al., 1993), information concerning the mode of action of the corresponding immunity proteins is extremely scarce. Only one report deals with the purification of the immunity protein against lactococcin A, LciA (Nissen-Meyer et al., 1993).

Recently, the immunity proteins against several *Escherichia coli* colicins were subjected to biochemical analyses. Only two of these, Cai and ImmE1, act against pore-forming colicins, ColA and ColE1, respectively (Geli et al., 1988; 1989a,b; 1992a,b; Song and Cramer, 1991; Song et al., 1991; Shirabe et al., 1993). The other immunity proteins are directed against colicins that act as DNases or RNases (Curtis and James, 1991; de Graaf and Klaassen-Boor, 1974; Masaki et al., 1991; Wallis et al., 1992a,b; Yajima et al., 1992). It has been established that the colicin immunity protein (Cai) protects cells against externally added colicin A (Geli et al., 1988). Cai is located in the cytoplasmic membrane with its N- and C-termini directed towards the cytoplasm (Geli et al., 1989a); however the N- and C-termini of ImmE1 are located on the cytoplasmic and periplasmic sides of the membrane, respectively (Song et al., 1991). The 113 residue ImmE1 protein structurally differs from Cai (178 residues) in that ImmE1 has only three transmembrane helices, whereas
Fig. 1. Construction of pKV2 (A) and its deletion derivatives (B).
A. Primers KOV3 and KOV4 were used to amplify the IciA gene, which was subsequently cloned between the BamHI and HindIII sites in pMal-c. The hybrid gene is shown together with the amino acid sequence at the junction between the Xa cleavage site and LciA.
B. Using primers KOV54, KOV55, KOV56 and KOV57, stop codons were introduced by PCR behind codons 80, 60, 40 and 20 of IciA, respectively. MBP: maltose-binding protein; ATG: start codon of IciA; TGA: stop codon of IciA; Xa c.s.: Factor Xa cleavage site; 1;98: amino acid residue number of LciA.
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Fig. 2. Western blotting and immunodetection with AbLciA1 of cell-free extracts (corresponding to 1 ml of culture) of *L. lactis* IL1403(pMB553; LciA⁺) (lane2) and IL1403 (LciA⁻) (lane3) after Tricine-SDS-PAGE. Lane 1: Rainbow molecular-weight marker (Amersham), the sizes (in kDa) of which are indicated in the left margin.

Cai has four.

It is believed that immunity proteins against pore-forming bacteriocins either prevent formation of pores, or block the pores once they have formed in such a manner that solutes can no longer leave the cell (Venema et al., 1992; Geli and Lazdunski, 1992a). It has been hypothesized that immunity involves a complex between the immunity protein and the bacteriocin, which is thought to be stabilized by electrostatic or polar interactions (Zhang and Cramer, 1993).

To unravel the mode of action of the lactococcin A immunity protein, monoclonal antibodies were raised against the protein. One of the monoclonal antibodies was used to locate LciA in the cell, and to determine its topology. Membrane vesicle-fusion experiments suggest that the immunity protein interacts with the putative bacteriocin receptor. A model is presented for the mode of action of the lactococcin A immunity protein.

RESULTS

Generation and isolation of the MBP-LciA fusion protein

Because of the lack of an assay for the lactococcin A immunity protein LciA, LciA was fused with the maltose binding protein (MBP) of *E. coli* (Maina et al., 1988). The fusion protein should be easy to purify by affinity chromatography on an amylose-resin column, provided that the conformation of the MBP moiety of the fusion had not changed drastically. The plasmid containing the hybrid gene pKV2 (Fig. 1A) was used to transform *E. coli* HS3018. One clone, containing the correct construct as verified by nucleotide sequence analysis, was selected and used for large-scale purification of the fusion protein. Usually, the yield of fusion protein was about 5 mg l⁻¹ of culture. As the protein was resistant to digestion by the protease factor Xa, the full-length fusion was used to raise monoclonal antibodies against LciA.

