

Oral Immunization of Mice with Lactic Acid Bacteria Producing *Helicobacter pylori* Urease B Subunit Partially Protects against Challenge with *Helicobacter felis*

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Background. The development of an efficacious vaccine against infection with *Helicobacter pylori*, the causative agent of chronic gastritis, peptic ulcer disease, and gastric adenocarcinoma, remains a challenge. Since the use of mucosal adjuvants is limited in human application, we have evaluated the potential of recombinant *Lactobacillus* strains producing *H. pylori* urease B (UreB) subunit to deliver this antigen to the gastrointestinal tract.

Methods. Mice were injected orally 3 times with a triple dose of recombinant *Lactobacillus plantarum* NCIMB8826, the recombinant isogenic cell-wall mutant (alr⁻ MD007 strain) expressing UreB, or a mixture of recombinant UreB and cholera toxin (rUreB/CT) as a control. Urease-specific seric immunoglobulin (Ig) G and IgA were measured by use of an enzyme-linked immunosorbent assay. After challenge with *Helicobacter felis*, stomach infection was examined by use of the rapid urease test and by polymerase chain reaction detection of *Helicobacter* genomic DNA.

Results. Intragastric immunization with both recombinant *Lactobacillus* strains and rUreB/CT elicited UreB-specific antibodies. After challenge, reduction of *H. felis* load in the stomachs of mice was observed only after immunization with the recombinant mutant strain MD007 or with rUreB/CT.

Conclusions. This is the first report of successful induction of partial protection against *H. felis* with a mucosal prime-boost regimen in which recombinant *Lactobacillus* strains were used as antigen-delivery vehicles.

Helicobacter pylori is recognized as a human-specific gastric pathogen that colonizes the stomachs of at least half of the world's population. Most infected individuals are asymptomatic, although, for a significant num-

ber, infection is associated with the development of duodenal and gastric ulcers and gastric cancers [1]. That natural immunity appears to be inadequate for clearing the infection questions the feasibility of producing an effective *H. pylori* vaccine [2]. Intragastric immunization with *H. pylori* virulence factors in combination with mucosal adjuvants, such as bacterial toxins, has been proven to eradicate the pathogen [3–6] and to prevent reinfection [7] in animal models. Notably, the urease B (UreB) subunit of urease, which exhibits substantial cross-reactivity between *Helicobacter* strains and species, has been extensively used as a protecting antigen in combination with cholera toxin (CT) or *Escherichia coli* heat-labile toxin [8, 9]. However, toxin-based adjuvants lead to numerous adverse effects, which preclude their applicability in humans [10]. Other approaches based on the use of attenuated *Salmonella* strains [11, 12] or nanoparticles [13] triggered a val-

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uable degree of protection in mouse models, yet efficacy in humans remains to be established. A key issue is the nature of the immune response required to eliminate *Helicobacter* infection; ambiguities remain with regard to the dispensability of antibody responses [14–16]. Antigen delivery should be designed to stimulate mucosal immunity with, possibly, a Th2 bias in the absence of proinflammatory adverse effects, because the early induction of a Th1 response causes damage in the host and is ineffective at clearing infection in humans [17].

Prevention of attachment of the pathogen and subsequent colonization at mucosal surfaces is best achieved when immunity is triggered after local stimulation. Different delivery systems satisfying this requirement are currently under development, with various avenues being explored for oral administration [18]. One such delivery system is based on live bacterial vectors, including nonpathogenic, noninvasive lactic acid bacteria (LAB) strains (which are generally recognized as safe) [19]. These carriers do not induce pronounced proinflammatory responses [19, 20], which renders them best suited for immunocompromised subjects, infants, and elderly individuals [21]. In the present study, the *Lactobacillus plantarum* NCIMB8826 strain was chosen for its capacity to resist passage through the stomach and to persist in mice for up to 1 week [22, 23]. Further, intragastric immunization using the recombinant strain expressing the potent immunogen tetanus toxin fragment C (TTFC) elicited both humoral and local protective antibody responses [24]. A mutant of *L. plantarum* NCIMB8826 (*alr*⁻ MD007 strain) affected in its cell-wall composition as a result of the inactivation of the alanine racemase (*alr*) gene [25] led to substantially enhanced immune responses in the same TTFC model [26].

