

Lactobacillus bulgaricus Proteinase Expressed in *Lactococcus lactis* Is a Powerful Carrier for Cell Wall-Associated and Secreted Bovine β -Lactoglobulin Fusion Proteins

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Lactic acid bacteria have a good potential as agents for the delivery of heterologous proteins to the gastrointestinal mucosa and thus for the reequilibration of inappropriate immune responses to food antigens. Bovine β -lactoglobulin (BLG) is considered a major allergen in cow's milk allergy. We have designed recombinant *Lactococcus lactis* expressing either full-length BLG or BLG-derived octapeptide T6 (IDALNENK) as fusions with *Lactobacillus bulgaricus* extracellular proteinase (PrtB). In addition to constructs encoding full-length PrtB for the targeting of heterologous proteins to the cell surface, we generated vectors aiming at the release into the medium of truncated PrtB derivatives lacking 100 (PrtB Δ , PrtB Δ -BLG, and PrtB Δ -T6) or 807 (PrtB Δ) C-terminal amino acids. Expression of recombinant products was confirmed using either anti-PrtB, anti-BLG, or anti-peptide T6 antiserum. All forms of the full-length and truncated recombinant products were efficiently translocated, irrespective of the presence of eucaryotic BLG sequences in the fusion proteins. *L. lactis* expressing PrtB Δ -BLG yielded up to 170 μ g per 10⁹ CFU in the culture supernatant and 9 μ g per 10⁹ CFU at the bacterial cell surface within 14 h. Therefore, protein fusions relying on the use of PrtB gene products are adequate for concomitant cell surface display and secretion by recombinant *L. lactis* and thus may ensure maximal bioavailability of the eucaryotic antigen in the gut-associated lymphoid tissue.

An inappropriate immunological response to bovine milk proteins, including β -lactoglobulin (BLG), has been put forward as a key factor in cow's milk allergy (34). Studies have shown that a reequilibration of this response toward a state of specific oral tolerance can be achieved upon oral administration of partially hydrolyzed cow's milk formula or BLG peptides obtained by tryptic hydrolysis (8, 22). Interestingly, the degradation of cow's milk proteins by lactic acid bacteria (LAB) used in food fermentation was also found to generate tolerogenic peptides, with a suppression of the specific lymphoproliferative response (32). In addition, certain LAB strains are more and more widely used as a vehicle for the delivery of heterologous proteins to the mucosal immune system (13, 20). Therefore, it can be hypothesized that the introduction of LAB capable of expressing full-length or BLG fragments may prove them to be a useful tool to aid in host protection against allergic sensitization to this dietary antigen.

Lactococcus lactis is a gram-positive, nonpathogenic bacterium that is widely used in industrial food fermentation. A noncolonizing bacterium, *L. lactis* has been shown to be quite resistant to gastric acidity when administered together with food, remaining metabolically active all the way through the digestive tract (3). Its use as an antigen delivery vehicle for mucosal immunization has been reported (1, 4, 26, 31). Induction of oral tolerance might require a large amount of target antigen, in contrast to immune modulators such as cytokines

(30, 31). Therefore, even though spontaneous bacterial lysis can lead to local antigen release, surface expression or secretion may improve the likelihood that the antigen is appropriately delivered to the mucosal immune system. The poor expression of eucaryotic gene products in LAB prompted us to explore their potential to produce fusion proteins carrying sequences from both bacterial and eucaryotic origins. Dairy LAB produce cell wall-bound extracellular proteinases that catalyze the first step in casein degradation (17). *Lactobacillus bulgaricus* harbors a proteinase (PrtB) whose constitutive expression when driven by a strong promoter makes it a good candidate for use as a fusion protein partner for surface expression of BLG on recombinant *L. lactis*.

In this study, we designed expression vector constructs that allowed highly efficient expression of full-length or truncated forms of PrtB with various secretion patterns. Insertion of BLG sequences in the PrtB constructs similarly permits translocation and secretion of fusion proteins by *L. lactis*. Our data shed light on anchoring properties of *L. bulgaricus* PrtB proteinase. A truncation just upstream of the degenerated cell wall sorting signal has been identified that allows recombinant *L. lactis* clones capable of concomitant delivery of cell wall-associated and secreted target protein to be obtained. Together, our results validate the use of LAB for high-yield expression of prokaryotic-eucaryotic fusion proteins.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. A *lac* *prtP* mutant of *L. lactis* strain MG1363 (9), obtained by plasmid curing, was grown at 30°C in M17 broth supplemented with 1% glucose (33). Stationary-phase cultures reached an optical density at 600 nm of approximately 3 U, with 1 U corresponding to 3.5 $\times$

