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Cell Wall Anchoring of the *Streptococcus pyogenes* M6 Protein in Various Lactic Acid Bacteria

J.-C. PIARD,[1]* I. HAUTEFORT,[2] V. A. FISCHETTI,[3] S. D. EHRLICH,[1] M. FONS,[2] AND A. GRUSS[1]

Laboratoire de Génétique Microbienne[1] and Unité d'Ecologie et de Physiologie du Système Digestif,[2] Institut de Biotechnologie, INRA, 78352 Jouy-en-Josas Cedex, France, and The Rockefeller University, New York, New York 10021[3]

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The M6 protein from *Streptococcus pyogenes* is the best-characterized member of a family of cell envelope-associated proteins. Based on the observation that the C-terminal sorting signals of these proteins can drive cell wall anchoring of heterologous unanchored proteins, we have cloned and expressed the *emm6* structural gene for the M6 protein in various lactic acid bacteria (LAB). The *emm6* gene was successfully expressed from lactococcal promoters in several *Lactococcus lactis* strains, an animal-colonizing *Lactobacillus fermentum* strain, *Lactobacillus sake*, and *Streptococcus salivarius* subsp. *thermophilus*. The M6 protein was efficiently anchored to the cell wall in all strains tested. In lactobacilli, essentially all detectable M6 protein was cell wall associated. These results suggest the feasibility of using the C-terminal anchor moiety of M6 for protein surface display in LAB.

Surface presentation of heterologous molecules in gram-positive bacteria is of increasing interest for applications in various fields of biotechnology. Major achievements concerning the surface display of heterologous antigens (25, 33), immunoglobulins (19), and enzymes (40) were recently reported. The absence of an outer membrane in gram-positive bacteria makes them particularly attractive for use in the exposure of bioactive molecules to the extracellular compartment.

Lactic acid bacteria (LAB) constitute a family of gram-positive bacteria which are extensively used in the fermentation of raw agricultural products and in the manufacture of a wide variety of food products (5). The burst of information concerning LAB genetics and metabolism, as well as the development of expression and secretion tools, has opened the door for new (non)alimentary applications of these bacteria, such as those described above (8, 29). The use of LAB as in vivo delivery vectors for biologically active molecules (e.g., antigens, enzymes, or biological peptides) is made attractive by their nonpathogenicity and ability to survive passage along an oral route down to the intestine. The fulfillment of this project necessitates a delivery system comprising a vehicule (in this case, a LAB species) and a system to present molecules at the cell surface. To do this, we examined the cell wall-anchoring potential of the M6 protein of *Streptococcus pyogenes*. M6 (49 kDa) is among the best characterized of the cell wall-anchored proteins and has already been successfully used to drive cell wall anchoring of recombinant fusion proteins to the surface of *Streptococcus gordonii* (25). More than 60 cell wall-anchored proteins have been identified in gram-positive organisms, and 2 such proteins, a cell wall proteinase and a clumping factor, were characterized in the model LAB, *Lactococcus lactis* (11, 17, 22). All these proteins share a rather similar C-terminal anchoring tail of about 35 amino acids, suggesting that the anchoring mechanism is conserved in gram-positive organisms. The anchoring structure includes an LPXTG motif followed by a stretch of about 23 hydrophobic amino acids and 6 to 7 mostly positively charged residues at the extreme C terminus (12). Recent studies on cell wall anchoring in *Staphylococcus aureus* suggest that the initial step involves export of the surface protein precursors across the membrane by a Sec-dependent mechanism and that once translocated, the hydrophobic domain and the positively charged C terminus anchor the protein through interactions with the membrane and the negatively charged phospholipids on the cytoplasmic face, respectively. The membrane-bound protein is positioned to allow processing of the LPXTG motif. A putative sortase enzyme(s) cleaves at the threonine and creates an amide linkage with the free amino group of the peptide cross-bridge in the cell wall (27, 34). Here we describe the cloning and expression of the *emm6* gene in several LAB and show that M6 can be highly expressed and efficiently cell wall anchored in several LAB which are unrelated to *S. pyogenes*.

