GroEL of *Lactobacillus johnsonii* La1 (NCC 533) Is Cell Surface Associated: Potential Role in Interactions with the Host and the Gastric Pathogen *Helicobacter pylori*

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Heat shock proteins of the GroEL or Hsp60 class are highly conserved proteins essential to all living organisms. Even though GroEL proteins are classically considered intracellular proteins, they have been found at the surface of several mucosal pathogens and have been implicated in cell attachment and immune modulation. The purpose of the present study was to investigate the GroEL protein of a gram-positive probiotic bacterium, *Lactobacillus johnsonii* La1 (NCC 533). Its presence at the bacterial surface was demonstrated using a whole-cell enzyme-linked immunosorbent assay and could be detected in bacterial spent culture medium by immunoblotting. To assess binding of La1 GroEL to mucins and intestinal epithelial cells, the La1 GroEL protein was expressed in *Escherichia coli*. We report here that La1 recombinant GroEL (rGroEL) binds to mucins and epithelial cells and that this binding is pH dependent. Immunomodulation studies showed that La1 rGroEL stimulates interleukin-8 secretion in macrophages and HT29 cells in a CD14-dependent mechanism. This property is common to rGroEL from other gram-positive bacteria but not to the rGroEL of the gastric pathogen *Helicobacter pylori*. In addition, La1 rGroEL mediates the aggregation of *H. pylori* but not that of other intestinal pathogens. Our *in vitro* results suggest that GroEL proteins from La1 and other lactic acid bacteria might play a role in gastrointestinal homeostasis due to their ability to bind to components of the gastrointestinal mucosa and to aggregate *H. pylori*.

Cell surface proteins have been extensively studied for many pathogenic bacteria as they mediate attachment to mammalian extracellular matrix components, thus favoring invasion of sub-epithelial tissues, and participate in mounting the host inflammatory response. In contrast, cell surface proteins from non-pathogenic bacteria and their roles in persistence in the host have not been thoroughly described.

Lactobacilli are natural inhabitants of the gastrointestinal tract of mammals and are considered potential probiotics. Several probiotics have been demonstrated to enhance gastrointestinal health by stimulation of host immunity and inhibition of pathogen adherence to mucus and epithelial cells (reviewed in references 7 and 44). The ability of lactobacilli to attach to epithelial cells (4, 5, 26, 36, 51) and mucins (27, 34, 43, 48–50) has been documented and is expected to be an important characteristic, enhancing intestinal persistence and antagonistic competition with pathogens, especially at the point of initial contact with the mucosa. Recently, the association of *Lactobacillus* species with Peyer's patches in mice has been described (40). However, at present, only a few molecules involved in attachment to mucus and to epithelial cells have been identified in *Lactobacillus* species (16, 42, 45).

Heat shock proteins of the GroEL class, also designated chaperones of the Hsp60 class, are a highly conserved group of proteins essential to all living organisms (19). Their key role consists of mediating protein folding within the cell to guarantee normal function (3, 6). Despite their designation, they are

expressed at all temperatures, but basal expression is enhanced by environmental stress, including elevated temperature, oxygen limitation, and nutrient deprivation. From its known functions, the GroEL protein is predicted to be located in the cytoplasm (19), which is supported by the fact that no member of the GroEL family possesses a secretion signal sequence or other recognizable motifs that would suggest its export. However, there are an increasing number of reports indicating an additional extracytoplasmic location of GroEL in pathogenic bacteria. Surface-associated Hsp60 has been reported in *Mycobacterium leprae* (14), *Salmonella enterica* serovar Typhimurium (9), *Clostridium difficile* (24), *Helicobacter pylori* (8, 39, 52), *Legionella pneumophila* (11), and *Haemophilus ducreyi* (10). Interestingly, in those mucosal pathogens for which Hsp60 is suggested to be surface exposed, the protein is also implicated in attachment and/or immune modulation activities (8–10, 24, 25, 56, 57).

We report here that, in addition to its cellular functions (55), the *Lactobacillus johnsonii* La1 GroEL protein can also be found at the bacterial surface and possesses activities that could contribute to its probiotic properties, including attachment to mucus and epithelial cells, stimulation of cytokine secretion in macrophages and epithelial cells, and the ability to mediate aggregation of the gastric pathogen *H. pylori*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *L. johnsonii* La1 (NCC 533) from the Nestlé Culture Collection (Lausanne, Switzerland) and *Lactobacillus helveticus* ATCC 15009 (American Type Culture Collection) were grown under anaerobic conditions in De Man-Rogosa-Sharp broth (Difco) at 37°C for 2, 4, 6, and 8 h for La1 spent culture supernatant preparations or overnight for other assays.

