Oral vaccination of mice against *Helicobacter pylori* with recombinant *Lactococcus lactis* expressing urease subunit B

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**Abstract**

To determine whether a protective immune response could be elicited by oral delivery of a recombinant live bacterial vaccine, *Helicobacter pylori* urease subunit B (UreB) was expressed for extracellular expression in food-grade bacterium *Lactococcus lactis*. The UreB-producing strains were then administered orally to mice, and the immune response to UreB was examined. Orally vaccinated mice produced a significant UreB-specific serum immunoglobulin G (IgG) response. Specific anti-UreB IgA responses could be detected in the feces of mice immunized with the secreting lactococcal strain. Mice vaccinated orally were significantly protected against gastric *Helicobacter* infection following a challenge with *H. pylori* strain SS1. In conclusion, mucosal vaccination with *L. lactis* expressing UreB produced serum IgG and UreB-specific fecal IgA, and prevented gastric infection with *H. pylori*.

**Introduction**

*Helicobacter pylori* is a Gram-negative bacterium, specialized in the colonization of the human stomach (Warren & Marshall, 1983), which infects about 50% of the world’s population, causes gastric diseases ranging from gastritis to cancer and has been categorized by the World Health Organization as a class I human carcinogen. The standard treatment for *H. pylori* infections has depended on antibiotics in combination with proton pump inhibitors (Bazzoli et al., 2002). Antibiotic-based triple therapies are, however, not practical for global control due to the high cost, patients’ noncompliance and low effectiveness due to the development of antibiotic resistance among strains of *H. pylori* (Telford & Ghiara, 1996; Michetti, 1997). Vaccination against *H. pylori* is therefore one of the most effective ways to control *H. pylori* infection and, indeed, administration of oral bacterial antigens can protect mice against *H. pylori* infection (Ferrero et al., 1994; Marchetti et al., 1998; Kotloff et al., 2001; Nyström & Svennerholm, 2007).

*Helicobacter pylori* synthesizes a urease to buffer the pH of its immediate surroundings within the stomach. The role of urease in the pathogenesis of *H. pylori*-associated diseases is not limited to colonization as ammonia produced by the urease enters the *H. pylori* nitrogen metabolism and is eventually incorporated into proteins (Williams et al., 1996). Urease might also help to recruit neutrophils and monocytes in the inflamed mucosa and to activate production of proinflammatory cytokines (Harris et al., 1996). Moreover, urease (including subunit proteins UreA and UreB) is one of the main antigens recognized by the human immune response to *H. pylori*, and UreB seems to be more protective than UreA (Ferrero et al., 1994). Urease has been used in vaccination trials to prevent infection with *H. pylori* in mice (Del Giudice et al., 2001). Both oral and systemic immunizations were considered. The use of oral routes for immunization against infective diseases is desirable because oral vaccines are easier to administer and have higher compliance rates, and mucosal surfaces are the portals of entry for many pathogenic microbial agents. To render the vaccine effective, oral immunization requires the use of mucosal adjuvants, such as the cholera toxin or *Escherichia coli* heat-labile enterotoxin (LT). However, these are used only in animal models because of their toxicity. Several nontoxic derivatives were developed, with LTK63 and LTR72 being the most promising ones (Rappuoli...
et al., 1999), although their safety for humans is still being investigated.

Recently, commensal and nonpathogenic bacteria have been developed as mucosal vaccine delivery vehicles (Mercenier et al., 2000; Thole et al., 2000; Seegers, 2002; Nouaille et al., 2003; Wells & Mercenier, 2008). The risk of infection is low, which is advantageous, particularly for children, the elderly or immunocompromised individuals. In addition, as mucosal delivery vehicles, recombinant bacterial vaccine vectors offer several practical advantages, including avoidance of culturing large quantities of pathogens, no need to purify antigenic components or subunits and the ability to express immunogens in their native conformation.

*Lactococcus lactis* is a nonpathogenic, noncolonizing Gram-positive bacterium and has a long history of widespread use in the dairy industry to make cheese and other fermented foods (Adams & Marteau, 1995). Because *L. lactis* does not naturally colonize the intestines of humans or animals, it is perhaps more analogous to inert microparticle vaccine-delivery systems (Wells et al., 1996). To date, several bacterial and viral antigens have already been produced in *L. lactis* (Wells et al., 1993; Que et al., 2000; Enouf et al., 2001; Ribeiro et al., 2002; Dieye et al., 2003; Pei et al., 2005), and immunization with these strains elicits immune responses specific to heterologous antigens (Robinson et al., 1997, 2000; Thole et al., 2000; Seegers, 2002; Ribeiro et al., 2002; Dieye et al., 2003; Ramasamy et al., 2006; Bahey-El-Din et al., 2008). Note that a protective immune response depends not only on the antigen and the delivery vehicle but also on the location of the antigen (Norton et al., 1996). In some cases, antigen export may be of interest because it allows a direct contact between the antigen and the immune system (Ribeiro et al., 2002; Dieye et al., 2003).

