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Topology of LcnD, a Protein Implicated in the Transport of Bacteriocins from *Lactococcus lactis*

CHRISTIAN M. FRANKE, KEES J. LEENHOUTS,† ALFRED J. HAANDRIKMAN,‡
JAN KOK, GERARD VENEMA,* AND KOEN VENEMA

Department of Genetics, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands

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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.

Lactococcins 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^{+1} (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 one TMS between amino acid residues 21 and 44. To test this topological model, we constructed four in-frame translational fusions with β-galactosidase (LacZ) (8) and alkaline phosphatase (PhoA) (1, 8). The fusion proteins were encoded by pWV01 derivatives (20) under control of the strong lactococcal P32 promoter (21), and activities could thus be tested in both *Escherichia coli* and *L. lactis*.

By standard PCR techniques (7), unique restriction enzyme sites were introduced in *lcnD* to generate fusions with *lacZ* or *phoA*. In the topological model of LcnD (Fig. 1), the amino acids shown as black circles correspond to residues to which either β-galactosidase or alkaline phosphatase was fused. The activities of β-galactosidase and alkaline phosphatase fusions were measured (1, 10) in triplicate in cell-free extracts (CFE) and plate assays (Table 1).

LacZ fusions. In both *E. coli* and *L. lactis*, activities were highest when LacZ was fused to the N-terminal moiety of LcnD, whereas they were negligible when LacZ was fused to the C terminus (Table 1). Fusion of LacZ to LcnD at position 30 in the putative TMS led to a chimeric protein with an activity 50 times lower than that of a protein in which LacZ was fused upstream of the putative TMS (at position 21). This is the case in both organisms and supports the presence of a transmembrane segment at this position. All fusions of LcnD with LacZ show comparable relative activities in *E. coli* and *L. lactis*, which suggests similar topologies in both organisms.

PhoA fusions. Because of instability problems with the *lcnD-phoA* constructs, we placed these behind the inducible *E. coli lac* promoter (23). Plasmids expressing the LcnD-PhoA chimeras were unstable in *L. lactis*. Even integration of the constructs into the lactococcal chromosome to 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).

Electron microscopy. Cells from overnight cultures of *E. coli* expressing the LcnD-LacZ fusions were fixed at 0°C in 2.5% formaldehyde–0.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) and dehydrated in a graded ethanol series. Fixed cells were then embedded in Unicryl resin. Ultrathin sections were labeled with rabbit anti-LacZ antibodies, which were subsequently detected with immunogold-labeled goat anti-rabbit polyclonal antibodies. Electron microscopic images were obtained on a Philips CM10 transmission electron microscope (Philips, Eindhoven, The Netherlands). In *E. coli* WK6, hardly any label was detected (Fig. 3). In cells containing pMG57, little label is found, whereas cells expressing LcnD fused to LacZ at residue 21 or at position 30 show a considerable quantity of label in the cytoplasm, indicative of the presence of LacZ antigen in this cell compartment. In cells expressing LcnD fused to LacZ at position 80 or 474, label was found almost exclusively near the cytoplasmic membrane. Ap-

* Corresponding author. Phone: 31 50 3632092. Fax: 31 50 3632348.
† Present address: International Centre for Genetic Engineering and Biotechnology, Department of Molecular Pathology, Padriciano 99, I-34012, Trieste, Italy.
‡ Present address: Hercules European Research Centre, Nijverheidsweg 60, 3771 ME Barneveld, The Netherlands.