In this article, we assess the potential of both the wild-type (*wt*) *L. plantarum* NCIMB8826 strain and the isogenic *alr*⁻ MD007 strain producing *H. pylori* UreB as oral delivery vehicles in a mouse model of *H. felis* infection. In prophylactically treated mice, although both recombinant strains induced seric UreB-specific antibody to levels similar to those elicited by intragastric administration of recombinant UreB (rUreB)/CT, the recombinant *alr*⁻ MD007 mutant was more potent at reducing *H. felis* load than was the recombinant NCIMB8826 strain, as assessed by rapid urease test (RUT) and polymerase chain reaction (PCR) screening. We conclude that the recombinant *alr*⁻ MD007 mutant is a valid vaccine vehicle to trigger partial mucosal protection against *H. felis* used as a model gastric pathogen.

MATERIALS AND METHODS

Construction of recombinant *Lactobacillus* strains. The 1700-bp DNA fragment coding for UreB was amplified from the pTCP3 plasmid by PCR [27] and cloned into *Sfr*I-restricted

PCRscript vector (Stratagene), yielding pMEC120. The *ureB* insert was next cloned into a derivative of pGIT032 [28] previously cleaved with *Nco*I and *Pvu*II, yielding pMEC142 carrying the UreB-coding sequence under the control of the strong constitutive L-lactate dehydrogenase promoter of *L. plantarum*. Cloning intermediates were amplified in *E. coli* XL1Blue and JM110, and the final construct (pMEC142) was introduced in the *E. coli* strain M15. The pTG2247 plasmid [29] devoid of urease-coding sequence was used as a control. Transformation of pMEC142 plasmids into the *L. plantarum* NCIMB8826 strain [29] and the *alr*⁻ MD007 mutant [25] was performed as described by Josson et al. [30]. Production of UreB from the pMEC142 construct was targeted to the bacterial cytoplasm.

Bacterial strains and growth conditions. *L. plantarum* NCIMB8826 carrying pMEC142 (referred to as “8826/pMEC142”) was grown at 37°C in Man-Rogosa-Sharpe (MRS) broth (Difco) supplemented with 5 µg/mL erythromycin. *L. plantarum* NCIMB8826 carrying pTG2247 (referred to as “8826/pTG2247”) was grown at 37°C in MRS broth supplemented with 10 µg/mL chloramphenicol. Cultures were grown to midexponential phase (OD₆₀₀ of 0.8–1.0), and bacteria were collected by centrifugation (4000 g for 10 min), washed twice in PBS, and resuspended at a final concentration of 1 × 10¹⁰ cfu/mL in PBS or gavage buffer (0.5% casein hydrolysate, 0.2 mol/L sodium bicarbonate, and 0.5% glucose in PBS) for systemic or intragastric immunization, respectively. The *L. plantarum* *alr*⁻ MD007 mutant carrying pMEC142 (referred to as “MD007/pMEC142”) was grown in MRS broth supplemented with 5 µg/mL erythromycin and 200 µg/mL D-alanine (D-Ala). Before administration in mice, bacteria were starved for 4 h in the absence of D-Ala, which does not lead to growth arrest or a drop in viability [25]. Bacterial counts and resuspension were performed as they were for 8826/pMEC142 and 8826/pTG2247.

E. coli strains were grown in Luria broth medium containing 100 µg/mL ampicillin at 37°C under aeration. *H. felis* strain ATCC 49179 was grown biphasically under microaerophilic conditions at 37°C, as described elsewhere [7].