In the present study, the UreB antigen was expressed in *L. lactis* when the UreB fused to the signal peptide of the major *L. lactis* secreted protein 310mut2. The recombinant lactococci were used for oral immunization of mice. The results showed that mice immunized mucosally with the recombinant lactococcal vaccines were protected against gastric infection following a challenge with *H. pylori*.

**Materials and methods**

**Bacterial strains and growth conditions**

*Lactococcus lactis* ssp. *lactis* MG1363 was grown in M17 medium supplemented with glucose at 30 °C without agitation. pAMJ399-based plasmids were maintained in lactococcal strains on GM17 with 5 μg erythromycin mL⁻¹. *Escherichia coli* JM109 was grown at 37 °C in Luria–Bertani medium with 250 μg erythromycin mL⁻¹. The *H. pylori* strain was cultured on Columbia agar supplemented with 8% (v/v) defibrinated sheep blood, Dent selective supple-

ment (Oxoid) in a microaerophilic atmosphere (85% N₂, 10% CO₂, 5% O₂) at 37 °C for 3–5 days.

**DNA techniques and transformation**

Recombinant plasmid DNA from *E. coli* was isolated using the alkaline lysis technique. Plasmids were isolated from *L. lactis* using the alkaline lysis technique after incubation with lysozyme (20 mg mL⁻¹) for 20 min at 37 °C. Preparative portions were further purified with columns as recommended by the supplier (Qiagen, Hilden, Germany). Restriction enzymes, Taq DNA polymerase, T4 DNA ligase and buffer systems were supplied by Amersham Pharmacia Biotech, and DNA manipulations were carried out according to the instructions of the supplier. *Lactococcus lactis* ssp. *lactis* MG1363 was transformed by electroporation according to the method described by Holo & Nes (1989), with 0.03–0.5 μg of DNA per electroporation.

**Construction of ureB expression plasmid**

A fragment encoding the UreB sequence was PCR amplified from the DNA of *H. pylori* and cloned into BglII/Sall-cut pAMJ399, resulting in pAMJ399-ureB. The primers used were 5’-ureB (5’-AATCAGATCTAAAAAGATTAGCAGAAAAG-3’) for the coding strand and primer 3’-ureB (5’-TTACGTCGACCTAGAAAATGCTAAAGAGTTGC-3’) for the complementary strand.

**Biochemical analyses**

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a Tricine buffer as described by Schägger & Von Jagow (1987). For cell fractionation, 2-mL cultures of *L. lactis* MG1363 carrying pAMJ399-ureB or pAMJ399 at an OD₆₀₀ nm were pelleted by centrifugation at 6000 g for 10 min at 4 °C. The supernatant and cells were processed separately. The cell pellet was washed with 0.9% NaCl and resuspended in buffer A (50 mM Tris-HCl, pH 8, 5% glycerol, 0.15 M NaCl, 2 mM EDTA and 1 mM dithiothreitol) and disrupted by double passage through a French press. Cellular debris was removed by centrifugation (15 000 g, 4 °C). The soluble fraction was precipitated with 40% ammonium sulfate, resuspended in buffer A and then dialyzed against buffer A at 4 °C. The cell-free culture supernatant was collected as described above. Equal volumes of 5 × loading buffer were added to all samples. Ten microliters of each sample was separated on 10% SDS-PAGE, and then transferred to a polyvinylidene difluoride membrane (Millipore) by electrobloctting (0.35 A, 1 h). Nonspecific binding on the membrane was blocked overnight with 5% nonfat milk powder in Tris-buffered saline (TBS)/Tween-20 (TTBS) for 1 h at room temperature and incubated with a polyclonal human antiserum raised
against *H. pylori* for 1 h at 25 °C. After washing three times with TTBS, the blot membrane was incubated with a goat anti-human immunoglobulin G (IgG) alkaline phosphatase conjugate (Amersham Biosciences) for 1 h at 25 °C. After washing three times with TTBS, BCIP/NBT was used to visualize the bound antibody.