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TABLE 1. Primers used for amplification of *groEL* genes from different bacteria

Bacteria	Gene product	Restriction site	Primer sequence ^a
<i>L. johnsonii</i> NCC 533	La1 La1	NcoI SalI	5'-CATATACCATGGCTAAAGAGATTAAATTTTCTGAAAATGCAAGACACTC 3'-GTTTCTGTCGACCATTTCCCATACCCATACCTGG
<i>L. helveticus</i> ATCC 15009	GroEL GroEL	NcoI BamHI	5'-ATATATCCATGGCAAAAGATATTAAATTCTCAGAAAACGC 3'-ATATATGGATCCATTCCCATGGAGCACCTGC
<i>B. subtilis</i> NCC 199	GroEL GroEL	NcoI BamHI	5'-ATATATCCATGGCAAAAGAAATTAAGTTTGTAGTGAAGAAGC 3'-ATATATGGATCCATCATTCACCCATACC
<i>L. lactis</i> MG 1363	GroEL GroEL	NcoI BamHI	5'-ATATATCCATGGCAAAAGAAATTAATTTTCAAGTGATGC 3'-ATATATGGATCCATCATACCCATTCCC
<i>H. pylori</i> P1	GroEL GroEL	BamHI SalI	5'-CCGGGATCCCGGATGGCAAAAGAAATCAAATTTTCAG 3'-GAATTCGTCGACTTACATCATGCCACCCATGCTC

^a Restriction sites are given in boldface.

Lactococcus lactis strain MG 1363 (12) was grown in M17 (Oxoid) glucose at 30°C under anaerobic conditions. *Bacillus subtilis* NCC 199 was grown in brain heart infusion broth (Difco) at 30°C with shaking. La1 bacterial pellets were prepared from overnight cultures and centrifuged at 4,000 × g for 10 min at 4°C.

H. pylori strain P1 (20) was grown on 3.6% GC agar plates (Oxoid), supplemented with 1% Isovit (Biological Laboratories) and 10% horse serum (Biological Industries) and maintained in a microaerophilic atmosphere (85% N₂, 10% CO₂, 5% O₂) at 37°C for 48 h.

Escherichia coli strains XL1 Blue and BL21(DE3) codon plus RIL were obtained from Stratagene Inc., grown in Luria-Bertani (LB) medium at 37°C with shaking, and supplemented with 50 µg/ml kanamycin and 25 µg/ml chloramphenicol as required. *E. coli* strain M15(pREP4) was obtained from QIAGEN and grown on LB medium containing 25 µg/ml kanamycin.

Cryocultures of *Salmonella enterica* serovar Typhimurium strain SL 1344 (kindly provided by B. Stocker, Stanford University, California) and enteropathogenic *E. coli* strain E 2348/69 (kindly provided by J. Hacker, University of Würzburg, Würzburg, Germany) were grown overnight in 10 ml of LB medium at 37°C with shaking. One hundred microliters of the overnight culture was inoculated into 10 ml of fresh LB medium and grown again at 37°C overnight. The number of bacteria/ml was estimated by measuring the optical density at 600 nm (OD₆₀₀; 1 OD unit = 1 × 10⁸ bacteria/ml).

Cell culture. Nondifferentiated human adenocarcinoma HT29 cells (American Type Culture Collection) were cultured in glucose-containing Dulbecco's modified Eagle's medium (DMEM) as previously described (31, 53), and HT29-MTX (methotrexate treated) cells were grown according to Lesuffleur (32). Human peripheral blood mononuclear cells were isolated by density centrifugation on Ficoll (Histopaque-1077; Sigma) from fresh blood obtained from healthy donors. Cells were counted and seeded in 96-well Nunclon plates (Nunc) at 2 × 10⁵/well in RPMI 1640 medium (Life Technology) supplemented with 10% endotoxin-free fetal calf serum (Gibco). Monocytes were isolated by adherence to the plastic plates for 2 h at 37°C (29).

Expression of *groEL* genes in *E. coli*. The *groEL* genes of La1 (LJ0461; AE017198), *L. helveticus* (AF031929), *B. subtilis* (D10972), and *L. lactis* (AY029215) were amplified by PCR with specific oligonucleotides containing the appropriate restriction sites for cloning (see Table 1). PCR amplification was performed as described previously (16). The amplicon was digested and ligated into the expression plasmid pET-24d (Novagen), digested with the same restriction enzymes, and transformed into *E. coli* XL1 Blue. A positive clone was confirmed by DNA sequence analysis and transformed into the expression host BL21(DE3) codon plus RIL.

The amplicon of the *H. pylori* P1 *groEL* gene was digested with the corresponding restriction enzymes, and the fragment was ligated into the pQE-30 vector (QIAGEN) and transformed into *E. coli* XL1 Blue. The translated *H. pylori* P1 GroEL sequence showed 100% identity to the GroEL protein sequence of strain 26695 (P42383). The selected clone was finally transformed into *E. coli* M15(pREP4) (QIAGEN) for protein expression. The paired primers containing the restriction sites used to amplify the different *groEL* genes are shown in Table 1.

