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

Qing Gu[1], Dafeng Song[1] & Muyuan Zhu[2]

[1]Department of Biotechnology, Zhejiang Gongshang University, Hangzhou, China; and [2]Institute of Cell Biology and Genetics, College of Life Sciences, Zhejiang University, Hangzhou, China

Correspondence: Qing Gu, Department of Biotechnology, Zhejiang Gongshang University, No. 149 Jiaogong Road, Hangzhou 310035, China. Tel.: +86 571 888 573 37; fax: +86 571 870 546 77; e-mail: guqing2002@hotmail.com

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, 2004; Xin *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 supplement (Oxoid) in a microaerophilic atmosphere (85% N_2, 10% CO_2, 5% O_2) 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/SalI-cut pAMJ399, resulting in pAMJ399-*ureB*. The primers used were 5′-*ureB* (5′-AATCAGATCTAAAAAGATTAGCAGAAA AG-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 $OD_{600 nm}$ 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 polyvinylicific difluoride membrane (Millipore) by electroblotting (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 5×10^9 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 $1 \mu g \, mL^{-1}$ 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). End-point titers were determined as the reciprocal of the dilution factor of sample yielding background levels of $OD_{450 \, nm}$.

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 $1 \times 10^9 \, CFU \, mL^{-1}$. Vaccinated mice were challenged with 0.2 mL (2×10^8) *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, $50 \mu L$ of the gastric biopsy was placed in $500 \mu L$ 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 on to 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
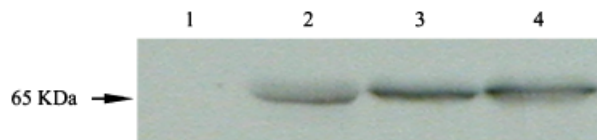

Fig. 1. Detection of the UreB expression in the supernatant of *Lactococcus lactis* by Western blot analysis. Immunoblotting of the protein extracts from cell-free medium of UreB expressor strains (pAMJ399-*ureB*) harvested in 12-h (lane 2), 18-h (lane 3) and 24-h cultures (lane 4). An extract from the control pAMJ399 strain (lane 1) harvested after 24 h was also tested. A polyclonal antiserum for detection of UreB was used as the first antibody.

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 2×10^8 *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 3×10^4 to 4×10^5 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 2×10^4 to 3×10^5 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
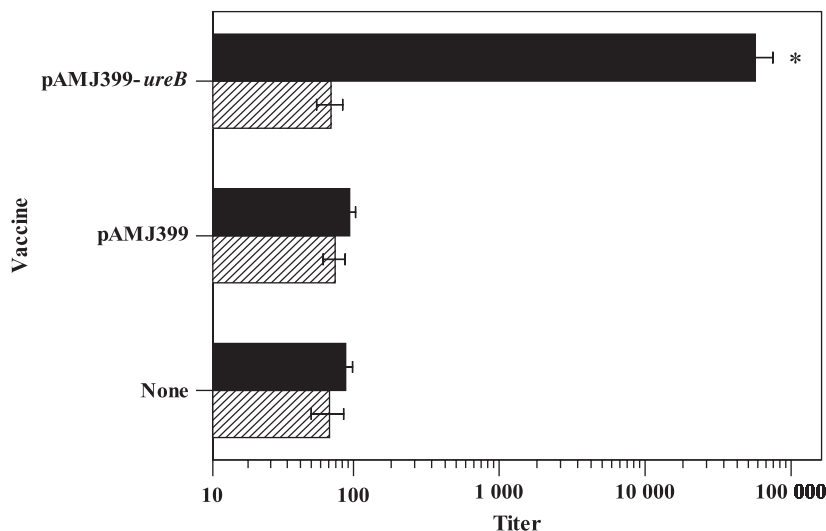