Identification of LciA-directed antibodies

After immunization of BALB/c mice with purified MBP-LciA protein and myeloma cell fusion, 17 hybridoma cell lines were obtained that reacted with the MBP-LciA fusion protein. Nine of these were negative against the MBP-LacZ fusion and should, therefore, produce antibodies against LciA. To test this, two were chosen, and designated AbLciA1 and AbLciA3. The two antibody preparations were tested in Western blots of cell-free extracts from *L. lactis* IL1403 (LciA⁺) and IL1403(pMB553; LciA⁻). Both clones AbLciA1 and AbLciA3 gave a clear signal with the strain containing the immunity protein but not with the negative control (Fig.

**Table 1.** Fractionation of LciA in the presence (*L. lactis* MG1363 (pMB553,pLP712)) or absence (*L. lactis* MG1363 (pMB563,pLP712)) of LcnA.

<table>
<thead>
<tr>
<th>Protein Fraction</th>
<th>MG1363(pMB553,pLP712)</th>
<th>MG1363(pMB563,pLP712)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant</td>
<td>PrtP 0 PCP 0 PrtM 0 LciA 0</td>
<td>PrtP 0 PCP 0 PrtM 0 LciA 0</td>
</tr>
<tr>
<td>Soluble cell-wall fraction</td>
<td>85 PrtP 9 PCP 0 PrtM 0 LciA 83</td>
<td>PrtP 8 PCP 0 PrtM 0 LciA 0</td>
</tr>
<tr>
<td>Cytosolic fraction</td>
<td>11 PrtP 78 PCP 0 PrtM 33 LciA 12</td>
<td>PrtP 81 PCP 0 PrtM 30 LciA 30</td>
</tr>
<tr>
<td>Membrane-associated fraction</td>
<td>4 PrtP 7 PCP 2 PrtM 33 LciA 6</td>
<td>PrtP 6 PCP 1 PrtM 25 LciA 25</td>
</tr>
<tr>
<td>Membrane fraction</td>
<td>0 PrtP 7 PCP 98 PrtM 34 LciA 0</td>
<td>PrtP 5 PCP 99 PrtM 45 LciA 45</td>
</tr>
</tbody>
</table>

The figures represent percentages of the total of the proteins indicated. Underlined: expected fractions for the control proteins. PrtP, cell wall-associated proteinase; PCP, pyrrolidonyl-carboxylyl peptidase; PrtM, proteinase maturation protein; LciA, immunity protein.

they were subjected to tricine-SDS-PAGE and subsequent Western blotting and immunodetection using AbLciA1. As shown in Table 1, the control proteins were present in the expected fractions. The distribution of LciA over the 5 fractions (Table 1, Fig. 3) shows that most (two thirds) of the protein was located in or associated with the membrane; the other one third was found in the cytoplasm. These data are similar to earlier observations (Nissen-Meyer et al., 1993). No LciA was detected in the supernatant or soluble cell-wall fractions. The location of LciA was independent of the presence of lactococcin A (compare L. lactis IL1403(pMB553) and IL1403(pMB563) in Fig. 3 (A and B) and Table 1).

The epitope in LciA is located in the C-terminus

Four deletion variants of the MBP-LciA fusion protein were constructed by the polymerase chain reaction (PCR) to map the epitope in LciA. Lysates of E. coli cells producing the truncated and full-size fusion proteins were subjected to SDS-PAGE (Fig. 4A). The protein patterns were analyzed by Western blotting and subsequent reaction with polyclonal antibodies against MBP (Fig. 4B) and AbLciA1 (Fig. 4C). Proteins of the expected size were produced in roughly equal amounts, as shown by their reaction with the anti-MBP polyclonal antibodies (Fig. 4A and B, lanes 2 to 6). Only the full-length fusion protein and the fusion protein containing the 80 N-terminal amino acids of LciA reacted with the monoclonal antibody AbLciA1 (Fig. 4C, lanes 1 and 2), indicating that the epitope in LciA is located between amino acid residues 60 and 80. Identical results were obtained when using AbLciA3.