Purification of recombinant GroEL proteins. Protein expression was induced with 100 µM isopropyl-β-D-thiogalactopyranoside (IPTG), and the six-His-tagged-fusion proteins were purified under native conditions by Ni²⁺-nitrilotri-

acetic acid affinity chromatography (QIAGEN) as described in the manufacturers' protocols. Briefly, bacterial lysates were loaded on a column equilibrated with lysis buffer containing 10 mM imidazole. After being washed with lysis buffer until the eluate reached an OD₂₈₀ of <0.01, proteins were eluted with a buffer containing 125 mM imidazole. The presence of protein in the different fractions was monitored by the Bio-Rad protein assay. Protein-containing fractions were pooled, equilibrated in phosphate-buffered saline (PBS) by passing through a PD10 column (Amersham), and finally stored at -20°C in the presence of 10% glycerol. Detection of lipopolysaccharide (LPS) contamination of all recombinant proteins was performed with the *Limulus* amoebocyte lysate endochrome test (Charles River Endosafe).

Determination of recombinant La1 GroEL (rGroEL) molecular mass by MALDI-MS. Mass spectrum was recorded on the Autoflex (Bruker) matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) time-of-flight mass spectrometer operating in the delayed-extraction linear positive-ion mode. Sinapinic acid (saturated solution in 40% acetonitrile containing 0.1% trifluoroacetic acid) was used as a matrix. One microliter of a 50% mix of matrix solution and protein (1 mg/ml) was deposited on the sample holder and allowed to dry at room temperature. External calibration was performed with the protein mixture (Bruker).

La1 rGroEL sequence analysis by nano-ESI-MS (MS/MS). La1 rGroEL protein (1 mg/ml) was dissolved in 50 mM ammonium bicarbonate (Sigma) and digested with 20 ng/µl trypsin (sequencing grade, Promega) or endoproteinase Arg-C (sequencing grade, Roche). The resulting digested peptides were desalted with ZipTip C₁₈ (Millipore) and analyzed by nano-electrospray ionization (nano-ESI)-MS (tandem MS [MS/MS]), as previously described (33). [Glu]-fibrinopeptide MS/MS data were used for mass calibration.

Detection of GroEL at the surface of La1. Detection of GroEL was performed by whole-cell enzyme-linked immunosorbent assay (ELISA). Maxisorb polyvinyl wells (Nunc) were coated with 100 µl of 0.3 × 10⁸ La1/ml in PBS buffer, pH 7.2, overnight at 4°C. After saturation for 1 h at room temperature with 1% bovine serum albumin in PBS buffer, pH 7.2, plates were incubated for 2 h at 4°C with rabbit anti-GroEL (Sigma) or anti-β-lactoglobulin antibody produced according to a well-described method (23). The antibody concentrations varied from 0.16 to 10.0 µg/ml in PBS-0.05% Tween 20. After three washes with the same buffer, plates were incubated for 1 h at 4°C with horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (Zymed) diluted 1/2,000 in PBS-0.05% Tween 20. After three additional washings, HRP enzymatic activity was revealed using the tetramethyl benzidine substrate kit (Pierce) and measured at 450 nm in a Dynatech MR 5000 microtiter plate reader. The supernatant from the first coating with La1 was carefully removed from wells and tested for bacterial lysis by measuring DNA (Ribogreen, quantitation kit; Molecular Probes) and the intracellular marker aldolase (28).

Protein preparation from La1 spent culture medium. La1 was grown as indicated for 2, 4, 6, and 8 h. Spent culture media were recovered by centrifugation at 4,000 × g for 20 min at 4°C. Aliquots of 50 ml were then passed through a 0.2-µm filter, the pH was adjusted to 7.0, and proteins were precipitated by slow addition of ammonium sulfate (Merck) to 60% with gentle shaking. After 30 min of incubation at room temperature, precipitated proteins were recovered by centrifugation at 12,000 × g for 12 min at room temperature and the pellets were

suspended in 1 ml of distilled water. Samples were loaded onto a PD10 column (Amersham) equilibrated in PBS. Elution was performed in PBS, and 1-ml fractions were collected and analyzed for protein content (Bio-Rad protein assay, dye reagent). Protein-containing fractions were pooled, divided into aliquots, and frozen at -20°C until further analysis. Protein preparations (20 μg) were loaded onto 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and transferred onto immunoPVDF (polyvinylidene difluoride) membranes (Bio-Rad), and the presence of GroEL was analyzed by Western blotting using a rabbit anti-GroEL (Sigma) as a primary antibody and a goat anti-rabbit immunoglobulin (Ig) conjugated to alkaline phosphatase (Sigma) as a secondary antibody, both at a dilution of 1/2,000. The alkaline phosphatase enzymatic activity was revealed by the 5-bromo-3-chloro-3-indolylphosphate-nitroblue tetrazolium substrate kit (Zymed) according to the manufacturer's instructions.

