

Peptide Surface Display and Secretion Using Two LPXTG-Containing Surface Proteins from *Lactobacillus fermentum* BR11

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A locus encoding two repetitive proteins that have LPXTG cell wall anchoring signals from *Lactobacillus fermentum* BR11 has been identified by using an antiserum raised against whole *L. fermentum* BR11 cells. The first protein, Rlp, is similar to the Rib surface protein from *Streptococcus agalactiae*, while the other protein, Mlp, is similar to the mucus binding protein Mub from *Lactobacillus reuteri*. It was shown that multiple copies of *mlp* exist in the genome of *L. fermentum* BR11. Regions of Rlp, Mlp, and the previously characterized surface protein BspA were used to surface display or secrete heterologous peptides in *L. fermentum*. The peptides tested were 10 amino acids of the human cystic fibrosis transmembrane regulator protein and a six-histidine epitope (His₆). The BspA promoter and secretion signal were used in combination with the Rlp cell wall sorting signal to express, export, and covalently anchor the heterologous peptides to the cell wall. Detection of the cell surface protein fusions revealed that Rlp was a significantly better surface display vector than BspA despite having lower cellular levels (0.7 mg per liter for the Rlp fusion compared with 4 mg per liter for the BspA fusion). The *mlp* promoter and encoded secretion signal were used to express and export large (328-kDa at 10 mg per liter) and small (27-kDa at 0.06 mg per liter) amino-terminal fragments of the Mlp protein fused to the His₆ and CFTR peptides or His₆ peptide, respectively. Therefore, these newly described proteins from *L. fermentum* BR11 have potential as protein production and targeting vectors.

Over the past several years investigation into the health-promoting, or probiotic, activity of certain strains of lactic acid bacteria in humans has been the subject of substantial research. Of particular interest are lactobacilli, which due to their nonpathogenic and mucosal surface-colonizing properties have been developed for recombinant protein delivery because they have the potential to express proteins at specific mucosal sites. For the most part, lactobacilli have been investigated as recombinant mucosal vaccines. There have been recent developments in the engineering of lactobacilli and other colonizing lactic acid bacteria so that they secrete or surface display single-chain Fv fragments with direct therapeutic effects (4, 16).

Lactobacillus secretion vectors have mainly utilized the secretion signals of proteins derived from bacteria other than lactobacilli, with examples including those of the M protein from *Streptococcus pyogenes*, Usp45 and PrtP from *Lactococcus lactis*, and protein A from *Staphylococcus aureus* (13, 31, 35). *Lactobacillus* secretion signals that have been used for extracellular heterologous protein production include the S-layer secretion signal from *Lactobacillus brevis* (33) and the α -amylase protein from *Lactobacillus casei* (16, 17). Protein surface display systems using lactobacilli can be divided into two classes, covalent and noncovalent anchoring systems. The covalent anchor system uses proteins that contain LPXTG cell wall sorting signals. The M protein has been used successfully (24, 27), while the PrtP proteins from *L. casei* and *Lactobacillus delbrueckii* have been used to anchor heterologous proteins

on *L. casei* (16, 18) and *Lactobacillus johnsonii*, respectively (34). Noncovalent surface attachment systems utilize either the S-layer protein from *L. brevis* or *Lactobacillus acidophilus* (3, 36) or BspA from *Lactobacillus fermentum* (14, 43).

Our research is directed towards the development of therapeutic protein secretion and surface display expression systems based on native surface proteins of the guinea pig vaginal tract isolate *L. fermentum* BR11. *L. fermentum* BR11 is our model organism because we have shown that it is amenable to genetic manipulation (31, 42) and because the *L. fermentum*-*Lactobacillus reuteri* group are one of the bacterial groups found on a number of different mucosal surfaces in mammals, including the intestine. Thus far, we have identified and characterized BspA from *L. fermentum* BR11, an abundant basic surface protein which is anchored to the cell surface noncovalently (41). Closely related homologs of BspA include Cnb from *L. reuteri* NCIB11951 (29), a 29-kDa protein from *L. fermentum* RC-14 (11), and MapA from *L. reuteri* 104R (28). These proteins probably interact with teichoic acids via electrostatic interactions due to their strong cationic properties, which are shared with the anchoring domain of lactobacillus S-layer protein subunits (2, 41). BspA has been used as a fusion partner to express and surface display peptides from human immunodeficiency virus and *Chlamydia* as well as a large glucosyltransferase enzyme (14, 43).

Here we report the identification and characterization of two tandem genes of *L. fermentum* BR11 encoding proteins that have features similar to those of repetitive LPXTG covalently anchored surface proteins and have similarity to adhesins from other gram-positive bacteria. It is also shown that functional elements of these two proteins can facilitate efficient secretion and cell surface anchoring of heterologous peptides

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spectively, using pMFT1 as a template (41). DNA encoding three copies of the CFTR peptide was amplified by using overlapping oligonucleotides CFTR-5'-Pst and CFTR-3'-Xho. The *bspA* gene and DNA encoding the BspA secretion signal used to generate His₆-CFTR-Rlp were amplified by using oligonucleotides BspA-Xba with BspA-Eco-stop and BspA-Eco-SS with BspA-SS-Pst-His, respectively. The *rlp* 3' end and terminator were amplified by using oligonucleotides Rlp⁺-Pst-Xho and Rlp-Sal-Apa with pPNG606 as the template. The *mlp* DNA fragment to generate Mlp-His₆ was amplified from pPNG606 by using oligonucleotides MlpN-Xba and MlpN-Xho-His. The *mlp* DNA fragment to generate Mlp-His₆-CFTR was amplified from pPNG607 by using oligonucleotides Mlprep-Xba and Mlprep-Pst-His, respectively. All PCRs were performed with the Expand high-fidelity system (Roche), except for the CFTR peptide PCR, with which Platinum *Taq* (Invitrogen, Carlsbad, Calif.) was used.