The epitope in LciA is located at the outside of the cytoplasmic membrane

Since membrane vesicles derived from an immune strain are immune to the action of lactococcin A (Van Belkum et al., 1991b), we reasoned that the functional immunity protein is located in the membrane. To determine the topology of the protein, right-side-out and inside-out vesicles were assayed in ELISA for binding of the antibody AbLciA1 (identical results were obtained when AbLciA3 was used). Table 2 shows that the monoclonal antibody did not react with inside-out vesicles of an LciA-producing strain. Western blot analysis revealed that the immunity protein was present in these vesicles (not shown). Right-side-out vesicles prepared from the same strain did react with the antibody, indicating that the LciA moiety containing the epitope is located at the outside of the cytoplasmic membrane.

In a separate experiment, membrane vesicles were treated with proteinase K before the binding assay was performed. Proteinase K-treated right-side-out vesicles of the LciA-producing strain failed to bind the antibody, indicating that the epitope is susceptible to the proteinase. Possible residual proteinase K activity after washing (less than 0.1 %) did not prevent binding of the antibody to LciA in the vesicles.
not react with the antibody (Table 2). Apparently, the bacteriocin shielded the epitope from the monoclonal antibody. This conclusion was corroborated by the observation that lactococcin A added externally to right-side-out vesicles derived from a strain producing only LciA almost completely blocked interaction of the monoclonal antibody with these vesicles (Table 2).

**Proteinase K treatment renders membrane vesicles insensitive to lactococcin A**

Sensitive as well as immune right-side-out vesicles were treated with proteinase K. Then, leucine uptake was examined in the absence and presence of lactococcin A and compared with uptake in non-proteinase K-treated vesicles. Residual proteinase K activity (0.1% of the added amount, as determined by the Azocoll assay) was unable to inactivate lactococcin A in these assays (not shown). As shown in Fig. 5B, immune vesicles incubated with proteinase K were not affected by bacteriocin treatment (0.12 µg mg⁻¹ vesicle protein), although the immunity protein was cleaved by proteinase K (see previous section). Proteinase K-treated sensitive vesicles, however, also became insensitive to the bacteriocin (Fig. 5A). First of all, these data show that proteinase K treatment did not affect the activity of the leucine carrier. Second, the data suggest that proteinase K, in addition to digesting LciA, also digested the bacteriocin receptor, previously inferred by Van Belkum et al. (1991b), making the membrane vesicles insensitive to the action of lactococcin A.

**The lactococcin A immunity protein interacts with the lactococcin A receptor**

So far, the results indicate that LciA is present in the cytoplasmic membrane and prevents the action of lactococcin A. However, this does not explain how the bacteriocin is inactivated. The presence of a proteinase K-sensitive component in the membrane vesicles, previously inferred by Van Belkum et al. (1991b), was confirmed by proteinase K analysis. Proteinase K treatment renders membrane vesicles insensitive to lactococcin A. In this section, we will show that proteinase K treatment renders membrane vesicles insensitive to lactococcin A.

**Table 2. Binding of anti-LciA monoclonal antibody AbLciA1 to membrane vesicles of *L. lactis***

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Right Side Out</th>
<th>Inside Out</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-protK</td>
<td>+protK</td>
</tr>
<tr>
<td>LcnA⁻, LciA⁻</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LciA⁺</td>
<td>++++</td>
<td>-</td>
</tr>
<tr>
<td>LcnA⁺, LciA⁺</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LciA⁺, LcnA added</td>
<td>+</td>
<td>nd</td>
</tr>
</tbody>
</table>

ProtK, proteinase K.

* a. Binding of AbLciA1: +, binding; -, no binding; nd, not determined.

**Fig. 4.** SDS-PAA (12.5%) gel (A) and Western blots (B+C) of cell-free extracts of *E. coli* HS3018 carrying: pKV2 (lane 1); pKV280 (lane 2); pKV260 (lane 3); pKV240 (lane 4); pKV220 (lane 5). Blots were used for immunodetection with polyclonal antibodies against MBP (B), or with AbLciA1 (C). Lane C6: prestained molecular weight marker (BioRad Laboratories); sizes (in kDa) are given in the margin.
A. It could do so by binding and, thus, neutralizing the bacteriocin, as has been shown for the colicin A and E1 immunity proteins Cai and ImmE1, or by interacting with 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 vesicles to accumulate leucine was examined. Right-side-out vesicles obtained from a strain producing LciA were labelled with R18 (approximately 4 mol% phospholipid phosphorus) and fused with unlabelled right-side-out vesicles obtained from a sensitive strain, in a ratio of 1:2. Several fusions were performed and the average percentage of fusion was 94%; leucine-uptake experiments were done with fused vesicles, in which the fusion efficiency was 97%.