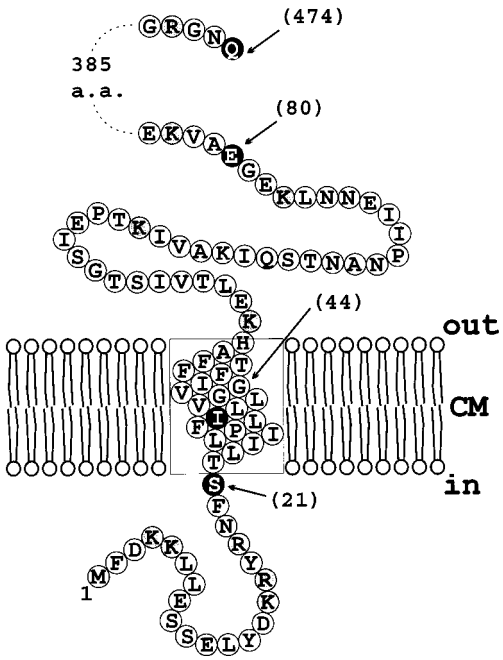

FIG. 1. Proposed model of LcnD. The black circles indicate the amino acid residues in LcnD to which β-galactosidase and alkaline phosphatase were fused. Their corresponding positions in LcnD are given in parentheses. Shaded circles, positively charged residues; CM, cytoplasmic membrane; a.a., amino acids.

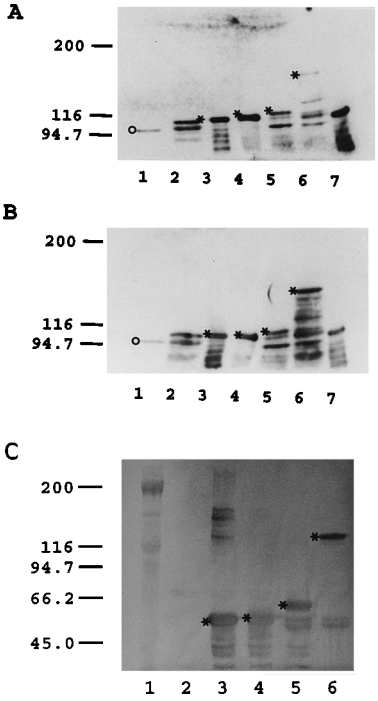

FIG. 2. Western hybridization with anti-β-galactosidase and anti-alkaline phosphatase antibodies of CFE of exponentionally growing (A and C) and overnight (B) cultures of *E. coli*. (A and B) *E. coli* WK6 carrying no plasmid, pMG57, and plasmids encoding β-galactosidase fused to LcnD at positions 21, 44, 80, and 474 (lanes 1 to 6, respectively); lanes 7, partially purified β-galactosidase. Approximately 80 and 40 μg of CFE from overnight and exponentially growing cultures were loaded, respectively. Lanes 3 and 4 contain 50 to 100 times less protein than the other lanes. ○, inactive ΔM15 β-galactosidase fragment encoded by the chromosome of *E. coli* WK6. (C) CFE of *E. coli* CC118 carrying no plasmid or plasmids encoding alkaline phosphatase fused to LcnD at positions 21, 44, 80, and 474 (lanes 2 to 6, respectively). CFE were prepared after 2 h of induction with IPTG. Lane 1, molecular size markers (Bio-Rad Laboratories); sizes are indicated (in kilodaltons) in the margin. LcnD-LacZ and LcnD-PhoA fusion proteins are indicated by an asterisk on the left of the protein bands in lanes 3 to 6.

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[a]

Plasmid or fusion[b]	Colony color[c]			Activity[d] (U/mg of protein)				
				β-Galactosidase				Alkaline phosphatase (*E. coli* EXP)
				E. coli		*L. lactis*		
	E. coli WK6, X-Gal	*E. coli* CC118, X-P	*L. lactis* MG1363, X-Gal	ON	EXP	ON	EXP	
None	White	White	White	113	15	19	7	0.2
pMG57	White	ND[e]	White	227	68	9	24	ND
Position 21	Blue	White	Blue	72,998	315,340	1,641	3,478	0.6
Position 44	Blue	Slightly blue	Blue	8,561	5,807	412	655	0.7
Position 80	White	Blue	White	61	76	27	12	5.9
Position 474	Slightly blue	Blue	White	406	65	9	14	2.2

[a] Activities were measured in CFE from overnight (ON) and exponentionally growing (EXP) cultures.
[b] pMG57 is a pWV01 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.
[e] ND, not determined.