Fig. 2. Serum anti-UreB IgG and IgA titers elicited by recombinant *Lactococcus lactis*. Groups of six mice were immunized orally with recombination *L. lactis* expressing UreB (pAMJ399-*ureB*) or a control strain (pAMJ399). On day 35 after initial treatment, blood samples were collected and UreB-specific serum IgG (solid bars) and IgA (striped bars) titers were measured by ELISA. Bars, mean titers; error bars, SD. *Highly significant ($P < 0.001$) difference from control groups.
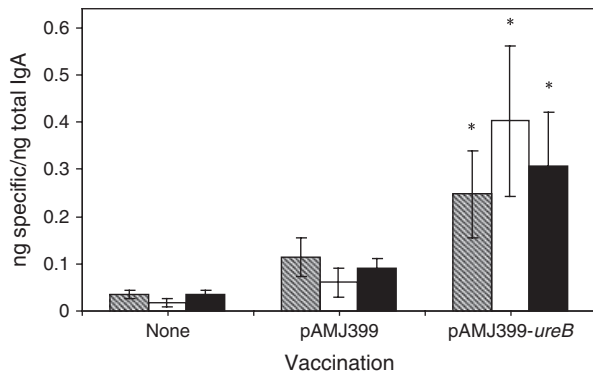
Fig. 3. Ratio of specific to total IgA in fecal pellets of mice immunized orally with lactococci expressing UreB (pAMJ399-*ureB*) or a control strain (pAMJ399). Fresh fecal pellets were collected from groups of six mice on days 6 (striped bars), 20 (open bars) and 38 (solid bars) after initial treatment. The samples were tested by ELISA for UreB-specific and total IgA. Bars, mean ratios; error bars, SD. *Highly significant ($P < 0.01$) difference from control groups.

Table 1. Protection of mice from *Helicobacter pylori* SS1 infection following immunization with live recombinant *Lactococcus lactis*

	Number (%) of mice colonized by *H. pylori*[†]		Log CFU g^{-1} tissue[‡]
Group	Urease	Histology	
MG1363 (pAMJ399-*ureB*)	0/10 (0)	1/10 (10)	4.56 ± 0.58*
MG1363 (pAMJ399)	9/10 (90)	10/10 (100)	6.25 ± 0.66
Control	10/10 (100)	10/10 (100)	6.69 ± 0.60

*Value significantly higher than those for both the infected control ($P < 0.001$) and the MG1363 (pAMJ399)-treated groups.

[†]Mice each received 2×10^8 CFU of *Helicobacter pylori* SS1. The presence of *H. pylori* infection in gastric biopsies was assessed at 1 month postinfection by urease activity and histological assay.

[‡]The numbers of *H. pylori* CFU recovered following homogenization of gastric biopsy samples. The results are presented as geometric means for 10 mice per group ± SD (for duplicate determinations).

with *H. pylori*. Our main aim is to study and perhaps modulate immune responses to food allergens with UreB as the protein model and recombinant *L. lactis* strains as the vehicle.

The rUreB-producing *L. lactis* strain was constructed to produce rUreB in an extracellular location under the control of an inducible promoter. rUreB could be detected in the supernatant of MG1363 (pAMJ399-*ureB*) by Western blotting experiments. Oral inocula of 5×10^9 recombinant *L. lactis* expressing UreB given on days 0, 7, 14, 21, 28 and 35 to BALB/c mice elicited anti-UreB serum antibody responses that were significantly different from those observed in the control groups. Following oral immunization, a rapid increase in UreB-specific IgA levels could be detected in fresh fecal samples, but this declined by day 38. Our results indicated that oral vaccination with a lactococcal vaccine could elicit mucosal immune responses in the intestine.

A major objective of this study was to investigate immunoprotection against *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 *H. pylori* after *H. pylori* strain SS1 challenge, although the antigen-specific serum IgG titers were detected in mice immunized with recombinant *L. lactis* constitutive expressing the *H. pylori* UreB gene. Our results suggest that UreB-specific fecal IgA was both necessary and sufficient to prevent gastric infection with *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). *Lactococcus lactis* has been described previously as an effective mucosal antigen-delivery system. Mucosal vaccination with recombinant *L. lactis* strains expressing CRR-produced CRR-specific salivary IgA and serum IgG, prevented pharyngeal infection with *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 *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.

Acknowledgements

References

Adams MR & Marteau P (1995) On the safety of lactic acid bacteria from food. *Int J Food Microbiol* **27**: 263–264.

Bahey-El-Din M, Casey PG, Griffin BT & Gahan CG (2008) *Lactococcus lactis*-expressing listeriolysin O (LLO) provides protection and specific CD8(+) T cells against *Listeria monocytogenes* in the murine infection model. *Vaccine* **26**: 5311–5321.