Structural stability of the recombinant pMEC142 plasmid in vitro. The 8826/pMEC142 strain was subcultured in selective medium (MRS broth supplemented with 5 µg/mL erythromycin). Ten successive cultures were performed by inoculating 10 mL of fresh medium with 10 µL of the previous culture every 24 h. Bacterial cells from cultures after 1, 5, and 10 passages were recovered and subjected to immunoblot analysis, as described below. Plasmid pMEC142 was extracted from 2 independent colonies originating from passages 1, 5, or 10 and served as a source of DNA for PCR and restriction analyses.

Western-blot assay. A total of 1 × 10⁸ recombinant bacteria (*L. plantarum* and *E. coli*) were lysed in reducing SDS-containing buffer [31], separated by PAGE, and transferred onto blotting membrane. After blocking for 1 h at 37°C with PBS–

0.5% Tween 20 (PBS-T) containing 5% skimmed milk (SM), detection of UreB was performed with specific monoclonal antibody (1:500 dilution) [32] in conjunction with alkaline phosphatase-conjugated rabbit anti-mouse IgG diluted 1:3000. UreB was detected by use of rabbit antiserum (1:2000 dilution in PBS-T containing 0.5% SM) [33], followed by horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:3000 dilution; Sigma).

Antigens. Recombinant UreB was obtained from *E. coli*, as described elsewhere [5]. CT was purchased from Calbiochem. Recombinant urease holoenzyme [7] or *H. felis* lysate was used to determine specific seric IgG and IgA antibody titers by use of an ELISA.

Systemic immunization. Eight-week-old female C57/Bl6 mice (Charles River Laboratories) were immunized by subcutaneous (sc) administration of UreB-producing *L. plantarum* 8826/pMEC142 or *E. coli* M15/pMEC142. Mice (groups of 5) received a single dose of 1×10^9 cfu of each recombinant strain 3 times, at 2-week intervals. Serum samples were recovered 7 days after the first and second booster injections.

Intragastric immunization and infection with *H. felis*. Specific pathogen-free female BALB/c mice were housed in microisolator cages, with free access to water and chow, as approved by the local veterinary office. Groups of 10 mice were injected intragastrically with 1×10^9 cfu of recombinant *Lactobacillus* strains in 200 μ L of gavage buffer 3 times for 3 consecutive days, at 3-week intervals (figure 1). Control mice were injected with MRS broth alone or with rUreB/CT (50 μ g/10 μ g). Feces were collected to examine the presence of recombinant strains and their capacity to maintain production of UreB. Twelve days after the last immunization, mice were infected with 5×10^7 freshly cultured *H. felis* by orogastric intubation under light anesthesia with isofluran (Baxter); this guarantees >90% infection in unvaccinated mice [7]. Mice were killed 2 weeks later, and stomachs were collected to examine the degree of protection.

Measurement of serum antibodies. Maxisorp immunoplates (Nunc) were coated overnight at 4°C with 0.5 μ g of recombinant urease holoenzyme or 1 μ g of *H. felis* lysate [34]. Mouse serum dilutions in Tris-buffered saline-T:0.5% SM were incubated for 2 h at 37°C. Urease-specific antibodies were detected by use of either rabbit anti-IgG or goat anti-IgA (α -chain specific) coupled with HRP (Sigma). Antibody dilutions yielding absorbance values 2-fold higher than those yielded by the preimmune serum were used to establish end-point titers.