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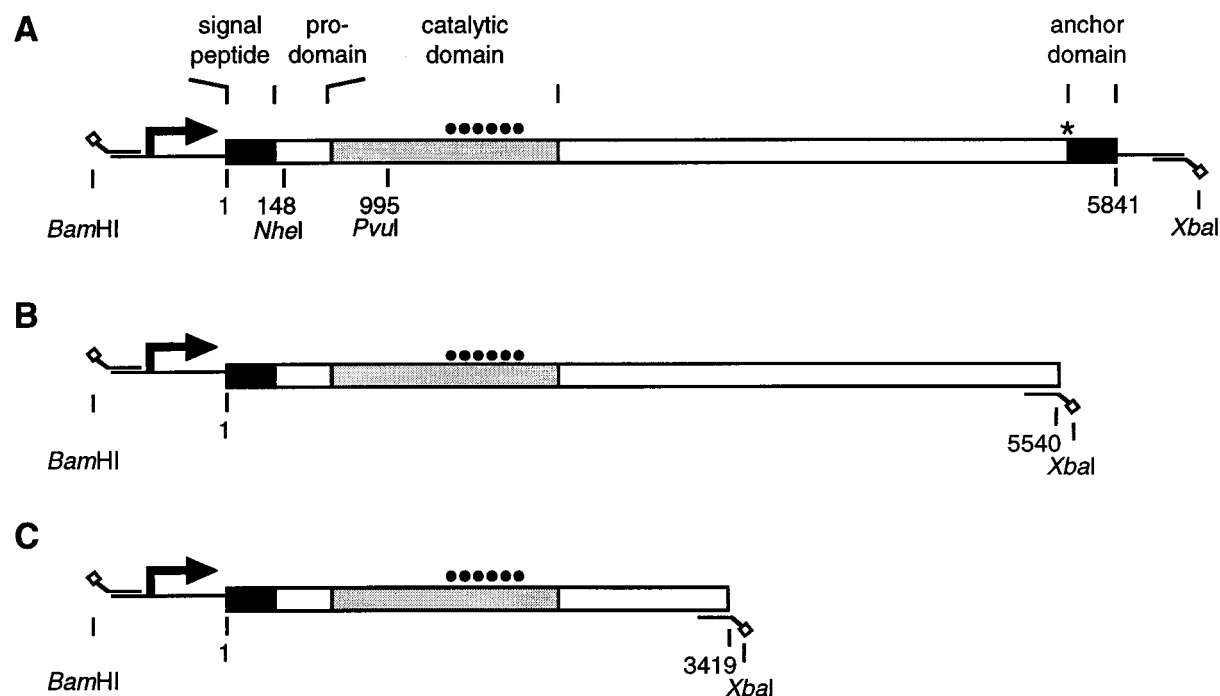


FIG. 1. Schematic representation of the *L. bulgaricus* *prtB* sequences inserted into pNZ124, encoding either the full-length proteinase (A), truncated *Pr*tB Δ (B), or *Pr*tB Δ (C). The first base of the translation initiation codon corresponding to nucleotide 2644 in the sequence corresponding to GenBank accession number L48487 was arbitrarily defined as number 1. The original *prtB* promoter (arrows) and the protease functional domains (boxes), including the degenerated anchoring signal (star) as well as the region recognized by rabbit anti-*Pr*tB antiserum (f-*Pr*tB; dotted line), are depicted. Unique *Bam*HI and *Xba*I restriction sites located on primers and used for cloning, as well as *Nhe*I and *Pvu*I sites subsequently used for insertion of BLG sequences, are indicated.

10^8 CFU/ml. *Escherichia coli* strain M15 containing plasmid pREP4 (Qiagen, Hilden, Germany) was grown routinely in Luria broth (Difco, Detroit, Mich.). Plasmids pNZ124 (23), pQE9, and pREP4 (Qiagen) were maintained by the addition of chloramphenicol (10 μ g/ml), ampicillin (100 μ g/ml), or kanamycin (25 μ g/ml), respectively.

DNA manipulations. Genomic *Lactobacillus delbrueckii* subsp. *bulgaricus* DNA was purified by the spooling method (10). The sequence encoding full-length *L. bulgaricus* *Pr*tB (11), truncated *Pr*tB Δ , and *Pr*tB Δ (Fig. 1) was cloned by DNA amplification and inserted into plasmid pNZ124 (23), yielding expression vectors pMD112, pMD116, and pMD115, respectively (Table 1). The coding sequence for mature BLG was obtained by DNA amplification using full-length *blg* cDNA as a matrix; forward primer AATCATAGCTAGCCTCATCGTCAC CCAGACC, which contained a *Nhe*I site; and reverse primer AATCATACGA TCGCGATGTGGCACTGCTCTCC, which contained a *Pvu*I site. The constructs encoding fusion proteins were obtained by insertion of this amplification

product between the unique *Nhe*I and *Pvu*I restriction sites in *prtB* and *prtB* Δ (Table 1). The sequence coding for BLG octapeptide T6 (IDALNENK) was directly introduced between the same sites by using complementary primers CTAGCATTGATGCTTTAAATGAAAATAAAAT and TTTATTTTCATTT AAAGCATCAATG. Recombinant *L. lactis* was obtained upon transformation by electroporation (12).