Transformation of *L. fermentum* BR11 and integration of plasmids. Transformation of *L. fermentum* BR11 with plasmids was done by a procedure similar to that described previously (42). Following outgrowth in the presence of a subinhibitory concentration of erythromycin, the cells were plated onto MRS agar containing 10 µg of erythromycin per ml and grown at 37 instead of 30°C. After overnight growth, colonies were inoculated into MRS liquid medium containing 10 µg of erythromycin per ml and incubated at 40°C.

Cell fractionation, protein extraction, and Western blot analysis. Cell extracts were prepared from early-stationary-phase cultures (optical density at 600 nm [OD₆₀₀], ~2.6), while supernatants were taken from exponentially growing cultures (OD₆₀₀, ~1.0). Two different whole-cell protein extraction methods were used. The first involved boiling cells in 2× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (32) for 5 min. The other involved suspending the cells in 20 mM triethanolamine-HCl (pH 7.6) containing 0.1 mM EDTA and sonicating (3 30-s bursts at 10 W) with a 36810 series Torbeo ultrasonic cell disruptor (Cole-Parmer, Vernon Hills, Ill.). Extractions of *L. fermentum* strains with 5 M LiCl were done as described previously (42). Supernatants were filtered through 0.2-µm-pore-size membranes (Schleicher and Schuell, Dassel, Germany) and then precipitated with 5% (wt/vol) ice-cold trichloroacetic acid, washed with acetone, and resuspended in equal amounts of 50 mM NaOH and 2× SDS-PAGE loading buffer. Prior to loading of SDS-PAGE, all samples were boiled for 5 min. Proteins were transferred to nitrocellulose, blocked, and then probed with an anti-His₆ monoclonal antibody (Qiagen, Hilden, Germany) at a 1:1,000 dilution. Following washes, the membrane was incubated with rabbit anti-mouse immunoglobulin-horseradish peroxidase conjugate (Dako, Glostrup, Denmark). The bound antibodies were detected by using a horseradish peroxidase chemiluminescence kit (Roche). To estimate levels of His₆ proteins in extracts, various amounts of His₆-labeled protein markers (Qiagen) were included alongside the samples. These markers have known quantities of His₆-containing proteins in each band, allowing densitometry to be done on films with the TotalLab version 1.11 package (Phoretix, Newcastle upon Tyne, United Kingdom).

Cell surface display assay. The assay of accessibility of the His₆ epitope on whole cells was done similarly to that described previously (43). Exponentially grown cells were washed with phosphate-buffered saline (PBS) and resuspended to an OD₆₀₀ of ~14 in PBS. One milliliter of the cell suspension was blocked with 1% casein in PBS for 30 min and then reacted with anti-His₆ antibody at a 1:1,000 dilution in PBS containing 1% casein for 30 min. Following two washes with PBS, the cells were suspended in rabbit anti-mouse immunoglobulin-alkaline phosphatase conjugate (Dako) at a 1:1,000 dilution in PBS containing 1% casein and incubated for 30 min. Following two further washes in PBS, the cells were suspended in 1 M Tris-HCl (pH 9). Several aliquots were taken and incubated with *p*-nitrophenyl phosphate substrate (Sigma, St. Louis, Mo.), and the absorbance at 405 nm was measured with an automated plate reader. In addition, 2 µl of each cell suspension was spotted onto nitrocellulose and then incubated with Western blue stabilized substrate for alkaline phosphatase (Promega).

Nucleotide sequence accession number. The sequence of the *rlp* and *mlp* locus has been deposited in GenBank under accession number AY240028.

RESULTS

Cloning and characterization of a locus encoding two putative surface proteins from *L. fermentum* BR11. We have previously identified the gene encoding BspA by screening a pUC18 genomic library with antiserum raised against whole *L. fermentum* BR11 cells (41). Several more positive clones from this screen were identified and confirmed by Western blot

analysis (data not shown). The plasmids from these clones were subjected to sequencing and were found to contain open reading frames (ORFs) encoding proteins similar to cell wall hydrolases, ClpC proteases (unpublished data), and the recently characterized mucus binding protein Mub from *L. reuteri* (30) (in pPNG601 and pPNG602). Two overlapping plasmids (pPNG601 and pPNG602) contained DNA similar to an internal region of *mub*, with the larger plasmid containing a 2.2-kb insert (Fig. 1). In order to clone a larger portion of this gene, termed *mlp* for Mub-like protein, a λ library was constructed and screened with a DNA probe containing part of the plasmid insert. Several positive plaques were isolated and were found to express large proteins reactive with whole-cell antiserum. These immunoreactive proteins were significantly larger than 200 kDa as determined by low-percent-polyacrylamide PAGE (data not shown). The 15-kb insert from one λ clone (λ8) was subcloned into two plasmids (pPNG606 and pPNG607) and fully sequenced (Fig. 1). The *mlp* gene was truncated at the 3' end in λ8, so the sequence of the last 1.2 kb of the gene was obtained by using a PCR-based gene walking procedure (see Materials and Methods and "Identification of multiple *mlp* genes in *L. fermentum* BR11" below). The organization of open reading frames at this locus is shown in Fig. 1.