Bazzoli F, Pozzato P & Rokkas T (2002) *Helicobacter pylori*: the challenge in therapy. *Helicobacter* **7**: 43–49.

Del Giudice G, Covacci A, Telford JL, Montecucco C & Rappuoli R (2001) The design of vaccines against *Helicobacter pylori* and their development. *Annu Rev Immunol* **19**: 523–563.

Dieye Y, Hoekman AJ, Clier F, Juillard V, Boot HJ & Piard JC (2003) Ability of *Lactococcus lactis* to export viral capsid antigens: a crucial step for development of live vaccines. *Appl Environ Microb* **69**: 7281–7288.

Enouf V, Langella P, Commissaire J, Cohen J & Corthier G (2001) Bovine rotavirus nonstructural protein 4 produced by *Lactococcus lactis* is antigenic and immunogenic. *Appl Environ Microb* **67**: 1423–1428.

Ferrero RL, Thiberge JM, Huerre M & Labigne A (1994) Recombinant antigens prepared from the urease subunits of *Helicobacter pylori* spp.: evidence of protection in a mouse model of gastric infection. *Infect Immun* **62**: 4981–4989.

Genta RM, Robason GO & Graham DY (1994) Simultaneous visualization of *Helicobacter pylori* and gastric morphology: a new stain. *Hum Pathol* **25**: 221–226.

Harris PR, Mobley HL, Perez-Perez GI, Blaser MJ & Smith PD (1996) *Helicobacter pylori* urease is a potent stimulus of mononuclear phagocyte activation and inflammatory cytokine production. *Gastroenterology* **111**: 419–425.

Holo H & Nes IF (1989) High-frequency transformation, by electroporation, of *Lactococcus lactis* subsp. *cremoris* grown with glycine in osmotically stabilized media. *Appl Environ Microb* **55**: 3119–3123.

Kotloff KL, Sztein MB, Wasserman SS, Losonsky GA, DiLorenzo SC & Walker RI (2001) Safety and immunogenicity of oral inactivated whole-cell *Helicobacter pylori* vaccine with adjuvant among volunteers with or without subclinical infection. *Infect Immun* **69**: 3581–3590.

Lamm ME (1997) Interaction of antigens and antibodies at mucosal surfaces. *Annu Rev Microbiol* **51**: 311–340.

Lee MH, Roussel Y, Wilks M & Tabaqchali S (2001) Expression of *Helicobacter pylori* urease subunit B gene in *Lactococcus lactis* MG1363 and its use as a vaccine delivery system against *H. pylori* infection in mice. *Vaccine* **19**: 3927–3935.

Mannam P, Jones KF & Geller BL (2004) Mucosal vaccine made from live, recombinant *Lactococcus lactis* protects mice against pharyngeal infection with *Streptococcus pyogenes*. *Infect Immun* **72**: 3444–3450.

Marchetti M, Rossi M, Giannelli V *et al.* (1998) Protection against *Helicobacter pylori* infection in mice by intragastric vaccination with *H. pylori* antigens is achieved using a non-toxic mutant of *E. coli* heat-labile enterotoxin (LT) as adjuvant. *Vaccine* **16**: 33–37.

Mercenier A, Muller-Alouf H & Grangette C (2000) Lactic acid bacteria as live vaccines. *Curr Issues Mol Biol* **2**: 17–25.

Michetti P (1997) Vaccine against *Helicobacter pylori*: fact or fiction? *Gut* **41**: 728–730.

Norton PM, Brown HW, Wells JM, Macpherson AM, Wilson PW & Le Page RW (1996) Factors affecting the immunogenicity of tetanus toxin fragment C expressed in *Lactococcus lactis*. *FEMS Immunol Med Mic* **14**: 167–177.

Nouaille S, Ribeiro LA, Miyoshi A, Pontes D, Le Loir Y, Oliveira SC, Langella P & Azevedo V (2003) Heterologous protein production and delivery systems for *Lactococcus lactis*. *Genet Mol Res* **2**: 102–111.