A DNA fragment encoding *Pr*tB amino acid residues 472 to 659 (f-*Pr*tB; Fig. 1) was obtained by DNA amplification using pMD112 as a matrix; forward primer GCGGATCCGGCTTGGGCGGTGCAGATG, which contained a *Bam*HI site; and reverse primer CGCAAGCTTGTGCGAAGTGTTTCATGGC, which contained a *Hind*III site. The amplification product was digested with *Bam*HI and *Hind*III and cloned into pQE9, which was cut with the same restriction enzymes. The resulting plasmid (pQE/*prtB*1414-1977) was cloned in *E. coli*.

Preparation of a polyclonal rabbit anti-*Pr*tB antiserum. The *Pr*tB fragment comprising residues 472 to 659 (f-*Pr*tB) fused to an N-terminal six-His tag was obtained upon induction with 1 mM IPTG (isopropyl- β -D-thiogalactopyranoside) of *E. coli* M15(pREP4) transformed with pQE/*prtB*1414-1977. Purification was performed under denaturing conditions on Ni-NTA agarose (Qiagen). Two rabbits were immunized intramuscularly at day 0, in the presence of complete Freund's adjuvant, with 100 μ g of f-*Pr*tB solubilized in 8 M urea. Subsequent immunizations were performed at days 14, 28, and 56, in the presence of incomplete Freund's adjuvant. Rabbits were bled at day 80, and antiserum reactivity was evaluated by both direct enzyme-linked immunosorbent assay (ELISA) and immunoblot analysis.

Preparation of a polyclonal rabbit anti-BLG antiserum. White New Zealand rabbits were injected subcutaneously with 2 mg of BLG, in the presence of Specol (Institute for Animal Science, Lelystad, The Netherlands) as an adjuvant. Booster injections were performed 3, 6, and 12 weeks later with the same material. Rabbits were bled 10 days after the last injection.

Preparation of rat antiserum specific to BLG peptide T6. Synthetic peptide CFKIDALNENKVSIR was conjugated via its sulfhydryl group to maleimide-activated keyhole limpet hemocyanin (Sigma, St. Louis, Mo.). Rats were injected subcutaneously four times at 2-week intervals with 20 μ g of conjugate in the presence

TABLE 1. Characteristics of the expression vectors and their protein products

Plasmid	Heterologous protein ^a
pMD112	<i>Pr</i> tB, amino acid residues 1-1947
pMD115	<i>Pr</i> tB Δ , amino acid residues 1-1140
pMD116	<i>Pr</i> tB Δ , amino acid residues 1-1847
pMD120	<i>Pr</i> tB-T6 = <i>Pr</i> tB but residues 49-332 were replaced with BLG peptide T6
pMD129	<i>Pr</i> tB Δ -T6 = <i>Pr</i> tB Δ but residues 49-332 were replaced with BLG peptide T6
pMD132	<i>Pr</i> tB-BLG = <i>Pr</i> tB but residues 49-332 were replaced with full-length BLG
pMD133	<i>Pr</i> tB Δ -BLG = <i>Pr</i> tB Δ but residues 49-332 were replaced with full-length BLG

^a =, is equivalent to.

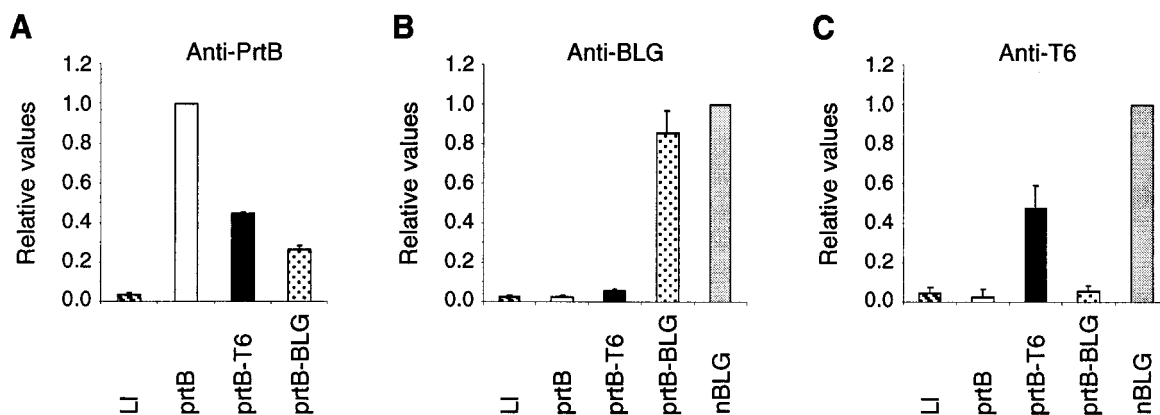


FIG. 2. Surface expression of anchored full-length heterologous proteins. Expression of *L. bulgaricus* PrtB, PrtB-T6, or PrtB-BLG by *L. lactis* (LI) was analyzed by ELISA using different antisera as indicated. nBLG and nonrecombinant LI were used as controls. Values represent means and standard deviations from four independent experiments.

of TiterMax (CytRx, Norcross, Ga.) as an adjuvant. Rats were bled 1 week after the last injection, and reactivity of the antiserum was checked in direct ELISA.