Purification of mucin-enriched preparations from HT29-MTX. The cell culture medium of 3-week-old HT29-MTX cells was replaced with serum-free medium for 16 to 24 h. Cell culture supernatants and mucus were collected by repeated gentle pipetting on cells to avoid damaging the monolayer. Mucins were purified by size exclusion chromatography, and the concentration and quality of the preparations were determined as previously described (16).

Assays of binding to mucins and HT29 cells. The capacity of different La1 recombinant proteins to bind to mucins and HT29 cells was tested at pH 7.2 and 5.0 as previously described (16) using 0.1 $\mu\text{g}/\text{ml}$ mouse anti-His Tag antibody (Penta. His antibody; QIAGEN) and rabbit anti-mouse Ig-HRP (Zymed) diluted 1/2,000 in PBS-0.05% Tween 20. HRP enzymatic activity was revealed and measured as described above.

La1 binding assays to HT29 cells in the presence of La1 rGroEL. Nondifferentiated HT29 cells (10,000 cells/well) were cultured for 5 days as described previously (16). They were equilibrated in acetate buffer, pH 5.0, and incubated in the same buffer containing 10 mg/ml bovine serum albumin (Sigma) for 30 min at room temperature with serial dilutions of rLa1 GroEL starting at 58 $\mu\text{g}/\text{ml}$ (1 nM). La1 bacteria (2.5×10^6 and 5×10^6 /well) labeled with 10 μCi of tritiated adenine/ml (17) were added, and this mixture was incubated for 30 min at the same temperature. Bound La1 was determined by radioactivity counting (17).

Measurement of cytokine secretion in HT-29 cells and macrophages. Cytokine secretion in HT-29 cells and macrophages was determined as described previously (16). A 5-day culture of HT29 cells or 2-h-adhered monocytes were washed twice with serum-free medium before the addition of the La1 recombinant proteins, native or heated at 100°C for 20 min, in the presence or absence of 2% human milk (HM) in DMEM. In some wells, MY4, murine anti-CD14 monoclonal antibody (Coulter Instrumentation Laboratory), and/or control mouse IgG2b Igs (Sigma) were also added at a final concentration of 20 $\mu\text{g}/\text{ml}$. Native or heated LPS from *E. coli* O55:B5 (Sigma) was used as a positive control at different dilutions. Cell viability was determined using a cytotoxicity kit (Roche Diagnostic). Detection of IL-8 release was performed as described previously (53).

Bacterial cell aggregation assays. The aggregation assay was adapted from Ensgraber and Loos (9). Bacteria were harvested and suspended in DMEM adjusted to pH 5.4. The number of bacteria was estimated by measuring the OD_{600} (1 OD unit = 1×10^8 bacteria/ml). The equivalent of 2.5×10^8 *H. pylori*, La1, *S. enterica* serovar Typhimurium, or *E. coli* cells was added to tubes containing 0, 0.1, 1.0, or 10 μg of *H. pylori* or La1 rGroEL proteins/ml of DMEM, pH 5.4, and further incubated for 1 h at 37°C . Sedimented bacteria were suspended by manual shaking and then by vortexing for 20 s at maximal speed to dissolve clumps. Ten microliters was loaded on a microscope slide and protected from drying with a coverslip. GroEL-mediated aggregation was assessed by microscopy (BH-2 Olympus) at a magnification of $\times 100$ and then photographed.

RESULTS

GroEL is present at the surface of *L. johnsonii* La1 and is released in the spent culture medium. To determine whether the GroEL protein of *L. johnsonii* La1 could be detected at the bacterial surface, intact cells from a fresh culture were analyzed by ELISA using an anti-GroEL polyclonal antibody that cross-reacts with La1 GroEL (Fig. 1A). This anti-GroEL antibody reacted strongly with La1, while controls made with the β -lactoglobulin polyclonal antibody showed no immune reactivity. To verify that the presence of GroEL at the surface was not due to cell lysis, the supernatants obtained after coating were checked for the presence of DNA and the enzymatic activity of the intracellular enzyme aldolase. Neither DNA nor

FIG. 1. (A) Localization of GroEL at the surface of La1. The presence of GroEL at the surface of La1 was determined by ELISA as described in Materials and Methods using a polyclonal anti-GroEL antibody as the primary antibody (●). An anti- β -lactoglobulin antibody (○) was used as a negative control. (B) GroEL release during logarithmic phase growth was monitored by Western blotting in La1 supernatants after concentration with 60% ammonium sulfate. The rGroEL protein was used as a positive control.

aldolase activity was detected, indicating that the cell surface location of GroEL was not due to cell lysis and that the bacteria had maintained their integrity on the ELISA plates.