Quantitative analysis of *H. felis* colonization. In method 1, the presence of *H. felis* in gastric tissue was assessed by use of RUT (Jatrox-test; Procter & Gamble) [5]. The cut-off value of RUT used to discriminate between infection and cure corresponded to the mean \pm 2 SDs of the absorbance values obtained with gastric tissues from naive mice. In method 2, 20 mg of stomach sample was cut into small pieces and homogenized in 180 μ L of ATL buffer (Qiagen). After digestion with 1.5 mg/mL proteinase K for 30 min at 55°C, 200 μ L of AL buffer (Qiagen) was added. The mixture was incubated for 10 min at 70°C, 200 μ L of 100% ethanol was added, and the precipitate was cleared by centrifugation. The lysate was extracted twice with phenol-chloroform (1:1), and the DNA was precipitated with 2.5 vol of ethanol before washing, drying, and final resuspension in water. A specific pair of primers for *H. felis* *flaB* (5'-TTCGATTGGTCCTACAGGCTCAGA-3' [sense] and 5'-TCTTGTGATGACATTGACCAACGCA-3' [anti-sense]) was combined with 1 μ g of DNA template, and real-time PCR amplification was performed as described by Stoicov et al. [35]. Standards were obtained accordingly, allowing for quantification of bacteria per microgram of gastric tissue [35].

Cytokine/chemokine expression in stomach samples. Total stomach RNA was extracted and reverse transcribed, as described elsewhere [36]. The cDNA was amplified by PCR with primers specific for tumor necrosis factor (TNF)- α , monocyte chemoattractant protein (MCP)-1, and RANTES. The samples were subjected to 40 PCR cycles, consisting of denaturation

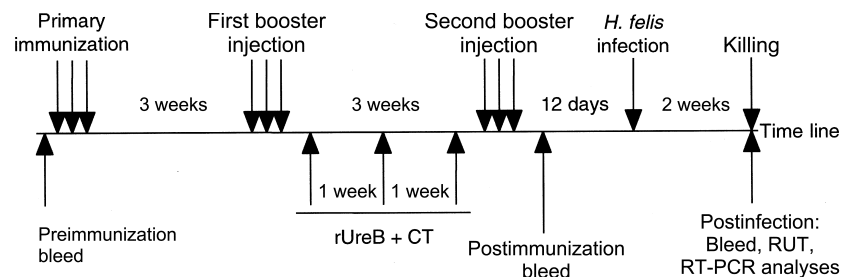


Figure 1. Experimental setting for the prophylactic immunization of mice with recombinant *Lactobacillus* strains (upper part of the time line). Priming and boosting injections were given at 3-week intervals. Control immunization was performed with recombinant urease B (rUreB) combined with cholera toxin (CT) (lower part of the time line). UreB-specific antibodies were measured before immunization, after the second booster injection, and after infection with *Helicobacter felis* at the time of killing of the mice. Presence of *H. felis* was examined by rapid urease test and real-time polymerase chain reaction (RT-PCR) analyses of genomic DNA recovered from the stomachs of mice.

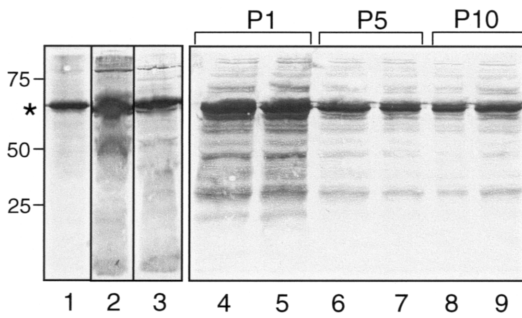


Figure 2. Production of urease B (UreB) and plasmid structural stability in the *Lactobacillus plantarum* NCIMB8826 strain. Production of UreB was assessed by use of whole bacterial lysates, separation by SDS-PAGE, and immunodetection. Lane 1, Five micrograms of purified *H. pylori* recombinant UreB; lane 2, *Escherichia coli* strain M15 carrying vector pMEC142; lane 3, *L. plantarum* 8826/pMEC142; lanes 4–9, UreB production by 8826/pMEC142 (2 independent clones) assayed after 1, 5, and 10 passages (Ps), respectively. The asterisk indicates UreB-specific signals.

(for 1 min at 94°C), annealing (for 1 min at 56°C–60°C), and extension (for 1 min at 72°C). Visualization and quantification of PCR products were performed as described elsewhere [37], and results were expressed as ratios to the groups given gavage buffer by use of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), for standardization between samples.

