Proteins of the lactococcin A secretion system: 
\( lcnD \) encodes two in-frame proteins

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Received 20 July 2001; received in revised form 27 August 2001; accepted 29 August 2001
First published online 12 October 2001

Abstract

Polyclonal antibodies were raised against LcnC and LcnD proteins of the \textit{Lactococcus lactis} bacteriocin lactococcin A secretory system to examine their cellular location and interaction. Two major reacting bands were detected by Western immunoblot with the anti-LcnD antibody: one of 52 kDa (LcnD) and another of 45 kDa, called here LcnD*. LcnD* was still detectable after removing the AUG start codon for LcnD. Chemical cross-linking analyses of membrane fractions of \textit{L. lactis} cells expressing the LcnC/D secretion machinery were performed. Our results indicate that LcnD is present in the secretion machinery complex as a dimer and is able to interact with LcnD* and LcnC. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Lactic acid bacterium; Bacteriocin; Lactococcin A; Protein secretion; \textit{Lactococcus lactis}

1. Introduction

Lactococcin A (LcnA) is a narrow specificity bacteriocin produced by certain strains of \textit{Lactococcus lactis} [1,2], a member of the lactic acid bacteria (LAB) family. These bacteria are of eminent economic importance because of their widespread use in food and feed fermentations and their potential to produce a number of substances with antimicrobial activity which can be used as biopreservatives.

Secretion and maturation of LcnA has been shown to depend on the two membrane proteins LcnC and LcnD that, together, form a Type I (\textit{sec}-independent) secretion system [3]. LcnC belongs to the family of ABC transporters, and LcnD is proposed to be the accessory protein [1]. The structural genes \textit{lcnC} and \textit{lcnD} were identified in an operon upstream of the bacteriocin operon on a plasmid [1], but homologues of \textit{lcnC} and \textit{lcnD} were also shown to be chromosomally located in \textit{L. lactis} IL1403, a strain that does not produce bacteriocin activity [4].

The membrane topology of LcnD conforms to that of accessory proteins of Type I transport systems: they are bitopic proteins with a short N-terminal part residing in the cytoplasm, one trans-membrane domain and the C-terminal part, representing the majority of the protein, at the extracellular side of the cytoplasmic membrane [5]. The function of the accessory proteins of ABC transporters in Gram-positive bacteria is unknown.

To be able to exploit the full potential of bacteriocins in food preservation, fundamental knowledge is needed not only of their mode of action and inhibitory spectra, but also of the secretory machinery. In this study, we describe a new factor involved in the secretory process and examine the protein interactions involved in LcnA secretion by protein cross-linking studies.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. \textit{L. lactis} was grown as standing cultures at 30°C in 2-fold diluted M17 broth [6], supplemented...
Table 1

Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant properties/genotype</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. lactis subsp. cremoris MG1363</td>
<td>Plasmid-free</td>
<td>[19]</td>
</tr>
<tr>
<td>L. lactis IL1403</td>
<td>Plasmid-free, chromosomally encoded secretion/maturation machinery for LcnA</td>
<td>[20]</td>
</tr>
<tr>
<td>E. coli C41(DE3)</td>
<td>BL21(DE3) derivative</td>
<td>[21]</td>
</tr>
<tr>
<td>L. lactis IL1403 Plasmid-free, chromosomally encoded secretion/maturation machinery for LcnA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MG1363</td>
<td>Plasmid-free</td>
<td></td>
</tr>
<tr>
<td>pKv4</td>
<td>Em&lt;sup&gt;R&lt;/sup&gt;, pIL253 derivative carrying lcnC, lcnD, lcnA, lciA, lcnB, lcnB</td>
<td>lab collection</td>
</tr>
<tr>
<td>pMG36A</td>
<td>Cm&lt;sup&gt;R&lt;/sup&gt;, pMG36 derivative carrying lcnD and lciA under P32</td>
<td>lab collection</td>
</tr>
<tr>
<td>pIcnD</td>
<td>pMG36A carrying lcnD under control of P32</td>
<td>this study</td>
</tr>
<tr>
<td>pIcnD&lt;sub&gt;A&lt;/sub&gt;</td>
<td>pMG36A carrying lcnD mutated in the AUG codon</td>
<td>this study</td>
</tr>
<tr>
<td>pL46</td>
<td>pMG36A carrying lcnD mutated in the 46th codon</td>
<td>this study</td>
</tr>
<tr>
<td>pET32</td>
<td>Histidine fusion vector</td>
<td>lab collection</td>
</tr>
<tr>
<td>pET32&lt;sub&gt;lcnD&lt;/sub&gt;</td>
<td>pET32 derivative carrying lcnD</td>
<td>this study</td>
</tr>
<tr>
<td>pET32&lt;sub&gt;lcnC&lt;sub&gt;200&lt;/sub&gt;&lt;/sub&gt;</td>
<td>pET32 derivative carrying lcnC&lt;sub&gt;200&lt;/sub&gt;</td>
<td>this study</td>
</tr>
</tbody>
</table>

*Em<sup>R</sup>: erythromycin resistance; Cm<sup>R</sup>: chloramphenicol resistance; P32: lactococcal constitutive promoter.

with 0.5% glucose (G1/2 M17). For L. lactis, chloramphenicol and erythromycin were used at final concentrations of 4 and 5 μg ml<sup>-1</sup>, respectively. Escherichia coli was grown in TY medium [7] at 37°C with aeration. Chloramphenicol and ampicillin were used at final concentrations of 10 and 100 μg ml<sup>-1</sup>, respectively. Isopropylthio-β-D-galactoside (IPTG) was used at a final concentration of 1 mM.