**Immunizations**

Recombinant strain MG1363 (pAMJ399-ureB) and control strain MG1363 (pAMJ399) were cultured as described above for 18 h. Groups of six female BALB/c mice were immunized orally with recombination *L. lactis* expressing UreB (harboring pAMJ399-ureB) or a control nonexpressor strain (harboring pAMJ399). A native, nonvaccinated strain was also included in the experiment. Oral doses of 5 x 10⁹ CFU were administered via an intragastric lavage on days 0, 7, 14, 21, 28 and 35. Serum samples were taken at intervals of 14 days and stored at −20 °C until use.

**Enzyme-linked immunosorbent assay (ELISA) for the detection of UreB-specific serum antibody**

ELISA plates were coated overnight at 4 °C with 1 µg mL⁻¹ recombinant purified UreB. Threefold serially diluted samples starting from 1:400 for serum were applied onto the plates and incubated for 1 h at 37 °C. Serum IgG and IgA were detected by peroxidase-labeled goat anti-mouse IgG (Amersham Biosciences) and anti-mouse IgA (Sigma). Endpoint titers were determined as the reciprocal of the dilution factor of sample yielding background levels of OD₄₅₀ nm.

**ELISA for the detection of UreB-specific mucosal IgA**

UreB-specific and total IgA in fecal pellets were quantified by ELISA. Portions of each plate were coated, respectively, with an anti-mouse IgA monoclonal antibody (Sigma) and UreB. In addition to the diluted samples, a dilution series of purified IgA was applied to each plate to provide a standard curve. After incubation with peroxidase-labeled anti-mouse IgA, the concentrations of UreB-specific and total IgA were determined from the standard curve. In order to address the possibility that increased IgA concentrations were the result of a polyclonal nonspecific response to mucosal stimulation, responses were expressed as the ratio of specific to total IgA.

**Challenge of vaccinated mice**

The plate-grown cultured *H. pylori* SS1 were harvested and resuspended in phosphate-buffered saline (PBS) to yield a concentration of 1 x 10⁹ CFU mL⁻¹. Vaccinated mice were challenged with 0.2 mL (2 x 10⁸) *H. pylori* SS1 instilled into gastric tissue. Food and water were given 4 h after the challenge.

**Assessment of *H. pylori* colonization of the mouse**

Four weeks after receiving the challenge, mice were sacrificed by spinal dislocation. The stomachs were washed twice in sterile 0.9% NaCl, and the gastric antrum from each stomach was assessed for *H. pylori* colonization by the detection of urease activity, quantitative culture and histological analyses of bacterial culture. Briefly, 50 µL of the gastric biopsy was placed in 500 µL of urea broth containing phenol red indicator. Urease positivity was determined by an increase in pH, indicated by a color change from yellow to red within 24 h at 37 °C. For *H. pylori* SS1 quantitative culturing, the stomach samples weighed, and homogenized in thioglycolate medium, serially diluted in PBS and plated onto Columbia agar plates with antibiotics. Bacterial counts were expressed as CFU per gram of gastric tissue. To histologically assess bacterial colonization, 4-µm-thick sections of the gastric antrum were cut and stained using the modified Steiner silver method (Genta et al., 1994). The degree of semi-quantitative analysis of blinded slides, whereby bacterial colonization was graded from 0 to 4, where 0 = no bacteria, 1 = 1–2 bacteria/crypt, 2 = 3–10 bacteria/crypt, 3 = 11–20 bacteria/crypt and 4 ≥ 20 bacteria/crypt.

**Statistical analysis**

Data were analyzed using the Social Sciences (SPSS) computer software. Student’s *t*-test was used to assess the differences of UreB-specific antibody levels in the different experimental groups and proportions of mice infected among groups of mice in the challenge experiment. *P* values of < 0.05 were considered statistically significant. *P* values of < 0.01 were considered highly significant.

**Results**

**Lactococcal production of *H. pylori* UreB**

To obtain a secreted form of UreB, the ureB gene fragment was fused in frame with the signal sequence of 310mut2, the major *L. lactis* secreted protein, and placed under the control of P170, resulting in pAMJ399-ureB. As a control, a plasmid pAMJ399 was also studied. These two plasmids were introduced into *L. lactis* MG1363, resulting in strains MG1363 (pAMJ399-ureB) and MG1363 (pAMJ399). Proteins were extracted and assayed by Western blotting after SDS-PAGE, using polyclonal antiserum to UreB. The results indicated that rUreB was produced in the supernatant of MG1363 (pAMJ399-ureB), which encodes the fusion between the signal peptide of 310mut2 and rUreB. In contrast, no band
corresponding to the UreB protein was detected in the supernatant of MG1363 carrying an empty plasmid (Fig. 1). Thus, L. lactis transformed with pAMJ399-ureB expressed a new, unaltered rUreB in the culture medium.