Bacterial strains, media, plasmids, and DNA manipulations. *Escherichia coli* DH5α (*recA*) (32) was grown in Luria broth (32) at 37°C, and *Lactococcus lactis* subsp. *lactis* IL1403 (6), *L. lactis* subsp. *cremoris* MG1363 *recA* (9, 15), *L. lactis* subsp. *lactis* JIM4454 (vegetal strain [18]) and *Streptococcus salivarius* subsp. *thermophilus* CNRZ302 (further designated *S. thermophilus* [CNRZ Culture Collection, Institut National de la Recherche Agronomique, Jouy-en-Josas, France]) were grown in M17 medium (41) at 30°C for lactococci and 37°C for thermophilic streptococci. The M6-producing *S. pyogenes* D471 (30) and its M6-deficient derivative JRS75 (28) were grown at 37°C in brain heart infusion broth (Difco, Detroit, Mich.) supplemented with 0.5% yeast extract or in Todd-Hewitt broth (Difco). Cultures of lactobacilli were grown in MRS medium (7) at 30°C for *Lactobacillus sake* 23K (1) or in MRS medium or APTG10 (31) at 37°C in anaerobic jars for *Lactobacillus fermentum* LEM83 (13). Liquid cultures of strains were grown without shaking. Where appropriate, antibiotics were added as follows: for *E. coli*, erythromycin (150 μg/ml) and ampicillin (100 μg/ml); for *L. lactis*, *L. sake*, and *S. thermophilus*, erythromycin (5 μg/ml), for *L. fermentum*, erythromycin (30 μg/ml). PCRs (30 cycles, with 1 cycle consisting of 30 s at 94°C, 1 min at 50°C, and 2 min at 72°C) were performed with a Perkin-Elmer Cetus (Norwalk, Conn.) apparatus. *Thermophilus aquaticus* DNA polymerase (Promega) was used as

* Corresponding author. Phone: 33-1-64 65 25 26. Fax: 33-1-34 65 25 21. E-mail: piard@biotec.jouy.inra.fr.

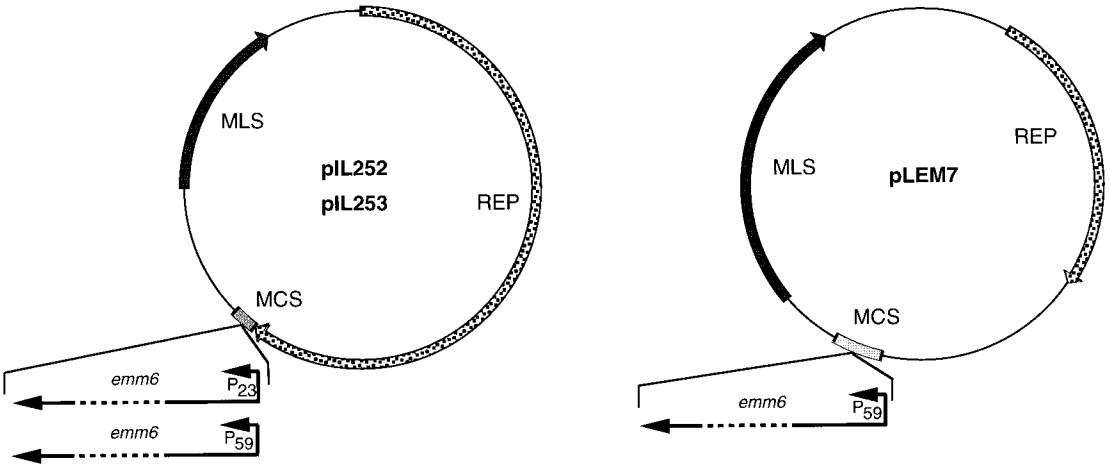

FIG. 1. *emm6* expression vectors for LAB. Vectors are based on pIL252 (low-copy-number) and pIL253 (high-copy-number) plasmids (39) and pLEM7 for *L. fermentum* (14). P_{23} and P_{59} are lactococcal promoters (42). These plasmids were used for cloning of the P_{23}:*emm6* and P_{59}:*emm6* cassettes, generating five *emm6*-expression vectors (see the discussion of cloning strategy in the text for details of constructions).