Direct ELISA. Bacteria of stationary-phase cultures were washed twice in Tris-buffered saline (50 mM Tris, 140 mM NaCl [pH 8.0] [TBS]), pelleted by centrifugation at $2,000 \times g$ for 5 min at 4°C, and resuspended in TBS. Approximately 10^8 CFU were directly coated into the wells of Maxisorp plates (Nunc, Roskilde, Denmark), as described previously (27). As a positive control, $3 \times$ crystallized BLG (Sigma) was used, coated at 5 ng per well. Plates were incubated uncovered at 37°C overnight. Dry wells were then washed nine times with phosphate-buffered saline containing 0.05% Tween 20 and were blocked with 0.5% fish gelatin (Sigma). PrtB-BLG fusion proteins were detected with either rabbit anti-PrtB (1:25,000), rabbit anti-BLG (1:10,000), or rat anti-T6 (1:400) antiserum for 1 h at 37°C. Horseradish-peroxidase-conjugated secondary antibodies (1:3,000; PharMingen, San Diego, Calif.) were developed with 1,2-phenylenediamine as a chromogen. Reactions were stopped with 1 volume of 2 M H_2SO_4 . Absorbance was measured at 490 nm, with 620 nm as the reference wavelength.

Competitive inhibition immunoassay. Bacteria were washed twice as described above and resuspended in TBS at 3.5×10^8 CFU/ml. Different volumes of the suspension were incubated for 90 min at 4°C in the presence of a 1:25,000 dilution of anti-PrtB antiserum in siliconized tubes blocked with 1% fish gelatin (Sigma). Bacteria were removed by centrifugation, and supernatant was transferred to wells coated with 10 ng of f-PrtB. In order to get a standard curve, tubes were coated with different amounts of f-PrtB, blocked with 1% fish gelatin, and incubated in the presence of anti-PrtB antiserum for 90 min at 4°C. Antiserum was then directly transferred as bacterial samples as described above. Plates were incubated for 1 h at 37°C, and detection was performed as described for the direct ELISA. The yields indicated in Table 2 take into account the size differences between the standard and heterologous proteins and are expressed in micrograms per 10^9 CFU.

SDS-polyacrylamide gel electrophoresis and immunoblot analysis. Culture supernatant or bacteria washed twice in cold TBS were mixed with 2× gel loading buffer (100 mM Tris, 200 mM dithiothreitol, 4% sodium dodecyl sulfate [SDS], 0.2% bromophenol blue, 20% glycerol [pH 6.8]). Samples were boiled for 3 min and the proteins were separated on SDS-12% polyacrylamide gels and transferred onto polyvinylidene dichloride membranes (Millipore, Bedford, Mass.). Membranes were blocked with 1% fish gelatin in phosphate-buffered saline containing 0.05% Tween 20, washed, and probed with anti-PrtB (1:10,000) or anti-BLG (1:7,500) antiserum for 1 h at room temperature. Horseradish-peroxidase-conjugated goat anti-rabbit immunoglobulin G (1:3,000; PharMingen) was used as a secondary antibody and was detected by incubation with ECL chemiluminescent substrate (Amersham Pharmacia Biotech, Uppsala, Sweden).

Statistical analysis. Standard deviations were calculated using the function STDEV from the Excel 98 application for Apple Macintosh (Cupertino, Calif.).

RESULTS

Construction of LAB vectors for expression of *L. bulgaricus*

PrtB and fusion proteins. In order to obtain antigen cell surface display or secretion, we isolated the *prtB* gene by PCR and generated expression vectors encoding the full-length proteinase (PrtB) or C-terminal (PrtB θ and PrtB Δ) truncation mutants (Fig. 1). For expression of fusion proteins, sequences encoding parts of the PrtB and PrtB θ pro- and catalytic domains were replaced with full-length BLG or peptide T6 sequences, yielding the vectors listed in Table 1. The growth characteristics of nonrecombinant *L. lactis* and all transformants were found to be very similar (data not shown).