Nyström J & Svennerholm AM (2007) Oral immunization with HpaA affords therapeutic protective immunity against *H. pylori* that is reflected by specific mucosal immune responses. *Vaccine* **25**: 2591–2598.

Pei H, Liu J, Cheng Y, Sun C, Wang C, Lu Y, Ding J, Zhou J & Xiang H (2005) Expression of SARS-coronavirus nucleocapsid protein in *Escherichia coli* and *Lactococcus lactis* for serodiagnosis and mucosal vaccination. *Appl Microbiol Biot* **68**: 220–227.

Que YA, Haefliger JA, Francioli P & Moreillon P (2000) Expression of *Staphylococcus aureus* clumping factor A in *Lactococcus lactis* subsp. *cremoris* using a new shuttle vector. *Infect Immun* **68**: 3516–3522.

Ramasamy R, Yasawardena S, Zomer A, Venema G, Kok J & Leenhouts K (2006) Immunogenicity of a malaria parasite antigen displayed by *Lactococcus lactis* in oral immunisations. *Vaccine* **24**: 3900–3908.

Rappuoli R, Pizza M, Douce G & Dougan G (1999) Structure and mucosal adjuvanticity of cholera and *Escherichia coli* heat-labile enterotoxins. *Immunol Today* **20**: 493–500.

Ribeiro LA, Azevedo V, Le Loir Y, Oliveira SC, Dieye Y, Piard JC, Gruss A & Langella P (2002) Production and targeting of the *Brucella abortus* antigen L7/L12 in *Lactococcus lactis*: a first step towards food-grade live vaccines against brucellosis. *Appl Environ Microb* **68**: 910–916.

Robinson K, Chamberlain LM, Schofield KM, Wells JM & Le Page RW (1997) Oral vaccination of mice against tetanus with recombinant *Lactococcus lactis*. *Nat Biotechnol* **15**: 653–657.

Robinson K, Chamberlain LM, Lopez MC, Rush CM, Marcotte H, Le Page RW & Wells JM (2004) Mucosal and cellular immune responses elicited by recombinant *Lactococcus lactis* strains expressing tetanus toxin fragment C. *Infect Immun* **72**: 2753–2761.

Schägger H & von Jagow G (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal Biochem* **166**: 368–379.

Seegers JF (2002) Lactobacilli as live vaccine delivery vectors: progress and prospects. *Trends Biotechnol* **20**: 508–515.

Sutton P, Wilson J & Lee A (2000) Further development of the *Helicobacter pylori* mouse vaccination model. *Vaccine* **18**: 2677–2685.

Telford JL & Ghiara P (1996) Prospect for the development of a vaccine against *Helicobacter pylori*. *Drugs* **52**: 799–804.

Thole JE, van Dalen PJ, Havenith CE, Pouwels PH, Seegers JF, Tielen FD, van der Zee MD, Zegers ND & Shaw M (2000) Live bacterial delivery systems for development of mucosal vaccines. *Curr Opin Mol Ther* **2**: 94–99.

Warren JR & Marshall BJ (1983) Unidentified curved bacilli on gastric epithelium in active chronic gastritis. *Lancet* **1**: 1273–1275.

Wells JM & Mercenier A (2008) Mucosal delivery of therapeutic and prophylactic molecules using lactic acid bacteria. *Nat Rev Microbiol* **6**: 349–362.

Wells JM, Wilson PW, Norton PM, Gasson MJ & Le Page RW (1993) *Lactococcus lactis*: high-level expression of tetanus toxin fragment C and protection against lethal challenge. *Mol Microbiol* **8**: 1155–1162.

Wells JM, Robinson K, Chamberlain LM, Schofield KM & Le Page RW (1996) Lactic acid bacteria as vaccine delivery vehicles. *Antonie Van Leeuwenhoek* **70**: 317–330.

Williams CL, Preston T, Hossack M, Slater C & McColl KE (1996) *Helicobacter pylori* utilizes urea for amino acid synthesis. *FEMS Immunol Med Mic* **13**: 87–94.

Xin KQ, Hoshino Y, Toda Y *et al.* (2003) Immunogenicity and protective efficacy of orally administered recombinant *Lactococcus lactis* expressing surface-bound HIV Env. *Blood* **102**: 223–228.