recommended by the manufacturer. Subsequent DNA manipulations in vitro and in *E. coli* were performed by published methods (32). Plasmid DNA was extracted from *E. coli* by the alkali lysis method (2). The same method was used for *L. lactis*, except that the cell walls were digested in TES (10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 25% sucrose) plus lysozyme (5 mg/ml) for 10 min at 37°C prior to cell lysis. For *S. thermophilus*, *L. sake*, and *L. fermentum*, plasmid extraction was performed as above, except that exponential-phase cells were harvested and cell walls were degraded in TES plus lysozyme (5 mg/ml) plus mutanolysin (0.1 mg/ml). Plasmids were established by electroporation as described for *L. lactis* and *S. thermophilus* (24), *L. fermentum* (4), and *L. sake* (1).

Cell fractionation, extraction of the M6 protein, and Western blot analysis. Total protein extracts from cell cultures (containing supernatant) were prepared by a slightly modified procedure of Schneewind et al. (36). Briefly, to 1.6 ml of exponential-phase culture at a given optical density at 600 nm (OD_{600}) was added 400 μl of ice-cold 80% (wt/vol) trichloroacetic acid (TCA; 16% final concentration). The mixture was kept on ice for 20 min and then microcentrifuged at 4°C for 10 min at 11,500 × *g*. The resulting pellet was washed twice with 1 ml of acetone, dried in a vacuum centrifuge (Savant), and resuspended in 160 μl per OD_{600} unit of TES containing lysozyme (1 mg/ml), mutanolysin (0.1 mg/ml), and RNase (0.1 mg/ml) (TES-LMR). After a 30-min incubation at 37°C, the cells were lysed with 1% sodium dodecyl sulfate (SDS) and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) (12% polyacrylamide) analysis (23).

For cell fractionation, 2 ml of exponential-phase culture at a given OD_{600} was microcentrifuged at 4°C for 5 min at 4,300 × *g*. The supernatant and cells were processed separately. To evaluate the amounts of secreted and cell-associated M6, both cell and supernatant fractions were concentrated. The supernatant was filtered through 0.2-μm-pore-size filters (low protein retention; Sartorius, Göttingen, Germany) to remove bacteria, and proteins from 1.6 ml of the filtrate were precipitated with TCA (16% final concentration). The resulting pellet was dissolved in 80 μl per OD_{600} unit of 50 mM NaOH. The cell pellets were washed once with TES, and the cell walls were digested with TES-LMR as described above. The protoplasts were pelleted by a 10-min centrifugation at 2,500 × *g*, and proteins released from cell wall digestion were precipitated from the supernatant with 16% (final concentration) TCA. The resulting precipitate was suspended in 100 μl per OD_{600} unit of 50 mM NaOH. Equal volumes of 2× loading buffer were added to all samples. Extracts were subjected to SDS-PAGE (12% polyacrylamide). Electroblotting on polyvinylidene difluoride membranes (Millipore) and antibody reactions and detection (by enhanced chemiluminescence) were performed as recommended by the manufacturer. Quantitation of M6 protein was estimated by scanning Western blots and comparing the signals to those of known amounts of a purified M6 protein control. Amounts are presented as milligrams per liter of culture corrected to an $OD_{600} = 1$.