To determine whether GroEL was released from *L. johnsonii* La1 as described for other bacteria (52), La1 spent culture media collected during the logarithmic phase were concentrated by ammonium sulfate precipitation and analyzed by Western blotting (Fig. 1B). Increasing amounts of La1 GroEL accumulated in the supernatant up to 8 h of culture. No traces of DNA or aldolase activity were detected in the supernatants even following a 50-fold concentration of the samples, thus indicating again that bacterial integrity was preserved during culture.

Characterization of the La1 recombinant GroEL protein. To facilitate the study of the role of La1 GroEL, a recombinant His-tagged La1 GroEL protein was prepared in *E. coli*. The recombinant protein was analyzed by nano-ESI mass spectrometry. The peptides generated by tryptic digestion of the recombinant La1 protein showed 90% of protein sequence coverage of the predicted sequence, including the C-terminal end containing the six His residues, with no detectable con-

FIG. 2. Binding properties of La1 GroEL. The capacity of La1 rGroEL (●) to bind mucins (A and B) and to bind to HT29 cells (C and D) was tested at pH 5.0 (A and C) and 7.2 (B and D). Two other La1 recombinant proteins were tested in parallel: rLJ1080 (◆) and rLJ0752 (▲). Bound recombinant molecules were revealed using an anti-His tag antibody. Results are representative of three independent experiments and are expressed as the mean $\pm$ the range of variation ($n = 2$).

tamination. The N-terminus amino acid was identified as alanine after digestion of the protein by endopeptidase Arg-C (data not shown). All of these results were confirmed by the analysis of the intact protein by MALDI-MS. An ion observed at m/z 59,150 corresponding to the protonated protein indicated a molecular mass of 59,149 Da. The latter result confirmed the molecular mass of the predicted protein, 59,120 Da, and the nano-ESI-MS (MS/MS) analysis.

La1 GroEL binds to mucins and intestinal HT29 cells in a pH-dependent manner. To investigate whether La1 GroEL is implicated in the interactions of bacteria with the gastrointestinal mucosa, two sets of experiments were performed using the recombinant protein in combination with anti-His tag monoclonal antibody detection (Fig. 2). The first set monitored the capacity of La1 GroEL to bind to intestinal mucins. The La1 rGroEL showed strong binding to mucins obtained from HT29-MTX cells at pH 5.0 (Fig. 2A). In contrast, no specific binding was observed when the incubations were performed at pH 7.2 (Fig. 2B). Two other La1 recombinant proteins were tested in parallel in the assay: pyruvate kinase (LJ1080), also detected at the surface of La1 (unpublished results); and the lipoprotein LJ0752, one of the two glutamine ABC transporter solute binding proteins known to be located in the outer membrane of *E. coli* (13). rLJ1080 showed similar mucin binding capacity at both pHs tested (Fig. 2A and B), but at pH 5.0, the rLJ1080 binding potential was less than 50% of that of rGroEL (Fig. 2A). rLJ0752 did not bind to mucins at either pH (Fig. 2A and B). The latter result excluded the possibility of unspecific

binding mediated by the His tag present on the recombinant proteins.

The second set of experiments analyzed the capacity of La1 rGroEL to bind to HT29 cells (Fig. 2C and D). The binding pattern of rGroEL to HT29 was similar to that observed with

FIG. 3. La1 binding assays to HT29 cells in the presence of La1 rGroEL. Nondifferentiated HT29 cells were incubated with increasing concentrations of La1 rGroEL followed by a second incubation with 2.5×10^6 ^3H -adenine-labeled La1 bacteria/well. Bound La1 cells were determined by radioactivity counting. Results are expressed as percentage of binding in the absence of La1 rGroEL (mean $\pm$ standard deviation; $n = 6$).

FIG. 4. Stimulation of IL-8 secretion from HT29 cells by La1 rGroEL. IL-8 release from HT29 cells was measured by ELISA after stimulation for 24 h with increasing concentrations of La1 GroEL or LJ1080 recombinant proteins or *E. coli* LPS in the absence (open bars) or presence (solid bars) of 2% HM as a source of sCD14. DMEM with or without HM was used as a negative control. Where indicated (dotted bars), cells were simultaneously treated with anti-CD14 (MY4) or IgG2b antibodies or heat treated (ϕ). IL-8 secretion was measured in triplicates, and the results are expressed as the mean $\pm$ standard deviation. The figure shows one representative experiment of two independent experiments.

mucins: i.e., strong at pH 5.0 and weak at pH 7.2. The interaction of rLJ1080 with HT29 cells was similar to that of rGroEL (Fig. 2C and D), while rLJ0752 showed no binding to HT29 cells at either pH as observed for mucins.

Influence of La1 rGroEL on the binding of La1 to HT29 cells. To determine whether La1 rGroEL was able to interfere with the binding of La1 to cells like intestinal HT29 cells, we performed the binding assays in the presence of increasing amounts of La1 rGroEL. La1 rGroEL induced a decrease of La1 binding from 50 to 35% at concentrations ranging between

0.9 and 3.6 $\mu\text{g/ml}$, followed by an increase up to 141% at a concentration of 14.4 $\mu\text{g/ml}$ (Fig. 3).