Mlp is predicted to have a 46-amino-acid secretion signal, a 957-amino-acid N-terminal nonrepetitive sequence, an internal repetitive region consisting of five imperfect repeats of 275 to 280 amino acids each and five imperfect repeats of 371 to 407 amino acids each, and a typical C-terminal gram-positive bacterial cell wall sorting signal containing the sequence LPQTG (Fig. 1). Mlp has similarity to several hypothetical proteins from the unpublished *Lactobacillus gasseri* genome which have potential LPXTG cell wall sorting signals (Lgas0953 and Lgas0817) and to some without LPXTG cell wall sorting signals (Lgas0292 and Lgas0954) (Table 2). Mlp also has strong similarity to the human and bovine hr44 proteins and similarity to the *L. lactis* YwfG protein (Table 2).

Upstream of *mlp* is an ORF which potentially encodes another cell wall-anchored surface protein (Fig. 1). The protein product has similarity to the surface protein Rib from *Streptococcus agalactiae* (Table 2) and therefore has been termed Rlp, for Rib-like protein. The partial sequence obtained shows that Rlp contains at least nine imperfect amino acid repeats of between 69 and 100 amino acids (average of 80 amino acids) and a typical C-terminal gram-positive bacterial cell wall sorting signal containing the sequence LPQTG (Fig. 1). Rlp also has similarity to the R28 putative adhesion from *S. pyogenes*, the Esp surface protein from *Enterococcus faecalis*, the α protein from *S. agalactiae*, several hypothetical proteins from the unpublished *L. gasseri* genome containing LPXTG type cell wall sorting signals (Lgas1358, Lgas0230, and Lgas0701), and biofilm-associated surface protein Bap from *S. aureus* (Table 2). Therefore, this chromosomal locus in *L. fermentum* BR11 appears to encode at least two repetitive surface proteins that have similarity to adhesion, pathogenicity, or colonization determinants.

Identification of multiple *mlp* genes in *L. fermentum* BR11. PCR was carried out with chromosomal DNA as a template and primers which flank the 3' end of *mlp* (Fig. 1). Partial sequencing of the amplified 1.3-kb DNA fragment revealed the presence of a number of nucleotide polymorphisms. An exam-

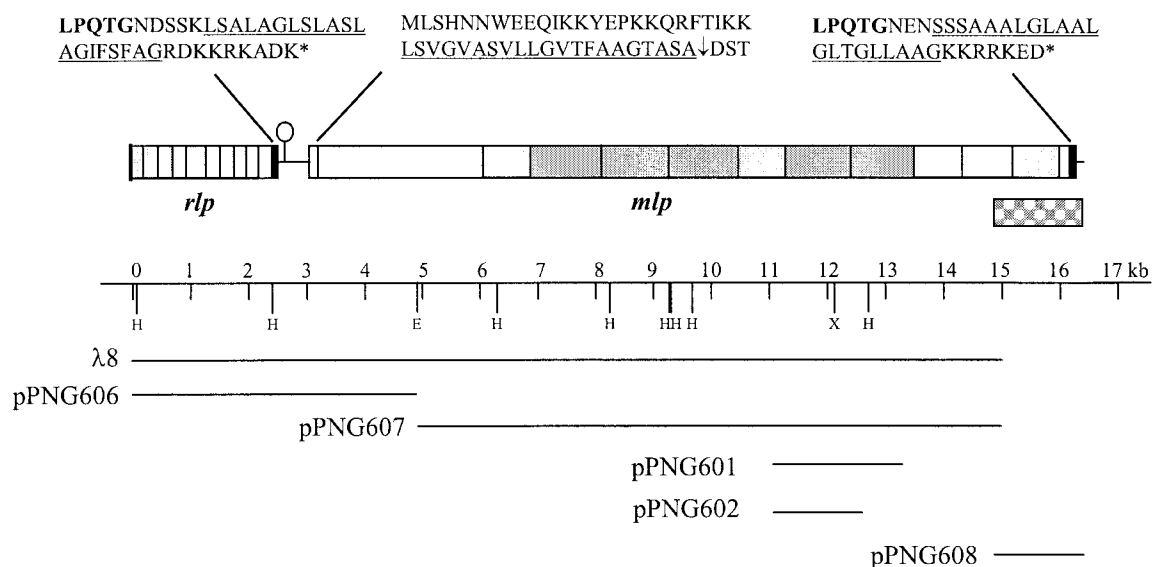


FIG. 1. The 16,401-bp DNA region, showing the arrangement of *mlp* and *rlp*, and the λ and plasmid clones used to determine the sequence. The letters above the boxes indicate the cell wall sorting (black boxes) and secretion signals of the encoded proteins. The underlined sequences indicate hydrophobic domains. The shaded boxes indicate the encoded amino acid repeat regions, with light and dark boxes indicating the different-sized repeats in Mlp. The lollipop indicates a potential stem-loop structure. The checkered box indicates the DNA region which was amplified by PCR to determine the 3' end sequence of *mlp* and to identify multiple *mlp* genes. The oligonucleotides Mlp-Paw-C and Mlp-C-check (Table 1) were used to amplify this region.