The results are presented in Fig. 5C and, first of all, show that labelled immune vesicles fused to immune, unlabelled vesicles were unaffected by lactococcin A (0.12 µg mg⁻¹ vesicle protein). This observation indicates that the immunity protein was still active after the fusion procedure. Leucine uptake of fused immune and sensitive vesicles in the absence of lactococcin A was normal (Fig 5C, ◁). However, uptake was completely blocked in the presence of lactococcin A (0.05 µg mg⁻¹ protein, Fig 5C, ▲). The lactococcin A concentration in the latter experiment was approximately one third of that used when immune vesicles were mutually fused, to compensate for the dilution of LciA in the fused membranes. As the presence of 3% unfused sensitive vesicles could not account for the observed block in leucine uptake, the fused vesicles had become sensitive to lactococcin A. Two conclusions can be drawn from these data: (i) the immunity protein in the fused vesicles cannot protect the fusion vesicles from the action of lactococcin A and, therefore, does not bind lactococcin A; and (ii) the receptors contributed by the sensitive vesicles render the fusion vesicles sensitive to lactococcin A. From this we conclude that LciA directly interacts with the lactococcin A receptor.

**DISCUSSION**

Monoclonal antibodies directed against LciA were isolated using the maltose-binding protein methodology (Maina et al., 1988). Although the LciA-moiety (11 kDa) is only one fifth of the fusion protein (55 kDa), 9 of the 17 monoclonal antibodies against the fusion were directed against LciA. On Western blots, the two that were selected reacted with a protein of approximately 10 kDa. The mobility of LciA in tricine-SDS-PAGE agrees well with its molecular size as lciA encodes a product of 11 kDa and the protein is not processed (Nissen-Meyer et al., 1993).

The cell fractionation studies showed that most of LciA is located in or at the membrane, independent of the presence or absence of lactococcin A. This is in agreement with the observation that membrane vesicles prepared from an immune strain are not inhibited in uptake of amino acids by lactococcin A (Van Belkum et al., 1991b). Therefore, we conclude that the functional immunity protein against lactococcin A is the membrane-located molecule.

The binding of the monoclonal antibodies to right-side-out
Net al. has been proposed by Ojcius transmembrane protein. Such insertion of proteins in the amphiphilic helix by virtue of its interaction with another α to 47 might span the cytoplasmic membrane as an residues 48 to 98, is at the outside of the cell. Residues 29 in a model for LciA topology in which the C an of amino acids from residues 29 to 47 in LciA could form the receptor for lactococcin A. Van Belkum et al. the bacteriocin, indicating, as was previously proposed by the immunity protein in the cytoplasmic membrane and that the hydrophilic side of the helix (black area) is tightly associated with a hydrophilic area in the receptor (light 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. Because of the presence of LciA, the bacteriocin is prevented from successful insertion into the cytoplasmic membrane to form pores. N= N-terminus.

vesicles of a strain producing LciA confirms the presence of the immunity protein in the cytoplasmic membrane and indicates that the epitope is located at the outside of the cell. As the epitope mapped between amino acid residues 60 and 80, the C-terminus of LciA is at the outside of the cell, which is in accordance with the observation that the epitope can be cleaved off by proteinase K. When inside-out vesicles are treated with proteinase K and analyzed by Western blotting and immunodetection, the size of LciA is the same as that in non-treated membrane vesicles. This indicates that, if present, the intracellular LciA moiety is either quite small or not accessible to proteinase K. In addition to removal of the C-terminal part of the immunity protein, proteinase K treatment of the membrane vesicles prevented the action of the bacteriocin, indicating, as was previously proposed by Van Belkum et al. (1991b), that a membrane protein acts as the receptor for lactococcin A.