Cloning strategy. The *emm6* structural gene for the M6 protein was amplified by PCR from pVV3:M6Δ as the matrix (21) with the coding-strand primer P1 (5′-AAATCGATAAC ATAAGGAGC-3′) and the complementary-strand primer P2 (5′-GTTGTTTAGTTTGTGACCTCC-3′). The amplified fragment comprises the promotorless entire *emm6* sequence including a Shine-Dalgarno sequence and the 3′-end inverted repeats presumably involved in transcriptional termination (20). The 1.6-kb PCR fragment was made blunt with DNA polymerases T4 and Klenow and inserted into the *Sma*I site of pBluescript SK^+II (pBS-SK^+; Stratagene). The resulting plasmid (pVE5201) was established in *E. coli* DH5α. To express *emm6*, the *Kpn*I-*Sac*I *emm6* fragment isolated from pVE5201 was recloned into *Kpn*I-*Sac*I-cut pJDC9:P_{23} and pJDC9:P_{59} (kindly provided by N. Galleron, Laboratoire de Génétique Microbienne, Institut National de la Recherche Agronomique, Jouy-en-Josas, France). The *Bam*HI-derived P_{23}:*emm6* and P_{59}:*emm6* cassettes were cloned in *Bam*HI-digested pBS-SK^+ to generate pVE5206 and pVE5207, respectively. The DNA sequences of both the 1.6-kb PCR fragment and the promoter regions were verified. The low- and high-copy-number lactococcal vectors, pIL252Δ*Xba*I and pIL253Δ*Xba*I, respectively, were fused via their *Sma*I sites to *Sma*I-cut pVE5206 or pVE5207. The pBS-SK^+ portion of the resulting plasmids was subsequently deleted through a *Sac*I digestion to generate the plasmids represented in Fig. 1. *E. coli* and *L. lactis recA* derivatives were used as cloning recipient strains to reduce the chances of deletion formation within direct repeats present in the *emm6* gene (20). For expression studies of *emm6* in *L. fermentum*, the pLEM7 vector derived from a *L. fermentum* native plasmid was used (14). The *Bam*HI-P_{59}:*emm6* cassette

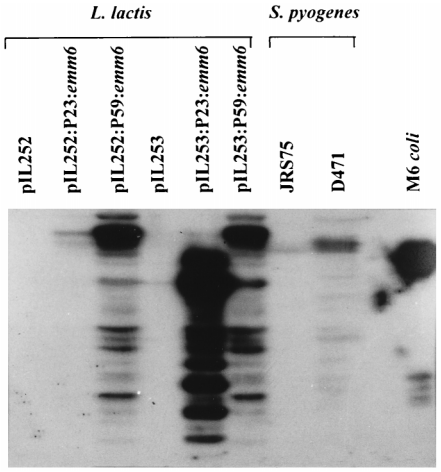

FIG. 2. Expression of M6 in *L. lactis*. Western blot analysis was performed on total protein fractions of *L. lactis* MG1363 *recA* containing *emm6* expression vectors and of *S. pyogenes* by using an anti-M6 monoclonal antibody. The plasmids present in each *L. lactis* strain are indicated above the wells. *S. pyogenes* D471 is a natural M6-producing strain, and *S. pyogenes* JRS75 is an M6-deficient derivative of D471. M6 protein (5 μg) purified from *E. coli* (denoted M6 *coli*) was used as a reference.

from pVE5207 was inserted into the *Bam*HI site of pLEM7 to generate the plasmid schematized in Fig. 1.

Expression of *emm6* in *L. lactis*. Four plasmids containing the *emm6* gene downstream of lactococcal promoters (Fig. 1) were established in *L. lactis* MG1363*recA*, and expression levels of the *emm6* gene were assessed by Western blot analysis of total protein extracts of clones, using M6 polyclonal antibodies or a monoclonal antibody directed toward the C repeat region (upstream of the cell wall anchor) of the M6 protein. Positive signals were observed with extracts of M6-producing *S. pyogenes* D471 but not of the M6-deficient derivative (JRS 75) or of *L. lactis* negative control strains (containing pIL252 or pIL253 without insert [Fig. 2]). Relative and absolute amounts of M6 protein produced (taking only the major band into account) were estimated by scanning Western gels and by including dilutions of known amounts of purified M6 (derived from *E. coli*) as a reference. In *L. lactis*, M6 levels varied according to the combination of vector and promoter used (Fig. 2). The pIL252:P_{23}:*emm6* vector resulted in the smallest amounts of M6, in keeping with low reported promoter activity of P_{23} (42). Expression of M6 from pIL252 (low-copy-number plasmid) in combination with promoter P_{59} resulted in about 5 mg of M6 per liter culture. This represents about a sevenfold-higher M6 production than that by *S. pyogenes* strain D471. The use of pIL253 (high-copy-number plasmid) did not improve M6 production; the expression vector pIL253:P_{23}:*emm6* gave rise to a truncated form of M6, while pIL253:P_{59}:*emm6* gave an expression level close to that of pIL252:P_{59}:*emm6*. We therefore used pIL252:P_{59}:*emm6* in subsequent experiments.