ple of part of the sequence trace obtained with the downstream primer (Mlp-C-check) is shown in Fig. 2. To ensure that this was not due to misincorporations occurring during the PCR, another PCR was carried out and the product was partially sequenced. The same polymorphisms were also clearly present in this sequence trace. Polymorphisms also are present in a nonrepetitive region (around the LPXTG sorting signal); therefore, they are not due to Mlp-Paw-C oligonucleotide mispriming in different repeats. This result demonstrates that *L. fermentum* BR11 contains multiple *mlp* genes and that they are very conserved, at least at the 3' end. The amplified DNA from the PCR was cloned into pGEM-T, and partial sequence

analysis of six plasmids revealed four different sequence types. At least two *mlp* sequence types have a 100% identical overlapping sequence (94 bp) with the 3' end of pPNG607, which therefore makes it impossible to ascertain which is the correct 3' sequence of the *mlp* gene cloned in pPNG607. Of the 900 bp sequenced from these two sequence types, only two nucleotides are different and there is no change in the amino acid sequence. Due to the high similarity of these two sequence types, the full sequence of one of the inserts (in pPNG608) was arbitrarily assigned as the 3' end of *mlp*.

Cell surface display of the CFTR and His₆ peptides by using BspA and Rlp. The cell wall sorting signal of Rlp was com-

TABLE 2. Proteins similar to Mlp and Rlp

Protein (no. amino acids)	Similar proteins	% Identity/no. of amino acids	Function of similar protein if known	Reference
Mlp (4,427)	<i>Lactobacillus gasseri</i>			
	Lgas0953	28/3,825		
	Lgas0817	23/1,737		
	Lgas0292	26/602		
	Lgas0954	21/874		
	<i>Lactobacillus reuteri</i> Mub	21/2,038	Mucus binding	30
	<i>Homo sapiens</i> hr44	58/224		6
<i>Lactococcus lactis</i> YwfG	22/702		5	
Rlp (831)	<i>Streptococcus agalactiae</i> Rib	38/747		44
	<i>Streptococcus pyogenes</i> R28	38/736	Adhesion to epithelial cells	37
	<i>Enterococcus faecalis</i> Esp	33/756	Biofilm formation	40
	<i>Streptococcus agalactiae</i> α	32/768		20
	<i>Lactobacillus gasseri</i>			
	Lgas1358	29/802		
	Lgas0230	29/745		
	Lgas0701	23/246		
<i>Staphylococcus aureus</i> Bap	22/528	Biofilm formation	7	

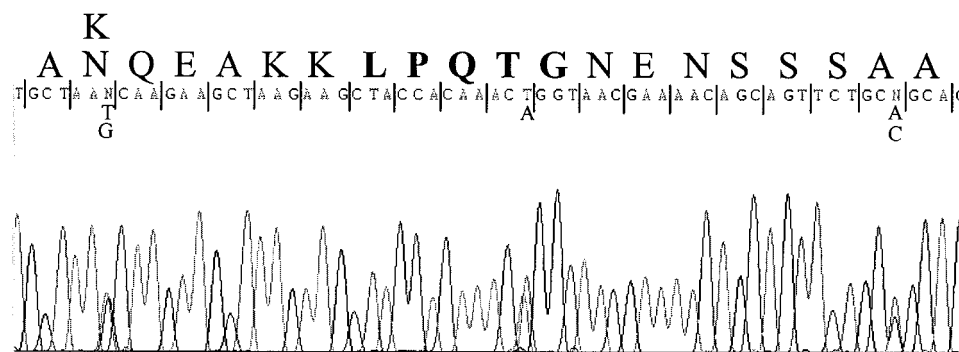


FIG. 2. Reverse complement of a DNA sequencing trace of the 1.3-kb *mlp* 3' PCR fragment (checked box in Fig. 1) with the Mlp-C-check oligonucleotide. Above the DNA sequence is the encoded amino acid sequence of the Mlp protein, with the LPQTG motif in boldface. Double peaks indicate polymorphic nucleotides, which are included under the assigned sequence. The first polymorphism from the left shows that the encoded amino acid is different in the two Mlp proteins.

pared with BspA in its ability to display heterologous peptides on the surface of *L. fermentum* BR11. The human CFTR peptide was used, since it has potential as a therapeutic agent because it has been shown to reduce uptake of *S. enterica* serovar Typhi into human intestinal epithelial cells (26). The His₆ peptide was also used to facilitate detection of the fusion proteins. To enable direct comparison, both constructs were integrated into the chromosome downstream of the *bspA* promoter (Fig. 3). The BspA fusion protein construct was generated similarly to that previously described (43). The Rlp fusion protein construct was placed downstream of a full-length and unaltered *bspA* gene which served as the homologous DNA for the chromosomal integration process. The last 161 amino acids of the Rlp protein were used as the cell wall anchor signal, and the BspA secretion signal was used to direct export of this Rlp fusion protein.