Statistical analysis. Antibody titers and *H. felis* copy numbers are reported as means ± SDs and were compared by use of Student's *t* test. RUT values were compared by use of the Mann-Whitney *U* test. *P* < .05 was considered to be statistically significant for differences between groups.

RESULTS

UreB production level and structural stability of the *L. plantarum* strains in vitro. We first evaluated the amount of UreB produced in vitro by the 8826/pMEC142 strain and compared it with that produced by positive controls consisting of 5 μg of purified rUreB and that produced from the pMEC142 plasmid in *E. coli* strain M15. By immunoblot analysis, a band of similar intensity was observed in all 3 lanes (figure 2, lanes 1–3), demonstrating that full-length UreB can be produced intracellularly in the NCIMB8826 strain with a yield close to that produced in *E. coli* (≥5 μg/1 × 10⁸ bacteria). As illustrated in figure 2, similar levels of UreB were observed by immunodetection along a time course covering 10 successive culture passages in selective medium, arguing for structural stability of the expression plasmid. PCR analysis of plasmid pMEC142 extracted from 2 independent clones confirmed that the plasmid was not rearranged during subculturing of the strain (data not shown). The segregational stability of the recombinant strains was examined by inoculating them at a 1:400 dilution in nonselective medium. After ~25

generations, >20% of the cells reaching late stationary phase retained the plasmid (data not shown).

Evaluation of the immunogenicity of the recombinant 8826/pMEC142 strain. Before conducting intragastric immunization, we first examined whether the amount of UreB produced by the model strain 8826/pMEC142 was sufficient to trigger a measurable antibody response when administered sc to mice. Injection of 1 × 10⁹ cfu resulted in UreB-specific IgG titers well above background levels obtained with sham-injected mice (figure 3). Specific antibody titers were not improved by the concomitant addition of alum as an adjuvant (data not shown). Mice injected with recombinant *E. coli* producing UreB exhibited 5-fold higher titers (figure 3), which is consistent with the higher intrinsic adjuvanticity of this expression host.

Intragastric immunization with recombinant *L. plantarum* strains and IgA and IgG responses. As outlined in figure 1, intragastric administration of recombinant *L. plantarum* strains was performed 3 times on 3 consecutive days. For practical reasons, priming and boosting injections were done with bacteria from the same culture batch kept in gavage buffer for 2 days at 4°C. No viability loss was observed for the recombinant *wt* strains after 48 h of storage, yet viable counts were reduced by 1 log for the MD007 strain at 48 h (data not shown). Notably, the relative abundance of UreB in bacterial lysates did not decrease during storage and was equivalent to 10 μg of rUreB (figure 4A). Previous experiments have shown that the survival capacities of the NCIMB8826 and MD007 strains in the mouse

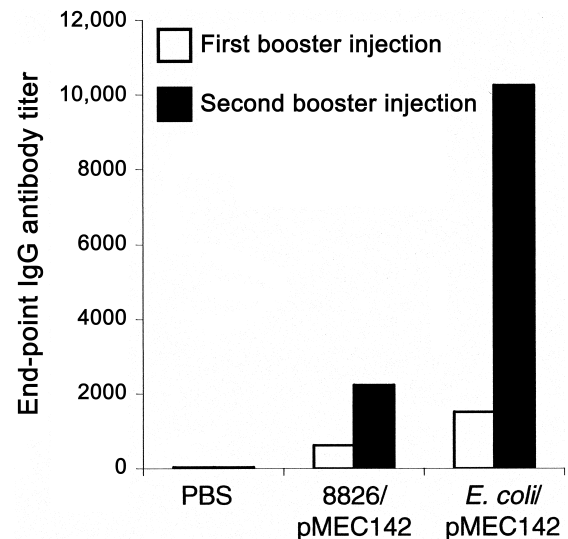


Figure 3. Capacity of *Lactobacillus plantarum* 8826/pMEC142 to deliver urease B (UreB) in vivo. Mice were injected subcutaneously, and serum IgG antibody titers specific for UreB were established by end-point dilutions (reciprocal of the last dilution giving an optical density higher than 2 times the background) after the first and second booster injections. Negative and positive controls included PBS and *Escherichia coli* carrying vector pMEC142, respectively.