Expression of full-length PrtB and fusion proteins in recombinant *L. lactis*. Expression and translocation of PrtB, PrtB-T6, and PrtB-BLG was assessed by direct ELISA using antisera reactive to either PrtB, BLG, or peptide T6. Recombinant *L. lactis* bacteria were found to harbor the heterologous proteinase (Fig. 2A). Specific antisera confirmed expression and surface display of BLG or peptide T6 in fusion proteins (Fig. 2B and C). Low measured activity values for anti-BLG versus PrtB-T6 (Fig. 2B) and anti-T6 versus PrtB-BLG (Fig. 2C) might be explained by the surface exposure or conformation of BLG or peptide T6 in the context of PrtB; alternatively, the presence of conformational alterations resulting from the drying of whole cells on a solid phase cannot be excluded. Specific signals exhibited characteristics similar to those of the signal obtained with 5 ng of native BLG (nBLG), validating the sensitivity and specificity of the assay. Together, these findings indicate that replacement of the prodomain and part of the catalytic domain of PrtB by heterologous amino acid stretches of different lengths still allows both efficient expression and translocation of the fusion protein.

Translocation and release of the various forms of PrtB and fusion proteins. In order to evaluate the translocation and release of heterologous products in recombinant *L. lactis* cultures, bacteria were pelleted and the supernatants were analyzed by Western blotting using anti-PrtB or anti-BLG antiserum. Unexpectedly, the supernatants of clones expressing full-length heterologous proteinases (PrtB, PrtB-T6, or PrtB-BLG) contained significant amounts of products specifically recognized by PrtB-specific antiserum. In addition, all bands

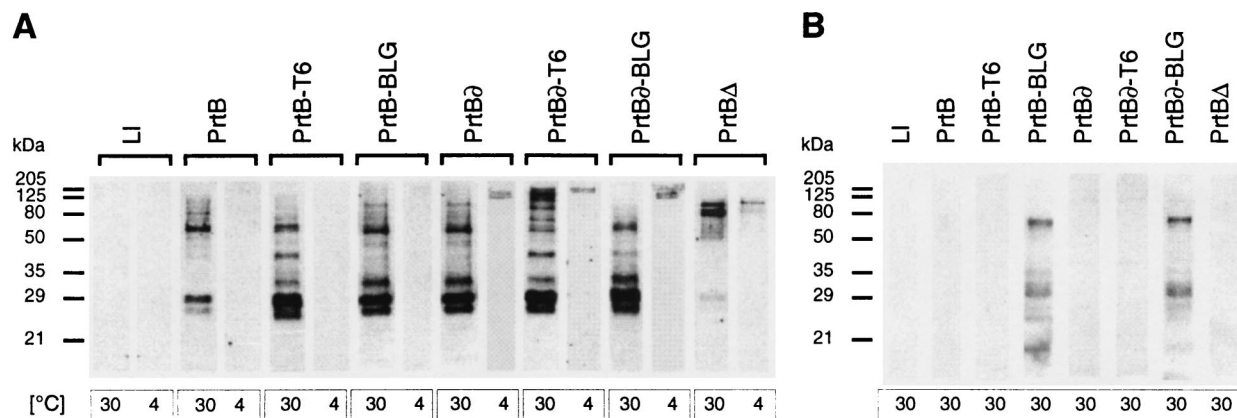


FIG. 3. Detection of heterologous proteins in the supernatant of recombinant *L. lactis* cultures. Lanes 30 show results for supernatants of stationary-phase cultures grown at 30°C. Lanes 4 show results for bacteria of stationary-phase cultures which were pelleted, resuspended at a ratio of 1:5 in fresh medium, and incubated at 4°C for 7 h. Proteins in supernatants were analyzed by Western blotting using anti-PrtB (A) or anti-BLG (B) antisera. Nonrecombinant *L. lactis* (LI) was used as control.

migrated faster than expected for intact fusion proteins, which ranged between 125 and 215 kDa (Fig. 3A). When these bacteria were washed and incubated at 4°C for 7 h, the degradation products were no longer observed, suggesting that the cell wall-anchored molecules are prone to temperature-sensitive proteolytic degradation (Fig. 3A). Expression vectors pMD115 and pMD116 and modifications thereof, encoding PrtB and fusion proteins truncated at the C terminus, were prepared with the aim of obtaining the secretion of heterologous products. The supernatant of PrtB Δ -expressing bacteria contained both high- and low-molecular-weight products which were reactive with anti-PrtB antiserum (Fig. 3A). However, only the largest products which were migrating as full-length fusion proteins were found in the supernatant of bacteria incubated at 4°C, suggesting that the signal observed at 30°C is due to both extracellular secretion and surface proteolytic degradation. While PrtB Δ and PrtB Δ -BLG yielded similar cleavage patterns, PrtB Δ -T6 exhibited more high-molecular-weight species. Finally, the large deletion in PrtB Δ was found to lead to the secretion of products of 90 kDa or larger, indicating that this truncated proteinase is more readily secreted and thus is much less susceptible to the effects of the proteolytic degradation which takes place at the bacterial surface.

BLG-containing products of different sizes were detected in the supernatant of *L. lactis* clones expressing either PrtB-BLG or PrtB Δ -BLG (Fig. 3B) as assessed by using rabbit BLG-specific antiserum. This observation suggests that oral administration of these clones ensure bioavailability of the eucaryotic antigen expressed as a fusion protein.