The majority of M6 protein isolated from *L. lactis* detected on SDS-PAGE migrated as a higher-molecular-weight species than M6 extracted from *S. pyogenes* D471. In both cases, the protein is larger than that observed for M6 protein purified from *E. coli*; the latter is not anchored, but there is evidence for protein cleavage near the LPXTG motif (29a). The difference between *S. pyogenes*- and *E. coli*-extracted M6 was attributed to the presence of cell wall fragments associated with the M6 protein, which markedly retards its migration compared to the *E. coli*-isolated M6 (10). Similar results were reported for the cell wall-anchored protein A of *Staphylococcus aureus* (35). We thus consider that the multiple banding pattern and the larger size of M6 observed in *L. lactis* extracts reflect differences in cell wall composition and indicate that the protein is anchored.

The M6 protein patterns also included degradation products. Partial proteolysis may be responsible for the smaller bands revealed by M6 antibodies and may also explain the multiple bands observed in *E. coli* (37) despite the absence of anchoring.

Taken together, these results show that the *emm6* gene can be efficiently expressed in *L. lactis* MG1363*recA* at levels superior to those detected in the original host, *S. pyogenes.*

Distribution of the M6 protein in *L. lactis* MG1363*recA*. To assess the anchoring efficiency of M6 protein in *S. pyogenes* and *L. lactis*, exponential-phase cells of *S. pyogenes* D471 or of *L. lactis* MG1363*recA* harboring pIL252:P_{59}:*emm6* were fractionated and M6 protein was assayed in both cell wall and supernatant fractions. About half of the *S. pyogenes* D471 M6 protein was released in the supernatant (Fig. 3A). A similar result was obtained when either brain heart infusion broth or Todd-Hewitt broth was used as the culture medium and irrespective of the presence of protease inhibitor during extraction of the M6 protein (data not shown). M6 protein could be detected in culture supernatants of *S. pyogenes* only after concentration of samples (which allowed us to compare equivalent amounts of cells and supernatant) and was not previously reported. Anchoring in *L. lactis* harboring pIL252:P_{59}:*emm6* appeared to be at least as efficient as in the *S. pyogenes* M6 producer. Although more M6 protein is present, the majority (about 80%) is anchored to the cell wall fraction of the lactococci (Fig. 3A). These observations indicate that the M6 protein precursor is recognized by the secretion machinery of *L. lactis* and that the

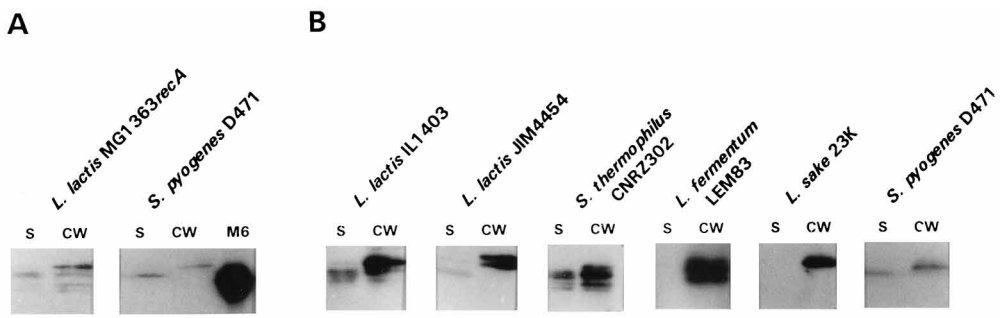

FIG. 3. Localization of M6 in different lactic acid bacteria. (A) Distribution of M6 in *L. lactis* MG1363*recA* and in *S. pyogenes*. (B) Expression of *emm6* and distribution of M6 in various LAB. Supernatant and cell wall fractions (see the text) were analyzed by Western blot analysis with anti-M6 polyclonal antibodies. S and CW refer to supernatant and cell wall, respectively. *S. pyogenes* D471 is a natural M6-producing strain. M6 protein (M6) (5 μg) was used as a reference.