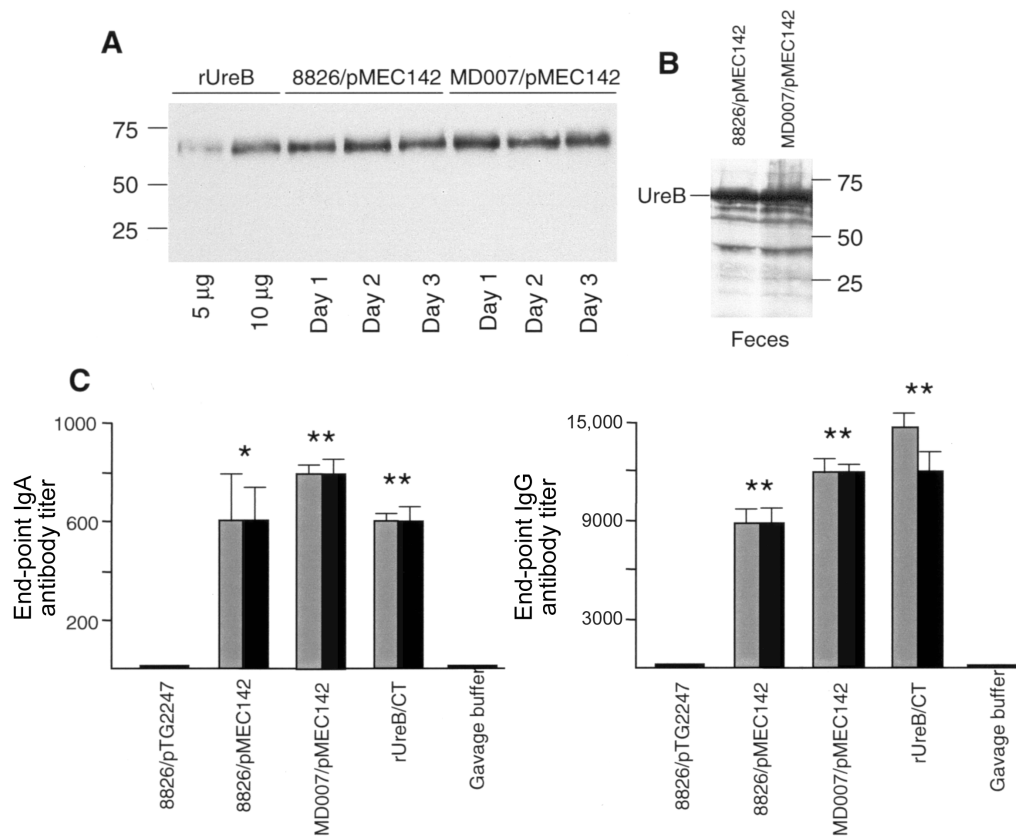


Figure 4. A, Western-blot analysis of urease B (UreB) production by the *Lactobacillus plantarum* 8826/pMEC142 and MD007/pMEC142 strains. A total of 100 μg of bacterial lysate was loaded per lane; 5 and 10 μg of purified recombinant UreB (rUreB) were loaded alongside to permit quantification. Days 1, 2, and 3 indicate the UreB content of cell lysates from bacteria administered at days 1, 2, and 3 of the first booster injection. Molecular weight markers are shown on the left side of the panel. B, Western-blot analysis of UreB production by 8826/pMEC142 and MD007/pMEC142 recovered from mouse feces, performed as described in panel A (1 representative colony of 10). C, Determination of anti-UreB-specific IgA and IgG responses after immunization. Serum titers were established by end-point dilutions after immunization (gray bars) and after infection with *Helicobacter felis* (black bars). Data were averaged from 5 mice randomly picked per group and are expressed as means ± SDs. * $P \leq .01$, by comparison with gavage buffer; ** $P \leq .001$, by comparison with gavage buffer. 8826/pMEC142, *L. plantarum* NCIMB8826 producing UreB; 8826/pTG2247, recombinant *L. plantarum* NCIMB8826 not producing UreB; gavage buffer, negative control; MD007/pMEC142, *alr*⁻ MD007 mutant producing UreB; rUreB/CT, a mixture of rUreB and cholera toxin serving as positive control.

gastrointestinal (GI) tract were equivalent (data not shown). We further verified that colonies recovered from feces continued to produce UreB (figure 4B).

The production of specific seric IgA and IgG antibodies indicated that mucosal delivery of the antigen was successful [38]. We thus evaluated production of these classes of antibodies to assess whether UreB delivered by *L. plantarum* was efficiently recognized in the GI tract. Mice that received the 8826/pMEC142 or MD007/pMEC142 strain or rUreB/CT intragastrically produced significant ($P < .01-.001$) levels of UreB-specific IgA, compared with preimmune mice (figure 4C, gray bars). Infection with *H. felis* did not induce production of more antibody during the 2 weeks preceding euthanasia of the mice (figure 4C, black bars). Levels of specific IgA barely exceeded background levels in groups receiving the 8826/pTG2247 strain or

gavage buffer. A similar response pattern was seen when production of UreB-specific IgG was assessed. We thus explored whether the onset of immune responses by recombinant LAB would lead to protection in a mouse model of *H. felis* infection.

Protection against *H. felis* infection in mice. Protection was evaluated 2 weeks after infection of immunized mice with *H. felis*, by use of RUT. The stomachs of mice given the *L. plantarum* MD007/pMEC142 mutant strain showed slower color development, compared with the stomachs of mice given the *wt* 8826/pMEC142 strain. This correlated with end-point values measured at 3 h, which showed a significant difference ($P < .05$) in the case of only MD007/pMEC142 (figure 5A). No reduction in *H. felis* load in the stomachs of mice was observed upon intragastric administration of the 8826/pTG2247 control strain or gavage buffer. As a validation of the immunization and

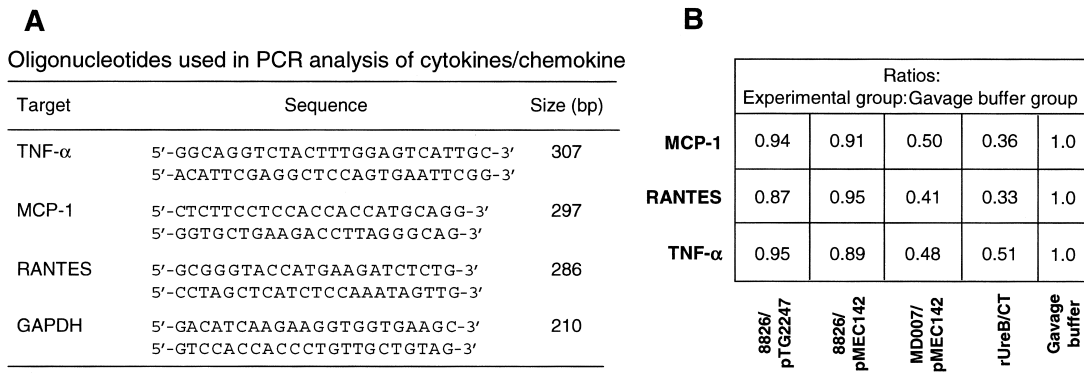


Figure 6. A, Sequences of primers used for real-time polymerase chain reaction (RT-PCR) amplification: tumor necrosis factor (TNF)- α , monocyte chemoattractant protein (MCP)-1, RANTES, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). B, Relative mRNA expression, determined by RT-PCR, in the stomachs of immunized mice and in those of control mice. Ratios were obtained by dividing the mean intensities of 6 individual samples in each immunization group with the mean level of expression measured in mice given gavage buffer. Quantification of fluorescent DNA signals in agarose gels was performed by use of the software Quantify1D (Advanced American Biotechnology).