Qualitative analysis of bacteria-associated heterologous proteins during biosynthesis and shortly after release. The observation of degradation products in the culture supernatant of bacteria grown at 30°C and expressing PrtB or PrtB Δ or fusion proteins thereof prompted us to assess whether these proteins were translocated to the cell surface as intact molecules. *L. lactis* bacteria were resuspended in reducing SDS-gel loading buffer, which led to the release of noncovalently anchored cell wall proteins (see Materials and Methods). Both full-length proteins (Fig. 4A) and a major degradation product

of 100 kDa were observed for PrtB, PrtB Δ , and PrtB Δ . In contrast, the fusions PrtB-BLG/T6 and PrtB Δ -BLG/T6 displayed full-length proteins only, a finding which argues for the presence of PrtB residues 50 to 332 in the 100-kDa product mentioned above. Analysis of culture supernatants recovered as soon as 30 min after inoculation showed a substantial enrichment in intact products for clones PrtB Δ and PrtB Δ and fusion proteins thereof (Fig. 4B). PrtB clones only released a

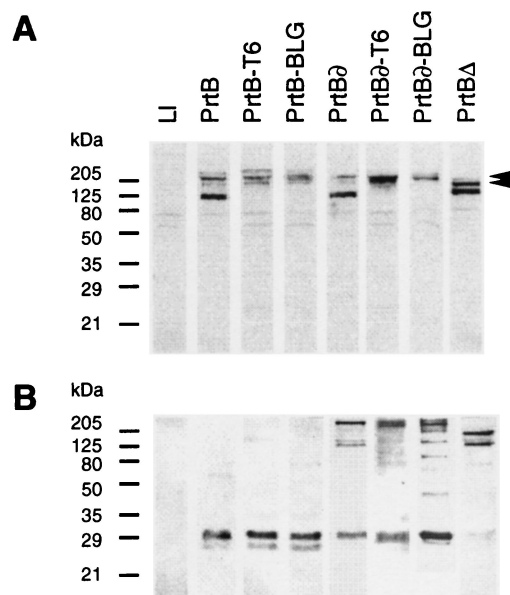


FIG. 4. Qualitative analysis of translocated and secreted heterologous proteins produced by *L. lactis* cultures. (A) Extensively washed bacteria of 14-h stationary-phase cultures were pelleted by centrifugation, resuspended in reducing gel loading buffer, boiled, and analyzed by Western blotting using anti-PrtB antiserum. Arrowheads indicate full-length proteins. (B) Bacteria of stationary-phase cultures were washed, resuspended at an optical density of 0.5 in fresh medium, incubated for 30 min, and removed by centrifugation. Proteins in supernatants were analyzed as described for panel A.

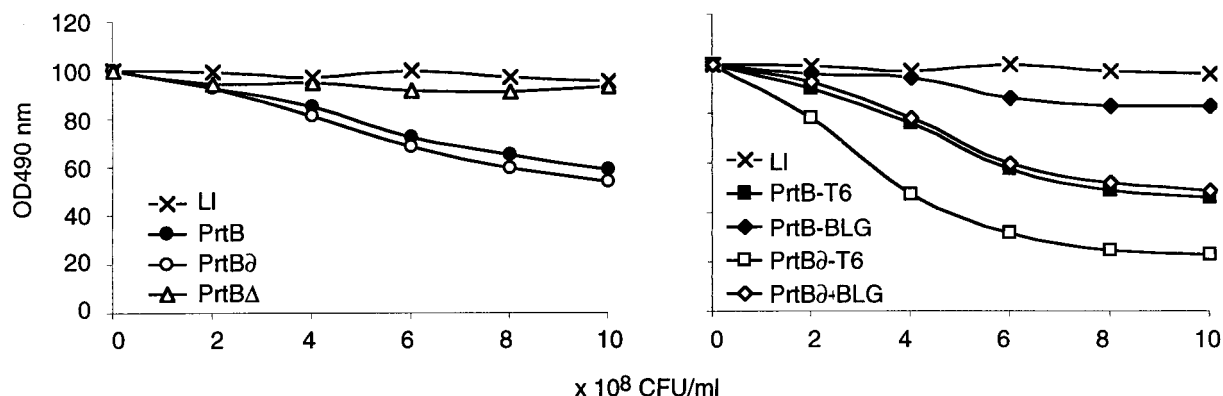


FIG. 5. Competitive inhibition ELISA on recombinant *L. lactis*. Defined amounts of cells as indicated on the x axis were incubated with anti-PrtB antiserum for 90 min on ice and removed by centrifugation. The depleted antiserum was then incubated in the wells of 96-well plates coated with 50 ng of f-PrtB standard. Values are expressed as a percentage of the signal obtained without cell competitors and were taken from a representative experiment ($n = 4$). For clear visualization of the data, curves are depicted in two independent panels and error bars were not drawn.