Protein structure computer programs predict that the stretch of amino acids from residues 29 to 47 in LciA could form an α-amphiphilic helix. All these observations can be united in a model for LciA topology in which the C-terminus, from residues 48 to 98, 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. Such insertion of proteins in the cytoplasmic membrane by means of α-amphiphilic helices has been proposed by Ojcius et al. (1991) and Sansom et al. (1991). Thus, the N-terminus of the molecule could be on the cytoplasmic face of the membrane.

Two inferences from the present study are pertinent to the question of how immunity against lactococcin A might work. These are: (i) lactococcin A addition prevents the interaction of the antibody with LciA, and (ii) LciA interacts with the bacteriocin receptor. These conclusions can only be accommodated if we assume that the receptor and the immunity protein are tightly associated in the membrane of immune cells. As the immunity protein contains an α-amphiphilic helix we envisage that the association between the immunity protein and the receptor is formed by the hydrophilic side of the helix to the receptor molecule. Lactococcin A can still bind to the LciA-occupied receptor: their close proximity would explain why the bacteriocin prevents the interaction of the immunity protein with the LciA-specific antibody. However, the binding of bacteriocin to its receptor does not result in the formation of pores when the immunity protein is tightly bound to the receptor. A model visualizing the immunity mechanism which accommodates the present findings is presented in Fig. 6.

The interaction between the immunity protein and the bacteriocin receptor resulting in immunity to lactococcin A also provides an explanation for the observation that only one third of the synthesized LciA molecules is present in the cytoplasmic membrane. Apparently, this portion is enough to interact with all receptors. The remaining two thirds reside in the cytoplasm and are in part associated with the cytoplasmic membrane. We envisage this to be a continuously available reservoir from which molecules can be drawn when new receptors are synthesized and inserted into the membrane, thus resulting in the immediate blocking of the receptor.

The mechanism conveying immunity to lactococcin A differs in a fundamental way from that of Cai and ImmE1. First of all, Cai and ImmE1 contain three and four membrane-spanning α-helices, respectively, whereas LciA has an α-amphiphilic helix. Second, in the colicin systems, no bacteriocin receptor molecules are present in the inner membrane and both immunity proteins directly interact with the corresponding bacteriocins (Zhang and Cramer, 1993).

The model proposed may also be relevant to immunity breakdown, the phenomenon whereby immune strains become sensitive at high amounts of bacteriocin. Immunity breakdown occurs with lactococcin A (Van Belkum et al., 1991b). Although other explanations cannot be ruled out, LciA and lactococcin A may compete for the same site on the receptor, and only at very high concentrations does the bacteriocin win this competition.

**EXPERIMENTAL PROCEDURES**

**Bacterial strains, plasmids, and growth conditions**

The bacterial strains and plasmids used in this study are listed in Table 3. L. lactis strains were grown in MRS (de Man et al., 1960) or in M17 (Terzaghi, et al., 1975) broth supplemented with 0.5% glucose or 0.5% lactose at 30 °C.
Table 3. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Relevant properties</th>
<th>Source/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strain</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactococcus lactis subsp. lactis IL1403</td>
<td>Plasmid free</td>
<td>Chopin et al. (1984)</td>
</tr>
<tr>
<td>subsp. cremoris MG1363</td>
<td>Plasmid free</td>
<td>Gasson (1983)</td>
</tr>
<tr>
<td>subsp. cremoris NCDO712</td>
<td>Donor in conjugations; carrying pLP712</td>
<td>Yanisch-Perron et al. (1985)</td>
</tr>
<tr>
<td><em>Escherichia coli</em> JM103</td>
<td></td>
<td>Freundlieb and Boos (1986)</td>
</tr>
<tr>
<td>HS3018</td>
<td>OmalE 444, malT'-1</td>
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</tr>
<tr>
<td><strong>Plasmid</strong></td>
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<td></td>
</tr>
<tr>
<td>pMB553</td>
<td>Em', containing the lactococcin A operon with lciA, lcnA</td>
<td>Van Belkum et al. (1991a)</td>
</tr>
<tr>
<td>pMB563</td>
<td>Em', containing lciA under control of P59</td>
<td>Van Belkum et al. (1991a)</td>
</tr>
<tr>
<td>pMal-c</td>
<td>Containing mbp-lacZ fusion gene</td>
<td>New England Biolabs</td>
</tr>
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<td>pKV2</td>
<td>Containing mbp-lciA fusion gene</td>
<td>This work</td>
</tr>
<tr>
<td>pKV220, 240, 260, 280</td>
<td>Containing mbp-lciA truncated fusion gene with N-terminal 20, 40, 60, 80 amino acids of LciA, respectively. Lac', PrtP', PrtM'</td>
<td>This work</td>
</tr>
<tr>
<td>pLP712</td>
<td></td>
<td>Gasson (1983)</td>
</tr>
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</table>