La1 rGroEL stimulates IL-8 secretion in intestinal HT29 cells. To investigate whether La1 rGroEL was able to interact with intestinal cells, we used the well-documented nondifferentiated human cell line HT29 known to secrete IL-8 in a soluble CD14 (sCD14)-dependent manner (31) and previously used by us to determine the response to La1 elongation factor Tu (EF-Tu) (16). La1 rGroEL induced a 4.2-fold increase of IL-8 secretion when added at a concentration of 10 $\mu\text{g/ml}$, but

FIG. 5. Stimulation of IL-8 secretion from macrophages by rGroEL proteins from different bacteria. Macrophages were isolated from healthy donors as described in Materials and Methods. IL-8 release was measured by ELISA after stimulation for 24 h with 1 $\mu\text{g/ml}$ rGroEL proteins from different bacteria or 1 ng/ml *E. coli* LPS. Open and solid bars represent two different donors. Where indicated, cells were simultaneously treated with anti-CD14 (MY4) or IgG2b antibodies or heat treated (ϕ). The figure shows one representative experiment of four independent experiments using macrophages from two different donors. La1, *L. johnsonii* NCC 533; Lhe, *L. helveticus* ATCC 15009; Bsu, *B. subtilis* NCC 199; Lla, *Lactococcus lactis* strain MG 1363; Hp, *H. pylori* strain P1. RPMI, medium alone used as a negative control.

only in the presence of human milk used as a source of sCD14 (Fig. 3), thus demonstrating that the La1 rGroEL-mediated increase of IL-8 is also CD14 dependent. The involvement of sCD14 was confirmed by the fact that IL-8 secretion returned to basal levels when La1 rGroEL was incubated in the presence of MY4 anti-CD14 antibodies. In contrast to that observed with *E. coli* LPS used as a positive control, heating of La1 rGroEL at 100°C completely abolished the GroEL-mediated increase in IL-8 secretion, indicating that the stimulation was not due to LPS contamination. Indeed, the amount of LPS of *E. coli* origin present in the recombinant protein preparation was determined as 1.8 ng per 10 µg of protein, which is not sufficient to induce the observed stimulation in IL-8 secretion. The stimulatory potential of La1 rGroEL was determined to be approximately 100 times lower than that of LPS. Even if rLJ1080 showed similar binding capacity to HT29 cells to rGroEL (Fig. 2B), it was unable to stimulate IL-8 secretion in the presence or absence of sCD14 (Fig. 3).

Bacterial GroEL proteins stimulate IL-8 secretion by isolated blood macrophages. The immunostimulatory capacity of La1 rGroEL was tested in macrophages purified from the blood of healthy donors (Fig. 5). Addition of 1 µg of La1 rGroEL protein resulted in 27- and 19-fold increases in IL-8 secretion in macrophages obtained from two different donors. The increase in IL-8 secretion was on the same order of magnitude as that induced by 1 ng of *E. coli* LPS used as a positive control. As observed when using HT29 cells, the presence of anti-CD14 antibodies completely blocked the induction of IL-8 secretion in response to either La1 rGroEL or *E. coli* LPS in macrophages. The immunostimulatory capacity of La1 rGroEL was compared to those of the rGroEL proteins from the three gram-positive bacteria *L. helveticus*, *B. subtilis*, and *L. lactis* and from the gram-negative bacterium *H. pylori*, after expression in *E. coli* and purification. Incubation of macrophages with 1 µg of the above rGroEL proteins resulted in an induction of IL-8 secretion similar to that observed with La1 rGroEL (Fig. 5). Addition of anti-CD14 antibodies blocked the induction of IL-8 secretion produced by the rGroEL obtained from gram-positive bacteria but did not alter the *H. pylori* rGroEL-mediated IL-8 increase. Heat treatment abolished the ability of all rGroEL proteins to induce IL-8, but as expected, it did not affect the stimulation of IL-8 secretion by *E. coli* LPS, thus indicating once more that the observed activity of the different rGroEL proteins was not due to endotoxin contamination.

La1 rGroEL induces aggregation of the gastric pathogen *H. pylori*. To investigate whether La1 rGroEL was implicated in the aggregation of *H. pylori*, bacterial cells were incubated with different concentrations of La1 rGroEL and aggregation was recorded. Incubation of *H. pylori* cells with 0.1 µg/ml of La1 rGroEL protein for 1 h induced a strong aggregation of the bacteria compared to that of *H. pylori* cells in the absence of La1 rGroEL (Fig. 6A). In contrast, La1 rGroEL was unable to induce “homologous” aggregation of La1 bacteria at 0.1