vehicle by different mucosal routes by use of TTFC as antigen [24, 42, 43]. Cell-wall mutants of this carrier that exhibited improved antigen-delivery capacities have recently been described [26]. We therefore used both the *wt* and the *alr*⁻ MD007 mutant strains in intragastric immunization experiments. Our data provide evidence that the recombinant strains that we constructed produced the antigen in microgram quantities in vitro. This indicates that, at the time of administration, antigen doses equivalent to those of purified rUreB/CT [5] were given to mice. Recombinant bacteria recovered from feces of immunized mice showed a preserved capacity to produce UreB, suggesting that sustained delivery of antigen occurs in vivo. We demonstrated that, although similar IgG and IgA antibody levels were obtained, the MD007/pMEC142 strain promoted a more pronounced reduction in *H. felis* load after challenge than did its *wt* counterpart. This was accompanied by a reduced expression of proinflammatory chemokines/cytokine involved in *Helicobacter* infection. The *wt* *L. plantarum* NCIMB8826 strain did not exhibit intrinsic protective properties in mice challenged with *H. felis*, as was demonstrated by the absence of an effect induced by the 8826/pTG2247 control strain. However, it is worth mentioning that probiotic-based interventions against *H. pylori* infections by use of different species of LAB have shown partial beneficial effects that deserve further examination [44, 45].

A variety of antigens have been produced in different LAB strains, belonging mostly to the *Lactococcus* and *Lactobacillus* genera [21]. Although seric or mucosal antibody responses have been measured, protection studies against the targeted pathogen were rarely performed or turned out to be unsuccessful [46, 47], with the notable exception of protective immunity demonstrated by use of the potent immunogen TTFC [48]. The ability of LAB carriers to induce protective immune responses depends on suf-

ficient antigen delivery in vivo and might be limited by their poor adjuvant properties. Optimization of LAB carriers requires selecting or defining the most-suitable LAB vehicle, the mode of antigen presentation (cytoplasmic, secreted, or cell-surface exposed), and the immunization regimen (route, dose, or timing). In this respect, a recombinant *Lactococcus lactis* strain producing cytoplasmic UreB was shown to be unable to induce protection against *H. pylori* in a mouse model [47]. Notably, the reported experimental setting led to very weak antibody responses. The authors concluded that the adjuvant effect of *L. lactis* was insufficient to trigger robust immune responses when used to deliver a weak immunogen, yet poor persistence of the strain in the GI tract might be considered too.

It is worth noting that, in mucosal immunization against *Helicobacter* species, the recombinant *alr*⁻ MD007 mutant turned out to be superior to CpG immunostimulatory oligodeoxynucleotides used as adjuvant [49], although it did not perform as well as CT. Our data fully corroborate the results obtained by use of the potent TTFC antigen and, thus, mark the *alr*⁻ MD007 mutant as a substantially improved delivery system that is also applicable to weak antigens. This likely results from enhanced in vivo release of antigen or improved antigen presentation to antigen-presenting cells; the 8826 and MD007 strains exhibit very similar persistence capacity in the mouse GI tract (data not shown). To the best of our knowledge, this is the first demonstration of the successful induction of partial mucosal protection against *H. felis* in mice by use of recombinant LAB as live antigen carriers. Coexpression of immunoregulatory cytokines [50, 51], combined with improved LAB vehicles, represents a valuable step toward further improvement of the prototype strains described in the present study.

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