2.2. Molecular cloning and plasmid construction

General molecular cloning techniques were performed essentially as described by Sambrook et al.[7]. DNA was isolated as described before [8,9]. Electrotransformation of L. lactis was performed as described earlier [10].

Deletion of the lcnD start codon was performed by PCR with oligonucleotides lcnD<sub>Δ</sub>-5'<sup>'</sup>-AAGCTTAAAGCTTTT-GATAAAAATTACTG-3'<sup>'</sup>; and lcnD<sub>(Asp718) 5'</sub>-GGTACCC-TCTACTGATTGCCTCTTCC-3'<sup>'</sup>, the resulting fragment was cloned in pMG36A. Site-directed mutagenesis of lcnD was performed by double-step PCR with the oligonucleotides lcnD(RBS) 5'<sup>'</sup>-CCGGAATTC-CATGGGTA CCTCTACTGATTGCCTCTTCC-3'<sup>'</sup>, lcnD<sub>(Asp718) 5'</sub>-GGTACCC-TCTACTGATTGCCTCTTCC-3'<sup>'</sup>, and lcnD<sub>(Asp718) 5'</sub>-GGTACCC-TCTACTGATTGCCTCTTCC-3'<sup>'</sup>. The mutated codon was designated the cytoplasmic fraction. The pellet was the membrane fraction.

Protein samples for SDS–polyacrylamide gel electrophoresis (SDS–PAGE) were prepared and loaded on gel as described earlier [12]. Proteins were blotted onto PVDF membranes. Polyclonal antibodies against LcnC and LcnD were used as primary antibodies; mouse horseradish peroxidase-conjugated antirabbit immunoglobulins were used as secondary antibodies. In vivo formaldehyde cross-linking was performed as previously described [13].

2.5. LcnA activity test

LcnA activity was determined by the dilution assay as described earlier [14], except that microtitre plates with wells containing 200 μl of G<sub>1/2</sub>M17 broth were used. L. lactis IL1403 was used as indicator strain: in each well of the microtitre plate, 100 μl of a 10<sup>4</sup>-diluted overnight cul-
ture of this strain containing approximately $10^4$ colony forming units was applied.

3. Results and discussion

3.1. Anti-LcnD antibodies react with two proteins

Anti-LcnD polyclonal antibodies were used to examine cell-free extracts of *L. lactis* MG1363(*plcnD*). After Western immunoblot analysis, two major reacting bands were detected, one of 52 kDa that corresponds to the previously identified LcnD [1] and another one of 45 kDa (Fig. 1, lanes 3 and 4). As both bands are absent in cell-free extracts of MG1363(pMG36A), used as negative control. The equivalent of 10 μl of cells (late exponential phase) were applied in each well. Sizes of LcnD and LcnD*, as determined from a comparison with the protein molecular mass marker on the same gel (not shown), are indicated in the right margin. Polyclonal antibodies raised against LcnD were used as primary antibodies.

Because LcnD is a monotopic membrane protein with the membrane-spanning domain formed by the amino acid residues 22–40 [5], it is likely that LcnD* lacks at least the first 40 residues of LcnD. LcnD* could have been formed either by specific degradation of LcnD or by alternative translation initiation of *lcnD* mRNA. In the latter case the alternative start codon should be located after the 40th codon.

3.2. LcnD* is not a degradation product of LcnD

To understand the origin of the LcnD* protein, the AUG start codon of *lcnD* was removed (Section 2). As expected, *L. lactis* carrying the mutated *lenD* gene on a plasmid, strain MG1363(*plnDΔ*), did not produce the 52-kDa protein LcnD (Fig. 1, lane 6). The strain did produce LcnD*, however, and this truncated protein was mostly present in the cytoplasmic fraction (Fig. 1, lane 7). This result indicates that LcnD* is not a proteolytic product of the larger protein, LcnD. Apparently LcnD* is produced from the *lcnD* mRNA by translation from an internal start codon.

We identified the UUG triplet of leucine 46 as a possible alternative translational start codon. This codon is immediately downstream of a putative Shine Dalgarno (SD) consensus sequence (Fig. 2). Direct evidence that the Leu46 UUG codon is the start codon of *lcnD* was obtained by site-directed mutagenesis. Using PCR the UUG codon was changed into UUA (Section 2), an alternative codon for Leu but not a start codon.