µg/ml (Fig. 6A) or even at a 100-fold concentration, 10 µg/ml (data not shown). Indeed, no difference was observed compared to La1 control cells. *H. pylori* rGroEL was unable to mediate any “homologous” *H. pylori* cell aggregation up to a concentration of 1 µg/ml or any visible aggregation of La1 bacteria at 1 µg/ml (Fig. 6A) or 10 µg/ml (data not shown). La1 rGroEL-mediated aggregation was not observed with either *S. enterica* serovar Typhimurium SL1344 or *E. coli* E 2348/69 at 0.1 µg/ml (Fig. 6B) or even at a 100-fold concentration, 10 µg/ml (data not shown). When testing the rGroEL proteins from the three other gram-positive bacteria, no aggregation of La1 cells was detected, while all induced *H. pylori* aggregation. We tested all rGroEL proteins on *H. pylori* cells in parallel at the concentrations of 0.1, 1, and 10 µg/ml (data not shown) and established a visual scale to compare their aggregation potentials: La1 rGroEL > *L. helveticus* rGroEL = *B. subtilis* rGroEL > *L. lactis* rGroEL.

DISCUSSION

We illustrate in this paper the localization of GroEL at the surface of *L. johnsonii* La1 and its capacity to attach to mucus and epithelial cells. The GroEL or Hsp60 class of proteins does not possess a secretion signal sequence or other recognizable motifs suggesting their secretion, nor does it possess a motif involved in anchoring of the protein to the outer membrane or cell wall. However, GroEL has been detected at the cell surface of many pathogenic bacteria (8–11, 14, 24, 39, 52), but to our knowledge, this is the first report describing the presence of GroEL at the surface of a lactic acid bacterium. We have recently shown that EF-Tu, which also possesses no identifiable secretion signal sequence to explain its translocation across membranes, is also present at the surface of La1 (16), indicating that GroEL is not the only “intracellular” protein of La1 that can be found associated with the cell wall of the bacterium. Indeed, EF-Tu like GroEL was originally thought to be restricted to the cytoplasm of bacteria but was further described as “an envelope-associated protein that can be released from the cell by osmotic shock” of sake spoilage lactobacilli (37). We have also found evidence of the presence of GroEL in the spent culture medium throughout the logarithmic growth phase. The lack of DNA or aldolase enzyme activity in the spent culture medium indicated that this is not due to the release of cytoplasmic components by nonspecific mechanisms such as autolysis, but rather occurs because some proteins are selectively transported to the bacterial surface and released into the supernatant. This result is not unexpected as it has been determined that a specific and selective mechanism or mechanisms are involved in the secretion of some *H. pylori* antigens, including GroEL (52).

The La1 GroEL was produced in *E. coli*, and the recombinant protein was used for further functional experiments. The comparison of the sequence and molecular mass of the recom-

FIG. 6. La1 rGroEL aggregating capacity. *H. pylori* and La1 (A) as well as *S. enterica* serovar Typhimurium SL1344 and *E. coli* E 2348/69 (B) in DMEM, pH 5.4, were incubated with no rGroEL as a control, with 0.1 µg of La1 rGroEL/ml, or with 1 µg of *H. pylori* rGroEL for 1 h at 37°C as indicated to the left of the panels. After elimination of bacterial clumps by manual shaking and vortexing, 10 µl of the preparation was loaded on a microscope slide and GroEL-mediated aggregation was assessed under microscope at a ×100 magnification and then photographed.

binant protein with those of the predicted protein confirmed authenticity. Our in vitro binding studies suggest that La1 GroEL might contribute to La1 attachment to mucus and/or intestinal cells in the host environment. Binding of rGroEL to mucins or intestinal cell lines was pH dependent: while a strong binding capacity was observed at pH 5.0, no binding occurred at pH 7.2. This is not a unique feature of GroEL. The pH dependency of this process had been observed not only for other mucus binding proteins (45), including La1 EF-Tu (16), but also for the attachment of intact lactobacilli to Caco-2 cells (2, 18). In particular, Blum et al. (2) showed that La1 adhesion to Caco-2 cells was pH dependent and higher at pH 5.0 than at pH 7.2. It has been postulated that the pH of the gut lumen is neutral but becomes gradually more acidic at the mucus-covered surface due to the sialic acid residues and sulfated content of the mucin. Binding at pH 5.0 would thus be more representative of physiological conditions (2). To further study the implication of GroEL in La1 attachment to components of the gastrointestinal mucosa, we performed binding assays to HT29 cells in the presence of La1 rGroEL. La1 rGroEL inhibits the binding of La1 to HT29 cells at concentrations ranging between 0.9 and 3.6 $\mu\text{g/ml}$ but promotes binding at higher concentrations. This increase in binding at a high concentration of rGroEL could be due to "homologous" aggregation of La1 in the presence of intestinal cells, a phenomenon not observed in the absence of intestinal cells. This would be similar to what has been described for *S. enterica* serovar Typhimurium GroEL, which induces aggregation of the bacterium only in the presence of colonic mucus (9).