30-kDa product reactive to anti-PrtB, which argues that rapid cleavage had taken place (Fig. 4B). These findings indicate that both full-length and truncated heterologous proteins are transported across the membrane as intact polypeptides but exhibit various degrees of stability after translocation or release in the supernatant.

Comparative cell surface displays of the different forms of heterologous proteins in *L. lactis*. We designed a competitive inhibition ELISA based on PrtB antiserum depletion by bacterial clones. Cultures of *L. lactis* were washed and then transferred to TBS at 0°C to abolish any proteinase release in the supernatant (data not shown), and anti-PrtB antiserum was added. Following incubation, bacteria were removed by centrifugation and the residual antiserum capacity for binding to f-PrtB was assessed. Different degrees of competition were obtained with bacteria expressing either PrtB or PrtBΔ and derived fusion proteins (Fig. 5), reflecting either differences in levels of expression or multiple modes of folding and epitope exposure in the bacterial cell wall. In contrast, competition with PrtBΔ-expressing bacteria resembled that with wild-type *L. lactis*, suggesting that the antiserum binding sites were embedded within the bacterial surface. Therefore, the assay is convenient for testing the surface availability of heterologous antigens in the context of whole bacteria.

Comparative quantitation of cell surface-exposed and secreted heterologous proteins produced in *L. lactis*. To determine the amount of heterologous protein displayed at the bacterial surface or released in the culture supernatant during stationary-phase growth, two analytical methods were employed, a competitive inhibition experiment and a direct ELISA. Both assays included f-PrtB as a standard. Amounts ranging from 3 to 10 μg of heterologous products per 10⁹ CFU were detected at the cell surface among the various clones (Table 2). As suggested by the Western blot analysis presented in Fig. 4A, surface expression of PrtBΔ or derived fusion proteins was equal to or exceeded the signal obtained with PrtB proteins. Culture supernatants were found to contain large amounts of heterologous products, ranging from 30 to 150 μg per 10⁹ CFU and corresponding to 10- to 20-fold-larger

amounts than those observed at the bacterial cell surface. Finally, the apparent weak display of PrtBΔ on the cell surface was most likely due to poor recognition by anti-PrtB antiserum, as this truncated product was previously shown to be efficiently translocated (Fig. 4A).

DISCUSSION

LAB are of increasing interest for the expression of heterologous molecules. The absence of an outer membrane in these gram-positive microorganisms makes them particularly attractive for the display or secretion of biologically active molecules such as antigens, enzymes, or cytokines (4, 19, 30, 31). In addition to being considered nonpathogenic, LAB show a remarkable ability to survive passage along the oral route, as documented by their active metabolism in most of the compartments of the gastrointestinal tract (3). Exposure of *L. lactis* bacteria in vitro to low pH and bile is well tolerated (15), and upon feeding of the organism to humans, a fraction of viable cells was recovered in feces (16). Together, these findings indicate the potential of the bacteria as an antigen vehicle but do not give a precise indication of the cellular location, the amount, and the molecular structure in which the heterologous protein should be expressed to exhibit optimal biological activity. To tackle this issue, we thus generated various *L. lactis*

TABLE 2. Production of heterologous proteins by recombinant *L. lactis*^a

Product	Cell surface	Supernatant
PrtB	5.4 ± 0.6	53.8 ± 21.3
PrtB-T6	7.4 ± 1.8	125.3 ± 29.1
PrtB-BLG	3.8 ± 1.7	28.9 ± 8.7
PrtBΔ	7.5 ± 0.8	70.0 ± 23.3
PrtBΔ-T6	9.4 ± 0.6	168.6 ± 31.5
PrtBΔ-BLG	8.8 ± 0.7	130.6 ± 21.2
PrtBΔ	0.5 ± 0.3	82.8 ± 26.3

^a Values represent the means and standard deviations from five independent experiments and are expressed in micrograms per 10⁹ CFU. Data were obtained from cultures incubated for 14 h at 30°C.

mutants capable of producing dozens of micrograms of recombinant fusion protein per 10^9 bacteria at the cell surface and in the culture supernatant.