**Molecular cloning, DNA sequencing, and synthesis**

General DNA cloning and manipulation techniques were carried out essentially as described by Sambrook et al. (1989). DNA sequencing was done on double-stranded DNA by the dideoxy chain-termination method (Sanger, et al., 1977), using the T7 sequencing kit and protocol (Pharmacia).

Synthetic oligo deoxyribonucleotides were synthesized with an Applied Biosystems 381A DNA synthesizer (Applied Biosystems). The sequences of the primers were as follows (the restriction endonuclease sites used for cloning are indicated underlined): KOV3: 5'-GGGGGAT-CCATCGAGGGTAGGATGAAAAAAAAACAA-3'; KOV4: 5'-AAGCTTAGAATAGTCGTCCCC-ACGACATAAT-3'; KOV9: 5'-GTGATCAACG-CCGCCACG-3'; KOV54: 5'-AAGCTT-AAAAGGGCGGTAGCCAACATAC-3'; KOV55: 5'-AAGCTT-CTAGTCAAGCGCCTTTTCTGC-3'; KOV56: 5'-AAGCTT-ATTAAAGGGCGGTAGCCAACATAC-3'; KOV57: 5'-AAGCTT-CTGATCAACG-CCGCCACG-3'.

**Analytical methods**

Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumine (BSA) as a standard. SDS-PAGE was performed as described previously (Laemmli, 1970). Tricine-SDS-PAGE was performed as described by Schäger and von Jakow (1987). The Rainbow marker (2.3-46 kDa, Amersham Int.) was used as a molecular-weight marker (MWM).

Pyrrolidonyl-carboxylyl peptidase (PCP) activity was measured by mixing 100 µl of a PCP-containing fraction and 100 µl substrate solution (2 mM pyroglutamyl-p-nitroanilide (Pyr-p-NA, Bachem) in 5 mM Tris.HCl (pH 7.0)) and continuously following the release of nitroanilide at 37 °C for 20 min at 410 nm.

Proteinase (PrtP) activity was measured by following the hydrolysis of the substrate methoxy-succinyl-arginyl-prolyl-tyrosyl-p-nitroanilide (MeOSuc-Arg-Pro-Tyr-p-NA, Bachem) in 5 mM Tris.HCl (pH 7.0) and continuously following the release of nitroanilide at 37 °C for 20 min at 410 nm.

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The proteinase maturation protein (PrtM) was detected using polyclonal antibodies (Haandrikman et al., 1991).
Proteinase K activity was measured using Azocoll (Sigma) as a substrate (Yang et al., 1984).

**Isolation of the maltose binding protein (MBP)-LciA fusion protein and construction of MBP-LciA deletion derivatives**

Using the primers KOV3 and KOV4 in a PCR with plasmid pMB553 as template, a PCR product was obtained in which the lciA gene was preceded by a BamHI-restriction site and a DNA sequence encoding the factor Xa recognition site, followed by a HindIII restriction endonuclease site (Fig. 1A). The PCR product was purified, digested with BamHI and HindIII and cloned in plasmid pMal-c (New England Biolabs).

