Topology of LcnD, a protein implicated in the transport of bacteriocins from Lactococcus lactis

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Lactococci are bacteriocins produced by *Lactococcus lactis* (15, 17, 18). They belong to the small heat-stable, non-lanthionine-containing membrane-active peptides characterized by a Gly-Gly-1-X Gly (X is any amino acid) processing site in the precursor. The proteins LcnC and LcnD are essential for extracellular activity of these bacteriocins, and a role in transport and/or maturation has been suggested (15). LcnC, a member of the ABC transporter family, is a putative transmembrane protein with several transmembrane sequences (TMS). LcnD does not show significant amino acid sequence similarity with other proteins. Computer predictions indicate that LcnD contains not show significant amino acid sequences similarity with other proteins.

Four in-frame translational fusions to both the reporter proteins β-galactosidase and alkaline phosphatase support a topological model of LcnD, a protein implicated in the transport of several bacteriocins from *Lactococcus lactis*, in which the N-terminal part is located intracellularly and one transmembrane helix spans the cytoplasmic membrane.

Plasmids expressing the LcnD-PhoA chimera in both *E. coli* and *L. lactis* were unstable in *L. lactis*. Even integration of the constructs into the lactococcal chromosome ensure a low copy number led to deletion formation, as judged by Southern hybridization, and no fusion products were identifiable in Western immunoblots (data not shown). When PhoA was fused in or upstream of the putative TMS, it showed no significant activity in *E. coli*, whereas the C-terminal fusion activities were clearly higher than background activity (Table 1). In all cases, the PhoA fusion proteins showed properties complementary to the LacZ chimeras. The fact that *E. coli* CC118 colonies expressing the LcnD-PhoA fusion in the putative TMS are slightly blue in the plate assay might indicate the presence of the TMS at this position.

**Western blot analysis.** Synthesis of fusion proteins was verified by Western hybridizations with Western-Light (Tropix, Bedford, Mass.) and nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate toluidinium (NBT/BCIP; Promega, Madison, Wis.) and anti-LacZ and anti-PhoA antibodies. Products of the expected sizes could indeed be detected, but breakdown products were also present (Fig. 2). A positive band in the CFE of *E. coli* WK6 corresponded to the size of β-galactosidase ΔM15 produced by this strain (Fig. 2A and B, lanes 1). *E. coli* WK6 containing pMG57, a plasmid carrying the entire *E. coli* lacZ gene without its ribosome-binding site (RBS) (19), showed an additional band similar in size to that of partially purified β-galactosidase. All strains carrying lcnD::phoA fusions were induced with IPTG (isopropylthiogalactopyranoside), and similar amounts of protein were produced (Fig. 2C).
parently, the LacZ moiety in these fusion proteins is associated with this membrane.

Topology of LcnD and homologs. Computer programs that predict protein topology, namely, those of Rao and Argos (11), Helixmem (2), Soap (5, 6), and Toppred 2.0 (14, 22), all arrive at the same model (Fig. 1). It is highly unlikely that an even number of TMS are missed by these computer programs, and we propose that our model of LcnD is correct.

The fact that E. coli colonies expressing the C-terminal LacZ fusion were slightly blue on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) plates after overnight incubation at 37°C seems in conflict with this model. Western hybridization (Fig. 2) shows that, in addition to full-size fusion protein, several breakdown products were present, one of which could explain residual LacZ activity. CFE with this fusion protein had to be concentrated 100-fold more than CFE of cells producing an intracellular LacZ chimera in order to obtain comparable signals in Western hybridization. This indicates that products of LacZ fused to parts of LcnD that are normally on

TABLE 1. Activities of the LcnD chimeras

<table>
<thead>
<tr>
<th>Plasmid or fusion</th>
<th>Colony color</th>
<th>Activityb (U/mg of protein)</th>
<th>Alkaline phosphatase (E. coli)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>E. coli WK6, X-Gal</td>
<td>E. coli CC118, X-P</td>
</tr>
<tr>
<td>None</td>
<td>White</td>
<td>White</td>
<td>White</td>
</tr>
<tr>
<td>pMG57</td>
<td>White</td>
<td>NDc</td>
<td>White</td>
</tr>
<tr>
<td>Position 21</td>
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<td>White</td>
<td>Blue</td>
</tr>
<tr>
<td>Position 44</td>
<td>Blue</td>
<td>Slightly blue</td>
<td>Blue</td>
</tr>
<tr>
<td>Position 80</td>
<td>White</td>
<td>Blue</td>
<td>White</td>
</tr>
<tr>
<td>Position 474</td>
<td>Slightly blue</td>
<td>Blue</td>
<td>White</td>
</tr>
</tbody>
</table>

a Activities were measured in CFE from overnight (ON) and exponentially growing (EXP) cultures.
b pMG57 is a pMV19 derivative containing a lacZ gene lacking an RBS and is the basic vector used for the lcnD-lacZ fusions. The fusion points are indicated in Fig. 1.
c X-Gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; X-P, 5-bromo-4-chloro-3-indolylphosphate. TY (12) agar plates were used for E. coli; glucose M17 (16) plates were used for L. lactis.
d One unit is defined as 1 ng of substrate converted per min.

ND, not determined.
the outside of the cytoplasmic membrane are susceptible to protease activity, conceivably because of incorrect folding. In *L. lactis*, such products were hardly detectable. The relative activities of all LcnD-LacZ fusion proteins in exponentially growing cells and overnight cultures were comparable except for the C-terminal fusion protein, the activity of which was significantly higher in the overnight culture. This result suggests more pronounced proteolytic degradation in overnight cultures, as was indeed shown by Western hybridization (Fig. 2B). It supports our notion of proteolytic breakdown leading to background LacZ activity.

From computer predictions, topological models have been developed for proteins forming dedicated transport systems for lactococcins (LcnC and LcnD) (15, 17), pediocin (PedC and PedD) (9), and hemolysin (HlyB and HlyD) (3). Each transport system has one member of the family of ABC transporters (LcnC, PedD, and HlyB, respectively). The accessory proteins HlyD (13) and PedC (2a) have a membrane topology similar to...
that of LcnD, and although they do not have amino acid sequence similarities, they seem to belong to a group of proteins with similar functions. All three are important for the production of active extracellular bacteriocin (LcnD and PedC) and hemolysin (HlyD). Their conserved genetic organization and topological structure suggest an important function in secretion of their respective allocrites.

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