We hypothesize that cell surface exposure and secretion of target protein are associated with an optimal antigenic bioavailability in the gut-associated lymphoid tissue. Previous studies have shown that an appropriate combination of promoter, signal sequence, and anchoring signal is a prerequisite for efficient surface expression in LAB. However, constitutive and inducible expression systems generally led to the production of microgram-level amounts of recombinant protein per liter of culture (1, 2, 18). We examined the potential of *L. bulgaricus* PrtB proteinase to serve as a protein carrier for a foreign sequence because (i) it is a large protein (1,947 amino acids) most likely prone to sustain changes in its primary structure and (ii) it is constitutively expressed at high levels on the surface of *L. bulgaricus* (5). Successful expression was achieved in *L. lactis*, as shown by the surface display and release in the supernatant of the heterologous proteinase. In mutants carrying BLG inserts which replaced part of the PrtB catalytic site, the presence and surface exposure of BLG inserts was also confirmed by ELISA using antisera directed either to full-length BLG or to peptide T6. The site of substitution for BLG coding sequences was thus adequate, since PrtB-BLG and PrtB-T6 were properly processed by the *L. lactis* secretion machinery and escaped intracellular proteolysis. Anti-BLG antiserum was not reactive, in both ELISA and immunoblot analysis, to T6-expressing clones, confirming previous reports of the low level of antigenicity of this BLG peptide (22, 28). The fact that anti-T6 antiserum, which recognizes native BLG in ELISA, failed to react with recombinant *L. lactis* expressing full-length BLG might reflect either epitope masking or subtle conformational changes in the fusion protein. Heterologous proteins associated to the cell wall migrated essentially as intact molecules, indicating that they were not a target for the cytoplasmic proteinase ClpP that contributes to the degradation of misfolded proteins in *L. lactis* (6).

The anchoring of PrtB at the bacterial cell surface does not rely on the LPXTG consensus motif found in a large variety of surface proteins of gram-positive bacteria (21) and therefore may depend on an as yet unelucidated mechanism. To bypass this lack of knowledge, we sought to get a source of secreted PrtB by expressing two types of PrtB proteins truncated at the C terminus. PrtB Δ finishes just upstream of the LPKKT anchoring motif (11), whereas PrtB Δ lacks the last C-terminal 807 amino acids comprising the 41-residue basic domain located upstream of the LPKKT motif putatively implied in cell wall anchoring (29). *L. lactis* expressing these two mutants was found to release anti-PrtB reactive products but was associated with distinct patterns. Most of PrtB Δ was secreted in the form of intact polypeptide. In the cell wall, the protein could be released upon SDS treatment but was not detected with anti-PrtB antiserum in the context of the whole bacterium, implying major conformational changes. This may also represent a plausible explanation as to why this clone does not grow in milk, in contrast to PrtB- and PrtB Δ -expressing clones (J.-E. Germond, unpublished data). This limitation led us to exclude PrtB Δ from further experiments.

Large amounts of small-sized products were identified in the supernatant of PrtB Δ -expressing clones during stationary-

phase growth. The latter supernatants contained integral fusion proteins shortly after inoculation or when the cultures were kept in conditions conducive to the reduction of protein degradation. In parallel, these clones expressed a significant anti-PrtB reactive cell surface signal. Their release in culture supernatant may therefore result from both extracellular secretion and proteolysis taking place at the cell surface, possibly due to less efficient translocation than that for PrtB Δ . Consistent with this, degradation products, surprisingly detected in the supernatant of PrtB-expressing clones, are most likely the result of the same proteolytic activity. As the lactococcal strain used in our study does not express its own extracellular proteinase PrtP, this proteolytic activity may be ascribed to the cell surface proteinase HtrA, recently reported to be responsible for the degradation of proteins during or after translocation (24). Importantly, in mutants carrying BLG inserts, anti-BLG reactive products migrating up to 70 kDa were identified in the culture supernatant, indicating efficacious shielding of BLG within the PrtB carrier.

The yield of cell surface protein was obtained using the novel competition assay described in Materials and Methods. PrtB Δ -expressing clones displayed larger amounts of heterologous protein than clones expressing the anchored version of the proteinase. PrtB-BLG-expressing clones exhibited the weakest surface expression, consistent with direct ELISA. With the release in the supernatant of 130 μ g of recombinant protein per 10^9 CFU within 14 h, the *L. lactis* recombinant expressing PrtB Δ -BLG ensured that more than 10 μ g of BLG was available as a fusion antigen. This corresponds to the yield of staphylococcal nuclease obtained in *L. lactis* by using an optimized propeptide (18). This is fivefold more than the yield of BLG alone obtained through the use of optimized expression vectors carrying the signal peptide from *L. lactis* Usp45 (1, 2), with the advantage that the protein is stabilized within the PrtB carrier. The dose of antigen administered orally and the frequency of feeding will be key factors in the regulation of the immune response to BLG (7). Indeed, while feeding with a high antigen dose triggers tolerance through anergy, minute doses are also efficacious for the inducement of active suppression mediated by so-called tolerogenic T cells (35). In addition, recombinant *L. lactis* strains expressing sufficient amounts of target protein could be administered in a volume compatible with a normal diet. Embedding of the BLG sequence in PrtB thus guarantees that the protein is not rapidly degraded and can possibly favor its appropriate presentation to antigen-presenting cells present in Peyer's patches (14) or along the epithelial surface (25). Whether the amount of antigen protein expressed in our system will be sufficient to elicit a mucosal immune response in vivo is presently being tested in a rat model of allergy.

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