Again, MG1363(*plcnD*) produced LcnD and LcnD* (Fig. 3). The protein extract from the MG1363 strain carrying plasmid pL46 with the *lenD* gene in which the UUG codon has been mutated contained the LcnD band, but not that corresponding to LcnD* (Fig. 3). Apparently LcnD* translation starts form the UUG codon in position 46.
3.3. Effect of lcnD mutations on LcnA production

_L. lactis_ IL1403 has a chromosomal copy of the _lcnC/D_ operon. The products LcnC* and LcnD*, as the truncated protein lacks the N-terminal TMS of LcnD. The gene of the accessory protein for colicin V secretion in _E. coli_, _cvaA*, also encodes two in-frame products, namely CvaA and CvaA* [15]. CvaA is an integral membrane protein [16]. CvaA* lacks the hydrophobic residues present in the N-terminal region of CvaA and is localised in the cytoplasm [15]. Although CvaA* is not essential for ColV secretion, it stabilises CvaA, thereby enhancing ColV secretion [15]. A model has been proposed in which CvaA* interacts with the secretion machinery from the cytoplasmic side of the membrane [15].

Formaldehyde cross-linking analysis of membrane fractions of _MG1363_(pKV4) cells expressing the _lcnC/D_ secretion machinery from the plasmid was performed to examine whether LcnC and LcnD interact.

46, which explains the cytoplasmic location of LcnD*, as the truncated protein lacks the N-terminal TMS of LcnD.

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3.4. LcnD, LcnD* and LcnC are organised in a membrane-associated complex

In conclusion, secretion of LcnA is dependent on the secretion machinery from the plasmid was performed to examine whether LcnC and LcnD interact.

Table 2

<table>
<thead>
<tr>
<th>Strain</th>
<th>Activity (AU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL1403</td>
<td>n.d.</td>
</tr>
<tr>
<td>IL1403(pKV4)</td>
<td>5120</td>
</tr>
<tr>
<td>IL1403(pMG36A)</td>
<td>1280</td>
</tr>
<tr>
<td>IL1403(plcnD)</td>
<td>1280</td>
</tr>
<tr>
<td>IL1403(pL46)</td>
<td>1280</td>
</tr>
<tr>
<td>IL1403(plcnDA)</td>
<td>160</td>
</tr>
<tr>
<td>n.d., not detectable</td>
<td></td>
</tr>
</tbody>
</table>

The differences in the amounts of LcnA produced by the various strains we used, indicate that LcnD* is not able to interact with the chromosomally encoded LcnD protein with the same affinity as it has for LcnD; indeed LcnD* has a negative effect on LcnA production in the strain _IL1403_(plcnD). The latter result is in favour of a chaperone function for LcnD*: the bacteriocin would be titrated away by LcnD* and is not available for the LcnC*/D’ machinery, unless isogenic LcnD is present in the membrane.

Formaldehyde cross-linking analysis of membrane fractions of _MG1363_(pKV4) cells expressing the _lcnC/D_ secretion machinery from the plasmid was performed to examine whether LcnC and LcnD interact.

On a Western blot probed with anti-LcnD polyclonal antibodies three additional bands were observed in the membrane fraction of formaldehyde-treated _MG1363_(pKV4) cells (Fig. 4A). None of these bands were, as expected, present in the extracts of _MG1363_. Molecular size comparisons suggest that the three bands may be due to interactions between LcnD and LcnD* (95 kDa), between two molecules of LcnD (110 kDa), and between two molecules of LcnD and one LcnC molecule (200 kDa). After anti-LcnD antibody stripping and re-probing with anti-LcnC polyclonal antibodies, a 200 kDa band cross reacted (Fig. 4B). This could be either a 2LcnD–LcnC complex, like it was in panel A, or an LcnC dimer, which would have approximately the same molecular masses. In addition, the anti-LcnC antibodies detected a band that was a few kDa larger than that of LcnC (Fig. 4B). This band may be the product of interaction between LcnC and LcnA. The latter could not be confirmed as, unfortunately, it has not been possible to make polyclonal nor monoclonal antibodies against LcnA due to the low immunogenicity of the bacteriocin (unpublished results).

In conclusion, secretion of LcnA is dependent on the two secretory proteins LcnC and LcnD. The specificity

![Fig. 3. Western hybridisation of cell-free extracts of MG1363(plcnD), MG1363(pL46) and MG1363 used as negative control. 15 µg of protein were loaded in each lane and run on a SDS 10% PAA gel. Sizes of LcnD and LcnD*, as determined from a comparison with the protein molecular mass marker on the same gel (not shown), are indicated in the left margin. Polyclonal antibodies against LcnD were used as primary antibodies.](image-url)
of LcnA production is also dependent on the presence of a cytoplasmically located LcnD* protein which originates from the same mRNA as LcnD but from a different internal translational start codon. The role of LcnD* in lactococcin secretion is still unknown. It may stabilise pre-LcnA, as proposed for other chaperones in type II or III secretion systems [17], or it could keep pre-LcnA in a secretion competent state, behaving like a molecular chaperone [18]. The results of chemical cross-linking analysis provide evidence for interactions between the dedicated export proteins of the LcnA secretion system.

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We would like to thank C.M. Franke for helpful discussions. M.V. was the recipient of a TMR fellowship of the European Comunity (Contract nr. ERBFMBICT960771).

References

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