Heat shock proteins from eukaryotic organisms (29, 30) and gram-negative bacteria (15, 30, 41, 46) are known to stimulate macrophages and gastric cells (56). We demonstrate here that rGroEL from the gram-positive bacterium *L. johnsonii* La1 is able to stimulate cytokine secretion in epithelial cells and macrophages by a CD14-dependent mechanism. Pyruvate kinase (LJ1080), another surface-exposed La1 protein, showed no stimulation of IL-8 secretion, indicating that this stimulatory property is not common to all La1 proteins present at the bacterial surface and able to bind to intestinal cells. All the other rGroEL proteins tested were shown to be as active as La1 rGroEL in the stimulation of IL-8 secretion from macrophages. La1 rGroEL, together with the rGroEL obtained from the three other gram-positive bacteria, exhibited a CD14-dependent mechanism as already described for human and chlamydial Hsp60 (30). Most of the Hsp60 proteins induce the production of proinflammatory cytokines by the monocyte-macrophage system via CD14/Toll-like receptor 2 (TLR2) and CD14/TLR4 receptor complexes leading to activation of NF- κ B (47). Interestingly, we observed that activation of IL-8 secretion by *H. pylori* rGroEL is independent of the presence of CD14, as described for *E. coli* GroEL (46). This in accordance with recent studies showing that *H. pylori* GroEL (or Hsp60) stimulates IL-6 secretion from macrophages through a mechanism that is independent of TLR2, TLR4, and myeloid differentiation factor 88 (15). In any case, all of the data obtained concerning the stimulation of IL-8 release by different rGroEL proteins reinforce previous work suggesting that heat shock proteins from different organisms may bind to different receptor complexes (21).

As mentioned before, *S. enterica* serovar Typhimurium GroEL participates in the aggregation of the bacterium to colonic mucus (9). Actually, we have demonstrated that both a fermented milk containing La1 (38) and a whey-based La1 culture supernatant (35) are able to decrease *H. pylori* infection levels in humans and also the number of *H. pylori* cells associated with the human gastric mucosa as determined by biopsy analysis (38). Therefore, we have hypothesized that La1 GroEL, through the aggregation of *H. pylori*, might contribute to the decrease of bacterial load by facilitating clearance of this pathogen during mucus flushing. We demonstrate in this article that La1 rGroEL does mediate aggregation of the gastric pathogen *H. pylori*. In contrast to the finding observed with *S. enterica* serovar Typhimurium GroEL (9), the presence of mucins is not required for the La1 rGroEL-mediated aggregation of *H. pylori* in vitro (Fig. 6). Furthermore, this effect on *H. pylori* seems to be specific as La1 rGroEL cannot aggregate the other gram-negative pathogenic bacteria tested, including *S. enterica* serovar Typhimurium (Fig. 6).

Even though all the gram-positive bacterial rGroEL proteins tested do aggregate *H. pylori*, we have observed some differences in their aggregation capacities. This may constitute another criterion for the selection of the most suitable probiotic to manage *H. pylori* infection.

We have shown here that La1 GroEL has a proinflammatory activity and that it is able to aggregate *H. pylori* cells in vitro. Even though at least two proinflammatory La1 proteins, GroEL and EF-Tu (16), are present at the bacterial surface, intact La1 bacteria do not stimulate IL-8 secretion from intestinal epithelium-like HT29 cells (54) and polarized Caco-2 cells (22) in the presence of sCD14 or peripheral blood mononuclear cells (1) in vitro. The global interpretation could be that the intestinal epithelial cells sense the bacterial surface with its proinflammatory components, like GroEL and EF-Tu, and its anti-inflammatory components, like lipoteichoic acid (53), process the information, and respond with a "consensus" pro- or anti-inflammatory response. In this sense, in vivo shedding of lipoteichoic acid from the La1 surface as a consequence of the stomach's acidic milieu may result in the exposure of the surface-associated proinflammatory molecules, thus favoring the activation of innate defenses. Unfortunately, the available clinical studies with La1—where cytokines were measured in biopsies from the gastrointestinal mucosa—were performed in patients with chronic inflammation (unpublished data) and therefore do not provide any support for our hypothesis.

We have never observed aggregation of *H. pylori* cells by intact La1 bacteria in vitro (unpublished results). The maximal amount of GroEL found in the La1 supernatants was 0.15 $\mu\text{g/ml}$ (representing 0.03% of the total GroEL content of the bacterial cell). This amount is not sufficient to induce *H. pylori* aggregation. In vivo, we have never observed persistent and live La1 cells in the gastric mucosa; therefore, we suggest that the acidic pH of the stomach will concomitantly induce the expression of the stress-sensitive GroEL and increase La1 death. This might finally result in the release of intracellular GroEL in large amounts, thus favoring *H. pylori* clearance due to its aggregating capacity as shown in vitro in this paper.

In any case, further studies will be required to determine the exact interplay of the La1 surface-exposed molecules within the gastrointestinal environment.

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