Western blot analysis with an anti-His₅ antibody showed that both fusion proteins were associated with the cells and also in the supernatant (Fig. 3). The predicted molecular mass of the Rlp fusion protein is 19 kDa; however, the band was approximately 28 kDa. The BspA fusion protein is 32 kDa, but the band was approximately 38 kDa. Fourteen percent of the BspA fusion protein was found in the supernatant, while 34% of the Rlp fusion protein was found in the supernatant (Table 3). The amount of protein associated with cells was estimated at 4 mg per liter for the BspA fusion protein and at 0.7 mg per liter for the Rlp fusion protein. Taking the sizes of the fusion proteins into account, cells expressing the BspA fusion contained approximately three times more His₆ peptide than cells expressing the Rlp fusion protein. The percentage of BspA fusion protein released from cells by using 5 M LiCl was 65%, compared with only 6% for the Rlp fusion protein (Table 3).

To test the ability of Rlp to display the heterologous peptides on the cell surface, a whole-cell enzyme-linked immunosorbent assay was used (43). The cells expressing the BspA fusion protein bound significantly more anti-His₅ antibody than wild-type cells, while the cells expressing the Rlp fusion protein bound significantly more anti-His₅ antibody than those expressing the BspA fusion protein (Table 3). A similar result was also seen when 2 μ l of the antibody-reacted cells was spotted onto nitrocellulose and incubated with substrate (data not shown). Therefore despite having ~3-fold-fewer cell-asso-

ciated molecules, the Rlp cell wall anchor fusion protein is much more efficiently recognized by antibodies than the BspA fusion protein on whole cells.

Secretion of the CFTR and His₆ peptides by using Mlp. To test Mlp as a secretion vector in *L. fermentum* BR11, two different constructs were made and integrated into the *mlp* gene such that they are under the control of the *mlp* promoter. The first fusion protein consisted of the Mlp putative secretion signal and the next 261 amino acids of the nonrepetitive amino-terminal region, as well as a His₆ peptide at the very carboxyl terminus (Fig. 4). The second fusion protein consisted of the Mlp putative secretion signal, 3,014 amino acids of Mlp including the nonrepetitive region and nearly six repeats, the His₆ peptide, and the CFTR peptide at the carboxyl terminus (Fig. 4). Western blot analysis revealed that both fusion proteins were secreted and also associated with the cells (Fig. 4). The small Mlp-His₆ fusion protein produced two bands in the SDS and supernatant extracts but only a single band in the sonication extracts (Fig. 4A). The bands resolved at approximately 40 to 45-kDa which is in contrast to its calculated size of 27-kDa (Fig. 4A). The large Mlp-His₆-CFTR fusion protein, which has a predicted molecular mass of 328 kDa, resolves as a single band which just enters the separating gel (Fig. 4B). For the cells expressing the small Mlp-His₆ fusion protein, only 3% of the His₆-containing protein was found in the supernatant, while cells expressing the large Mlp-His₆-CFTR fusion protein had 86% of the His₆-containing protein in the supernatant (Table 3). The level of the small Mlp-His₆ fusion protein detected in the supernatant was only 0.06 mg per liter, while 10.3 mg of the large Mlp-His₆-CFTR fusion protein per liter was detected in the supernatant (Table 3). When the sizes of these two proteins are taken into account, there was approximately 14 times more large Mlp-His₆-CFTR fusion protein than small Mlp-His₆ fusion protein detected in the supernatant. This result shows that secretion of a very large heterologous fusion protein by *L. fermentum* BR11 can be very efficient.

DISCUSSION

This paper describes the identification and characterization of two tandem genes from *L. fermentum* BR11 which encode proteins similar to surface proteins from other gram-positive