cell wall-anchoring mechanism is efficient in *L. lactis*. Based on an average of 4 mg of M6 in the cell wall of lactococci per liter of culture at $OD_{600} = 1$, the number of M6 molecules associated with the cell wall corresponds to about 5×10^4 per cell. The M6 protein detected in the supernatants of *S. pyogenes* and *L. lactis* could result from incomplete anchoring of the M6 protein and/or cell wall turnover. M6 protein detected in supernatants of either organism migrated faster on SDS-PAGE than do the cell wall-associated protein and at the same position as the protein extracted from *E. coli*, suggesting that M6 molecules present in the supernatant are processed at their C termini.

Expression of *emm6* in various LAB and distribution of the M6 protein. To assess whether the M6 protein could be expressed, exported, and anchored in other LAB hosts, plasmid pIL252:P_{59}:*emm6* was transferred to different subspecies of *L. lactis*, as well as to *S. thermophilus* and *L. sake*. In the experiment with *L. fermentum*, the P_{59}:*emm6* cassette was recloned into the recently developed cloning vector, pLEM7 (14). Cell wall and supernatant protein fractions were analyzed by Western blotting. Good anchoring of M6 was observed in all LAB strains tested (Fig. 3B). Size variation in anchored M6 between strains could be due to differences in peptidoglycan composition. The sizes of secreted M6 proteins also varied in different strains. This might reflect processing efficiencies of the C-terminal tail of M6 in different strains or heterogeneity of peptidoglycan-M6 products generated by cell wall turnover. Two groups of bacteria were distinguished with respect to anchoring efficiency. The cocci, including *S. pyogenes*, *L. lactis*, and *S. thermophilus*, all showed detectable M6 protein in the supernatants. We consider that detection of M6 in supernatants is not due to proteolytic activity, because *L. lactis* MG1363 and IL1403 and *S. thermophilus* CNRZ302 do not secrete cell wall proteases (26). In a second group including the *Lactobacillus* species *L. sake* and *L. fermentum*, anchoring appeared to be complete; when polyclonal anti-M6 antiserum was used, neither full-size protein nor degradation products of M6 could be detected in supernatants, even after overexposure of the films (data not shown). It is possible the lack of detection of M6 in the supernatants of *Lactobacillus* strains occurs because of either trapping of M6 in the cell wall fraction or proteolytic degradation in the supernatant. However, we consider both possibilities unlikely. First, the detection of anchored M6 protein involves extracellular release of M6 by lysozyme. Thus, free M6 is released once it is detached from the cell wall, which suggests that it is not trapped even if there is a capsule. In addition, recent studies indicate that *L. fermentum* LMG 8896 and 104R have no S-layer capsule (3). Second, no M6 degradation products were detected in envelope or supernatant fractions of *Lactobacillus* when examined on Western gels (data not shown). We propose that differences in anchoring capacities may reflect the nature of the peptide cross-bridge of the peptidoglycan (16) or the rate of cell wall turnover leading to the release of peptidoglycan-associated protein (38).

Taken together, these results show that the M6 preprotein signals specifying the export (i.e., via the leader peptide) and anchoring (i.e., via the C-terminal tail) of M6 to the cell wall are efficient in various LAB. These signals are currently being used to design a functional surface display system for LAB. In this regard, efficient anchoring of M6 protein in a digestive tract-colonizing strain (*L. fermentum*), as well as in noncolonizing strains (*L. lactis*, *S. thermophilus*, and *L. sake*), will be useful in comparative studies of protein antigen presentation.

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