Serum anti-UreB antibody responses following mucosal immunization

Groups of mice were immunized orally with L. lactis that expresses the UreB protein in the culture supernatant. Control mice were vaccinated in the same way with pAMJ399 strains of L. lactis. One week after the final vaccination, the level of IgG in the serum was elicited significantly ($P < 0.001$), while IgA responses observed with the vector control strain (pAMJ399) and in unvaccinated groups of mice were lower and did not differ significantly from those of the naive group (Fig. 2).

Mucosal antibody responses

Significantly elevated UreB-specific IgA responses could be detected in fresh fecal pellets from mice immunized orally with MG1363 (pAMJ399-ureB), but not in those of mice given the control strain (Fig. 3). On days 6 and 20 after oral inoculation, the means of ratios of specific to total IgA detected for the group given pAMJ399-ureB were seven- and 23-fold those for unvaccinated controls ($P < 0.01$). On day 38, the ratios from the pAMJ399-ureB were still higher than the control groups. No significant differences could be detected between mice vaccinated with the pAMJ399 control strain and unvaccinated groups at any of the time points.

Challenge with H. pylori

Four weeks after immunization, mice were challenged once with $2 \times 10^8$ H. pylori SS1. Four weeks later, all of 10 stomach biopsies from the immunized BALB/c mice were negative by urease assay, but all 10 were positive by colony counts. The 10 negative immunized mice had a range of H. pylori colonization from $3 \times 10^4$ to $4 \times 10^5$ bacteria $g^{-1}$ stomach. However, there was a significant difference in protection between the groups of mice immunized with the control groups ($P < 0.001$). Similarly, for the histological analysis, in a separate experiment, nine out of 10 protectively immunized mice that were negative by histology assay gave positive results by culture with a range of $2 \times 10^4$ to $3 \times 10^5$ bacteria $g^{-1}$ stomach (Table 1). Thus, the mouse that was just detectable by histology or urease assay had a level of infection near the midpoint of the protected group. From this we can conclude that the sensitivity of the urease assay and the histological method is about $10^5$ bacteria $g^{-1}$ stomach. The immunization used with live recombinant L. lactis-expressing UreB subunits reduced H. pylori colonization compared with control mice, suggesting that a protective immune response had been induced in the mice.

Discussion

This report shows that oral vaccination with recombinant L. lactis UreB producer strains prevented gastric infection
A major objective of this study was to investigate immunoprotection against \textit{H. pylori} infection on BALB/c mice by oral administration. Nine out of mice in the immunization study were negative by histology, although all animals were positive by culture. Similar results have been obtained by immunization with whole bacterial sonicate and cholera toxin (Sutton et al., 2000). In this study, we have shown that a major bacterial reduction in gastritis was seen in all the animals immunized.

The results of the work by Lee et al. (2001) showed that there was no protective effect against \textit{H. pylori} after \textit{H. pylori} strain SS1 challenge, although the antigen-specific serum IgG titers were detected in mice immunized with recombinant \textit{L. lactis} constitutive expressing the \textit{H. pylori} UreB gene. Our results suggest that UreB-specific fecal IgA was both necessary and sufficient to prevent gastric infection with \textit{H. pylori}. A potential role for cell-mediated immunity is the subject of future investigations.

An ideal recombinant vaccine vehicle should be capable of inducing systemic responses relevant for protection against a variety of pathogens and should also elicit IgA at mucosal surfaces to prevent the entry of pathogens into the body. Evidence suggests that the production of antigen-specific secretory IgA is best achieved by presentation of the antigen directly to the mucosal surface (Lamm, 1997). \textit{Lactococcus lactis} has been described previously as an effective mucosal antigen-delivery system. Mucosal vaccination with recombinant \textit{L. lactis} strains expressing CRR-produced CRR-specific salivary IgA and serum IgG, prevented pharyngeal infection with \textit{Streptococcus pyogenes} and promoted survival (Mannam et al., 2004). Using a constitutive strain expressing TTFC, Robinson et al. (2004) demonstrated that intragastric or intranasal administration induced elevated TTFC-specific IgA levels at several mucosal sites and a TTFC-specific T-cell response with a mixed profile of T-helper subset-associated cytokines in the intestine. The present study shows that \textit{L. lactis} has the potential to act as an effective mucosal delivery system for UreB. The plasmids used for secretory expression of UreB in this system provide a useful model for exploring the characteristics of this vaccine delivery system. However, before recombination LAB can be used in humans, it will be necessary to construct strains for human or animal use that will meet the safety requirements of the regulatory bodies. The existing food-grade expression systems should be further developed for this purpose.

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