The resulting plasmid, pKV2, encodes an MBP-LciA fusion protein under control of the IPTG inducible Tac-promoter (dIII and cloned in plasmid pMal-c (New England Biolabs). The HindIII restriction endonuclease site was followed by a DNA sequence encoding the factor Xa recognition site, resulting in MBP-fusion protein. The fusion protein was purified in a one-step procedure using affinity chromatography on an amylose resin (New England Biolabs). All steps were performed at room temperature. One liter of broth containing ampicillin was inoculated with 10 ml overnight culture of HS3018 (New England Biolabs). All steps were performed at room temperature, one liter of broth containing ampicillin was inoculated with 10 ml overnight culture of E. coli HS3018 containing plasmid pKV2. After 3 h of growth, IPTG was added to a final concentration of 100 µg ml⁻¹. After 1 h, cells were harvested by centrifugation, resuspended in 50 ml of lysis buffer (10 mM Na₂HPO₄, 30 mM NaCl, 0.25% Tween 20, 10 mM β-mercapto ethanol, 10 mM EDTA, 10 mM EGTA, pH 7.0), and disrupted using glass beads in the 'Shake it, Baby' (Biospec Products). After removal of cell debris by centrifugation, the cell lysate was applied to an amylose-column equilibrated with c-buffer (10 mM Na₂HPO₄, 0.5 M NaCl, 1 mM sodium azide, 10 mM β-mercapto ethanol, 1mM EGTA, pH7.0). The column was washed with 3 column-volumes of c-buffer containing 0.25% Tween 20, and then with 5 volumes of c-buffer. The fraction was subsequently eluted with c-buffer containing 10 mM maltose. Fractions were collected and analysed on SDS-PAGE for the presence of the fusion protein. Fractions containing the protein were pooled and dialysed overnight against phosphate-buffered saline (Sambrook et al., 1989).

Deletion variants of the full size mbp-lciA gene fusion were made by PCR. LciA contains 98 amino acids. Oligo-nucleotide primers were constructed to delete 18, 38, 58, and 78 C-terminal amino acids from LciA, resulting in MBP-fusion proteins with 80, 60, 40, and 20 amino acids of LciA, respectively (Fig. 1B).

**Monoclonal antibody production**

Two BALB/c mice were immunized by intraperitoneal injection with 100 µl (1 mg ml⁻¹) of the purified fusion protein in complete Freund’s adjuvants. The mice were boosted intravenously twice with 100 µg fusion protein with a 4-week interval. Fusions with myeloma cell line SP2/0 were performed as described by Kennett et al. (1980) with modifications of de Leij et al. (1983). Culture supernatants of growing hybridoma cells were examined for reactivity with the MBP-LciA fusion and with an MBP-LacZ fusion (encoded by plasmid pMal-c) by enzyme-linked immunosorbent assay (ELISA). Two cell lines that were positive with the MBP-LciA fusion but negative with the MBP-LacZ fusion, were chosen as the source of monoclonal antibodies. The antibodies were designated AbLciA1 and AbLciA3.

**ELISA, Western blotting, and immunodetection**

ELISA was performed as described previously (Laan et al., 1988). Plates were scanned in a TiterTek multiscan (Molecular Devices) at 490 nm.

Western blotting onto BA85 nitrocellulose (Schleicher & Schuell, Inc.) was performed as described by Towbin et al. (1979), except that blotting was done overnight at 25 °C, using a Bio-Rad mini trans-blot apparatus. Membranes were blocked with 2% BSA in solution A (10 mM Tris.HCl pH 8.0, 150 mM NaCl, 0.05% Tween 20) for 30 min at room temperature, after which they were exposed for 1 h at room temperature to monoclonal antibody diluted 100 times in 1% BSA in solution A. After washing three times with solution A, the membranes were incubated for 1 h at room temperature with peroxidase-conjugated rabbit anti-mouse immunoglobulins (Dako-immunoglobulins), diluted 200-fold in 1% BSA in solution A.

The second antibody was detected after reaction with substrate solution (0.6 mg ml⁻¹ diaminobenzidine tetrahydrochloride, 0.3% CoCl₂ in 10 mM Tris.HCl pH 7.4). The relative band intensities were estimated visually.

**Fractionation of L. lactis**

Fractionation of cells was performed as described by Baankreis (1992). To check the fractionation, the following marker proteins were chosen: PCP as an intracellular enzyme, PrtP as a cell envelope-associated enzyme, and PrtM as a membrane protein. Assays are described above. The following fractions were obtained: supernatant, soluble cell wall, cytosolic fraction, and membrane fraction. The last fraction was sonicated to obtain a membrane-associated and a tightly membrane-bound protein fraction (Baankreis, 1992).