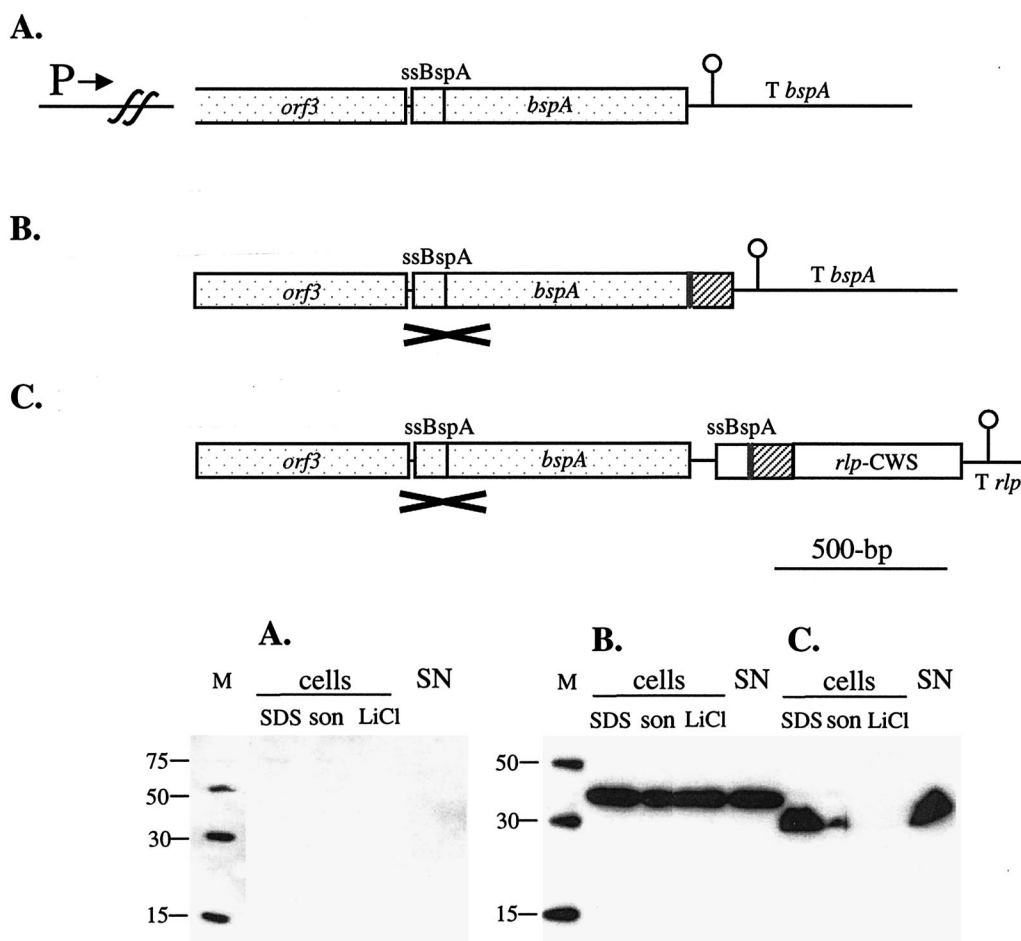


FIG. 3. Gene constructs used to integrate into the *L. fermentum* BR11 *bspA* locus (top panels) and Western blot detection of fusion proteins in cell extracts and in the supernatant with anti-His₆ antibody (bottom panels) for the native *bspA* genomic locus (A), the BspA-His₆-CFTR fusion protein (B), and the His₆-CFTR-Rlp fusion protein (C). In the diagrams in the top panels, the *bspA* upstream promoter (P→), the *bspA* terminator (T *bspA*), the *rlp* terminator (T *rlp*), and DNA encoding the BspA secretion signal (ssBspA), His₆ (grey box), three copies of the CFTR peptide (hatched box), and the cell wall sorting signal of Rlp (*rlp*-CWS) are indicated. The DNA region which is the site of single-crossover homologous recombination into the *bspA* locus is stippled and marked with a cross. In the bottom panels, the Western blots contained His₆ molecular mass markers (with sizes in kilodaltons) (lanes M); cell extracts prepared by boiling in 2× loading dye (lanes SDS), by sonication (lanes son), and with 5 M LiCl (lanes LiCl); and precipitated supernatant fractions (lanes SN). The amounts of cells or medium loaded in each lane are equivalent to 500 μl (lanes SDS), 50 μl (lanes son), 160 μl (lanes LiCl), and 625 μl (lanes SN) of culture.

TABLE 3. Levels and distribution of His₆-containing fusion proteins in *L. fermentum* strains

Vector type	Fusion protein (size in kDa)	Total (cell extract + supernatant) ^a	Protein (mg/liter of culture)				Surface display efficiency ^f (mean ± SD)
			SDS ^b	Son ^c	LiCl ^d	SN ^e	
Surface display	BspA-His ₆ -CFTR (32)	4.7	0.78	4.0	2.6	0.68	2.03 ± 0.01
	His ₆ -CFTR-Rlp (19)	1.1	0.47	0.71	0.04	0.37	10.01 ± 0.03
Secretion	Mlp-His ₆ (27)	2.2	0.46	2.1	0	0.06	ND
	Mlp-His ₆ -CFTR (328)	12.0	1.7	1.2	0	10.3	ND

^a Calculated by using the cell extract method which yielded the greatest amount of protein (either SDS or Son).

^b SDS, proteins extracted from cells by boiling in 2× SDS loading buffer.

^c Son, proteins extracted from cells by sonication.

^d LiCl, proteins extracted from cells by 5 M LiCl.

^e SN, proteins from supernatant.

^f Whole-cell ELISA results presented as the fold increase in OD₄₀₅ signal per OD₆₀₀ of cells ± over that obtained with wild-type *L. fermentum* BR11. The OD₄₀₅ signal per OD₆₀₀ of cells for *L. fermentum* BR11 was 0.0226 ± 0.0006. ^gND, not determined.