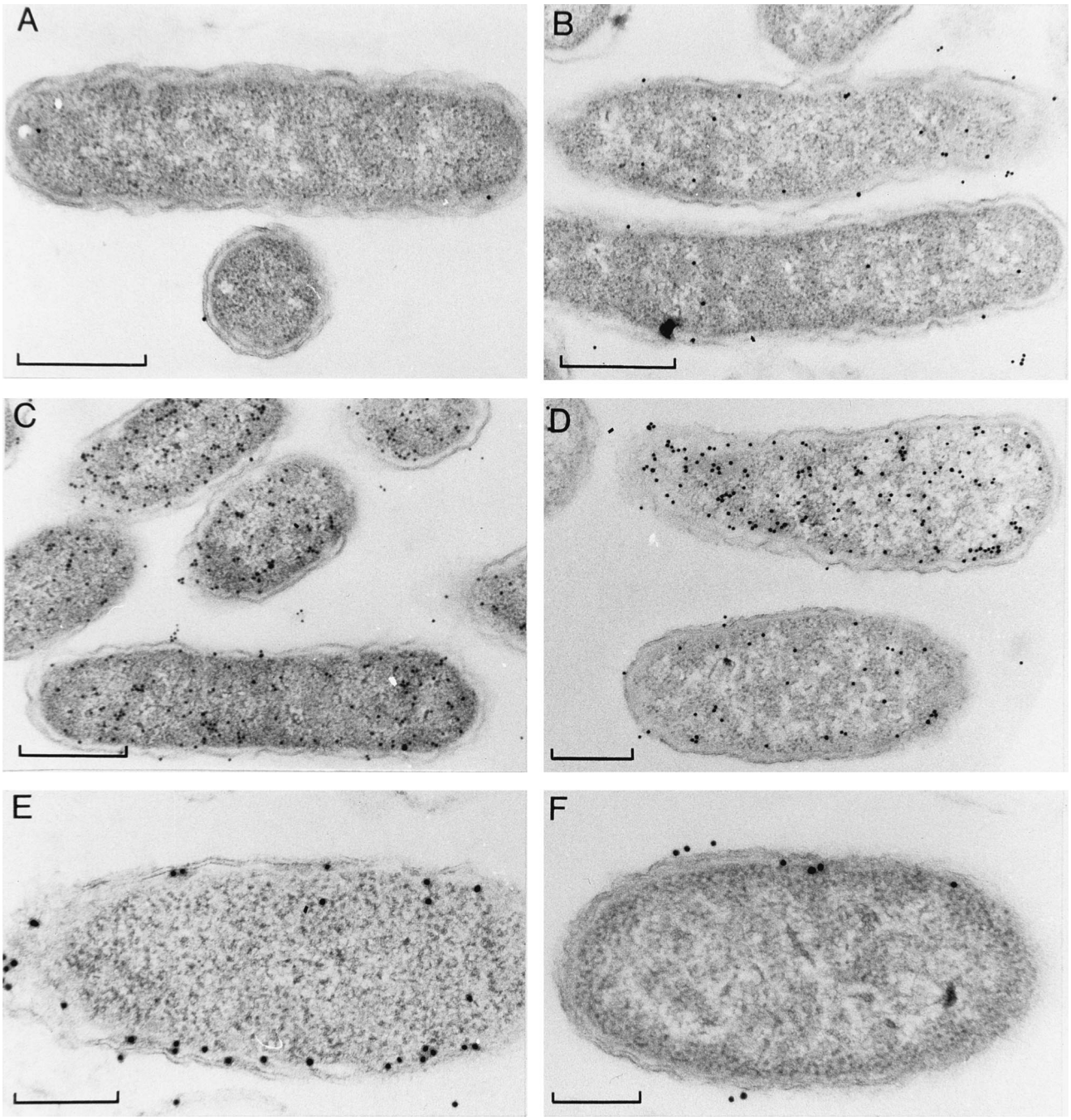

FIG. 3. Electron microscopy images of immunogold-labeled ultrathin sections of fixed overnight cultures of *E. coli* WK6 carrying (A) no plasmid, (B) pMG57, and (C to F) plasmids encoding β-galactosidase fused to LcnD at positions 21, 44, 80, and 474 (Fig. 1), respectively. The first antibody was rabbit anti-β-galactosidase. The second was goat anti-rabbit antibody labeled with 15-nm gold particles. Bars, 0.5 μm.

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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