Samples corresponding to 1 ml of culture were subjected to tricine-SDS-PAGE and blotted onto nitrocellulose filters which were used for immunodetection of LciA.

**Preparation of right-side-out and inside-out membrane vesicles**

Right-side-out membrane vesicles were prepared essentially as described by Otto et al. (1982). Inside-out vesicles were prepared according to a modified protocol of Kinoshita et al. (1984). Briefly, cells grown in 3 liters of MRS (supplemented with 0.5% glucose) to an optical density at 660 nm of 1.5 were harvested and washed in 100 mM potassium phosphate pH 7.0 (K-Pi). Cells were resuspended in the same buffer containing 1 mM dithiothreitol and 5 mM MgSO₄, Lysozyme (5 mg ml⁻¹) and mutanolysin (2.5 U ml⁻¹) were added and after 30 min incubation at 37 °C, the suspension was run through a French pressure cell at 8000
psi). The sample was treated for 15 min. at 37 °C with DNase and RNase (100 μg ml⁻¹ final concentration each), after which K-EDTA was added to a final concentration of 15 mM. The sample was centrifuged (15 min, 14 000 x g), and the upper two-thirds of the supernatant was collected and recentrifuged (60 min, 150 000 x g). The pellet containing the inside-out vesicles was resuspended in 50 mM K-Pi containing 5 mM MgSO₄ and 20% glycerol, frozen in liquid nitrogen and stored at -80 °C.

Proteinase K treatment of membrane vesicles was performed as follows: after proteinase K addition (0.2 mg mg⁻¹ vesicle protein) and incubation at 37 °C for 30 min, the vesicle suspension was centrifuged, washed three times with, and ultimately resuspended in, a suitable buffer for further experiments.

Assay of monoclonal antibody binding to membrane vesicles

To a quantity of membrane vesicles corresponding to 5.4 μg protein, NET-gel buffer (50 mM Tris.Cl (2-amino-2-(hydroxymethyl)-1,3-propanediol), 150 mM NaCl, 0.1% Nonidet R40, 1 mM EDTA, 0.25% gelatin, 0.02% sodium azide, pH 7.5) was added to a final volume of 50 μl. Then, 5 μl of an AbLciA1- (or AbLciA3-) containing culture supernatant of hybridoma cells was added, and the suspension was incubated at 0 °C for 1 h. Next, 2 μl of peroxidase-conjugated rabbit anti-mouse antibody (DAKO-Immunoglobulins) was added, and incubation was continued for another hour at 0°C. The suspension was centrifuged (4 °C, 15 000 x g) in a microcentrifuge. The pellet was washed three times with NET-gel buffer containing 0.5 M NaCl and 0.1% SDS and resuspended in 50 μl NET-gel buffer. A 10 μl sample of this suspension was diluted in 100 μl substrate (0.2 mg ml⁻¹ o-phenylenediamine, 0.0045% H₂O₂ in 50 mM sodium phosphate, pH 5.6) in a microtitre plate and allowed to react for 10 min at room temperature. The reaction was stopped by the addition of 100 μl 1N H₂SO₄. Plates were scanned in a Titertek multiscan (Molecular Devices Corp.) at 490 nm. When purified lactococcin A (a gift from Dr I.F. Nes, Laboratory of Microbial Genetecology, Ås, Norway) was added, the membrane vesicles were washed three times with NET-gel buffer after incubation with lactococcin A at 0 °C for 1h.

Fusion of membrane vesicles and leucine uptake experiments

Fusion of membrane vesicles was quantitated with the octadecyl rhodamine B-chloride (R18) fusion assay (Hoekstra et al., 1984). Membrane vesicles obtained from a strain producing only LciA were labelled with R18 as described (Driessen et al., 1986). Labelled vesicles were fused with unlabelled vesicles obtained from the sensitive strain IL1403, or with unlabelled immune vesicles, in a ratio of 1:2. R18 fluorescence was measured using excitation and emission wavelengths of 560 nm and 590 nm, respectively, in a Perkin Elmer LS50 spectrophotofluorometer. Leucine-uptake experiments were performed as described previously (Van Belkum et al., 1991b).

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REFERENCES


Chapter V

Mode of action of LciA