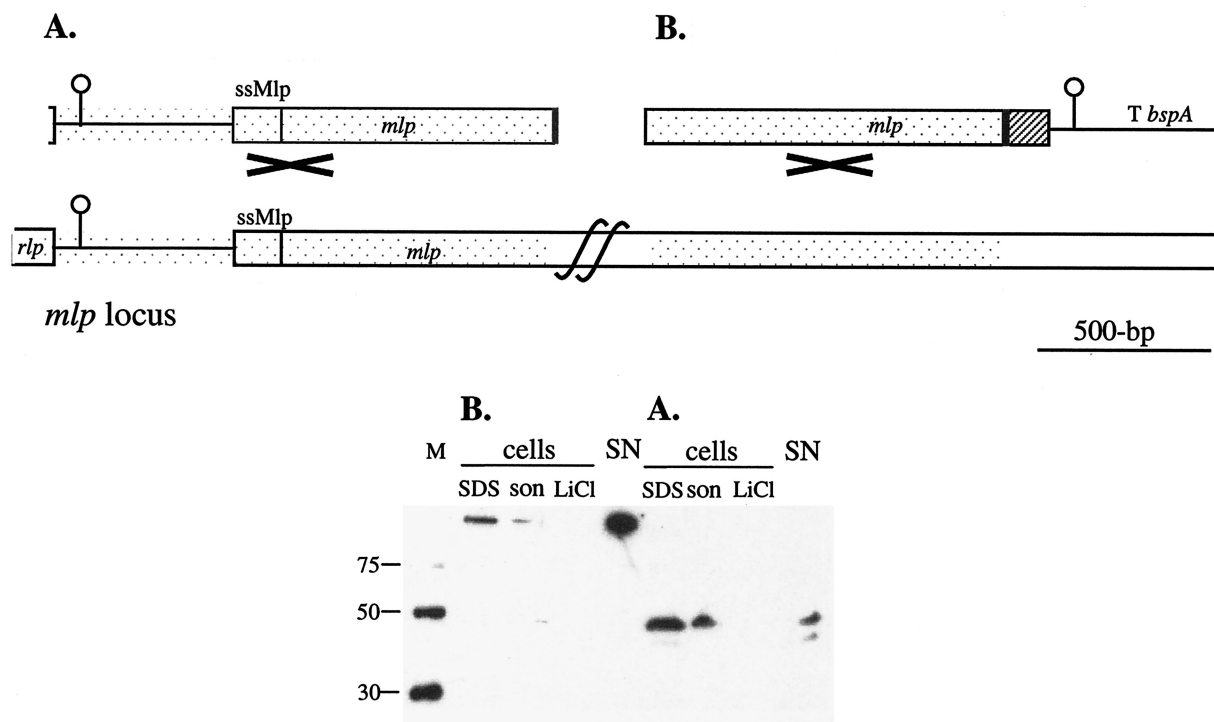


FIG. 4. Gene constructs used to integrate into the *L. fermentum* BR11 *mlp* locus (top panels) and Western blot detection of fusion proteins in cell extracts and in the supernatant with anti-His₆ antibody (bottom panels) for the Mlp-His₆ fusion protein (A) and the Mlp-His₆-CFTR fusion protein (B). In the diagrams in the top panels, the *bspA* terminator (*T bspA*) and DNA encoding the Mlp secretion signal (ssMlp), His₆ (grey box), and three copies of the CFTR peptide (hatched box) are indicated. The DNA region which is the site of single-crossover homologous recombination into the *mlp* locus is stippled and marked with a cross. Due to the large size of *mlp*, the full length of the gene could not be shown. In the bottom panels, the Western blots contained His₆ molecular mass markers (with sizes in kilodaltons) (lanes M); cell extracts prepared by boiling in 2× loading dye (lanes SDS), by sonication (lanes son), and with 5 M LiCl (lanes LiCl); and precipitated supernatant fractions (lanes SN). The amounts of cells or medium loaded in each lane are the equivalent to 500 μl (lanes SDS), 50 μl (lanes son), 160 μl (lanes LiCl), and 625 μl (lanes SN) of culture.

bacteria and the construction of protein surface display and secretion systems by using these two proteins. Although Mlp and Rlp have not definitively been shown to be surface localized, they do contain features very typical of cell wall covalently anchored surface proteins, including amino acid repeat regions, LPXTG cell wall anchoring signals, and, in the case of Mlp, a putative secretion signal (21). The functions of Mlp and Rlp are as yet unknown; however, due to their similarity to putative adhesin proteins such as Mub and R28, respectively, they may function as colonization determinants. Like the case of Mlp in *L. fermentum* BR11, a number of other gram-positive bacteria have multiple copies of cell wall-anchored surface proteins, including the CshA and CshB as well as the SspA and SspB proteins of *Streptococcus gordonii* (8, 19).

Mlp is one of the largest bacterial surface proteins thus far characterized; it is predicted to have 4,427 amino acids and a molecular mass of 473 kDa. Mlp is more similar to Lgas0953 from *L. gasseri* than to Mub from the much more closely related *L. reuteri*. It is possible that multiple Mub homologs which are more closely related to Mlp exist in *L. reuteri*. Although *L. fermentum* BR11 contains multiple genes with similarity to *mub*, we have been unable to demonstrate binding of *L. fermentum* BR11 cells to pig gastric mucin (M1778; Sigma) by using a method similar to that used previously to demonstrate *L. reuteri* and Mub binding (30). This is probably not due

to *L. fermentum* BR11 *mlp* being silent, since part of this gene was initially cloned from a pUC18 genomic library by using an antiserum raised against whole cells. It is possible that Mlp may bind to a different receptor than Mub, since there is reasonable divergence between the two protein sequences. Mlp contains larger repeats than Mub (275 to 407 amino acids long in Mlp and 183 to 197 amino acids long in Mub), and Mlp contains an approximately 60-amino-acid proline-rich region (around 30% proline) in every repeat which is not present in Mub. This proline-rich region is present in at least some repeats of the Lgas0953 protein and is also in the human and bovine hr44 proteins.

The discovery of Rlp represents the first report of a Rib family protein present in a nonpathogenic organism. Rlp contains nonidentical repeats, unlike Rib and α from *S. agalactiae* and R28 from *S. pyogenes*, which contain identical repeats. The repeats of Rib, α, and R28, however, are not identical between proteins (37). The repeats from the streptococcal proteins are 79 to 82 amino acids long, while *L. fermentum* BR11 Rlp repeats range from 69 to 100 amino acids in length, with the majority (six of nine) ranging from 73 to 79 amino acids in length. The size of Rlp is not known; however, the sizes of the putative Rlp homologs from *L. gasseri* suggest that it may be another very large surface protein (Lgas1358, Lgas0203, and Lgas0701 are 2,549, 2,456, and 268 amino acids long, respec-

tively). Attempts to clone the 5' end of *rlp* have been unsuccessful. Similarly, the cloning of *rib* in high-copy-number plasmids in *E. coli* was found to be unstable (44). Rlp has similarity to the R28 protein, which has been implicated in promoting adhesion of *S. pyogenes* to epithelial cells (37), and also to Esp from *E. faecalis* and Bap from *S. aureus*, both of which have been shown to be involved in biofilm formation (7, 40). It is therefore probable that Rlp may function as an adhesin and/or may be involved in biofilm development.

The carboxyl-terminal 161-amino-acid region of Rlp containing a cell wall anchoring signal has been shown to be a significantly better peptide surface display vector than the previously developed noncovalently anchored BspA system. This was despite there being threefold more cell-associated BspA fusion molecules than Rlp fusion molecules. This may be because either (i) a major portion of BspA is located not on the outside of the cell wall but instead between the cytoplasmic membrane and the peptidoglycan or (ii) a significant amount of the BspA fusion is washed away from the cells during the wash steps of the whole-cell enzyme-linked immunosorbent assay. Interestingly, a very closely related BspA homolog from *L. fermentum* RC-14 can be removed from cells by suspension in PBS for 2 h, suggesting that BspA is easily washed from cells even by unconcentrated salt solutions (11). Both the Rlp and BspA fusion proteins were detected in concentrated supernatants. This may be due to cell wall turnover, cell lysis, or limiting sortase enzyme in the case of the Rlp fusion protein (9). A number of LPXTG-containing proteins from their native host have been detected in supernatants. These include M6 from *S. pyogenes* and Mub from *L. reuteri* (9, 30). Recently, a closely related homolog of BspA (MapA from *L. fermentum* 104R; accession number AJ293860) has been detected in supernatant fractions as well as cell surface protein extracts (28). The absence of multiple bands in the protein extracts of cells expressing the BspA and Rlp fusion proteins suggests that the BspA secretion signal is very efficient in driving secretion of heterologous proteins.

Large and small Mlp fragments were tested as potential secretion fusion partners. The significant difference in secretion efficiency between the small and large Mlp fusion proteins is probably due to stability of the His₆-containing proteins in the supernatant in the presence of cell surface and secreted proteases. Indeed, no His₆-containing large Mlp-His₆-CFTR fusion protein could be detected from overnight stationary-phase culture supernatants, indicating degradation of at least the His₆ peptide. Mlp fusion protein associated with the cell appears to be more stable than that in the supernatant, since the large Mlp-His₆-CFTR protein could be detected in overnight stationary-phase culture cell extracts (data not shown). Similarly, when *L. fermentum* BR11 was used to express and secrete the amino terminus of protein A, most (80%) of the protein was found associated with cells and not in the supernatant (31). The small Mlp-His₆ fusion protein migrated as a 45-kDa protein in SDS-PAGE, a molecular mass which is much greater than the predicted 27 kDa. This may be due to glycosylation of this protein. Like for Mub, the mature 144 amino acids of Mlp are rich in serine (23%), which may be potential O-linked glycosylation sites. Recently, the LPXTG-containing Fap1 fimbrial adhesin of *Streptococcus parasanguis*

was confirmed to be a glycoprotein containing sugar residues that are O linked to serine amino acids (38).

Levels of heterologous protein produced by lactobacilli are reported to be 2 to 4 mg per liter with M6 as a cell surface fusion protein (9), 3 mg per liter with BspA as a fusion protein (43), and 10 mg per liter with M6 or protein A as a secretion fusion protein (13, 31). The number of cell-associated BspA fusion protein molecules detected in this study is similar to that reported previously, while the number of cell-associated Rlp fusion protein molecules is ~3-fold lower. This suggests that the *bspA* promoter is strong and able to drive expression of high levels of protein from a single chromosomally integrated gene, compared with other expression systems which are plasmid encoded. The number of cell-associated and secreted Mlp fusion protein molecules is two- to fourfold lower than the number of cell-associated and secreted BspA fusion protein molecules. This suggests that the Mlp promoter is also highly active. Recently, *L. brevis* and *L. acidophilus* S-layers have been investigated for the cell surface display of heterologous peptides by modification of the chromosomal S-layer genes (3, 36). Although S-layers are expressed at very high levels, a major limitation of S-layer-based expression systems is that many fusion partners disrupt the ability of the S-layer subunit to polymerize into the S-layer lattice. In contrast, LPXTG and BspA protein surface display systems have been proven to be versatile and to be capable of accommodating very large fusion partners (12, 14).

A number of therapeutic proteins which interfere with attachment of pathogenic organisms to host surfaces have been characterized (15). It has been shown that a peptide corresponding to the pathogen receptor region of the human CFTR protein, which was inserted into the BspA, Rlp, and Mlp fusion proteins, binds to lipopolysaccharides of *S. enterica* serovar Typhi and *P. aeruginosa* and can reduce their internalization by human intestinal or airway epithelial cells (25, 26). Further work is needed to examine the ability of CFTR peptide-expressing *L. fermentum* strains and purified CFTR fusion proteins to bind to lipopolysaccharide and prevent pathogen internalization.

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