Expression of *Helicobacter pylori* urease B on the surface of *Bacillus subtilis* spores

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*Helicobacter pylori* infection is a major risk factor for chronic gastritis, digestive ulcers, gastric adenocarcinoma and lymphoma. Due to the decreasing efficacy of anti-*H. pylori* antibiotic therapy in clinical practice, there is renewed interest in the development of anti-*H. pylori* vaccines. *Bacillus subtilis* is non-pathogenic and can produce endospores, which can survive under extreme conditions. These features make the *B. subtilis* spore an ideal vehicle for delivery of heterologous antigens to extreme environments such as the gastrointestinal tract. In this study, we displayed *H. pylori* urease B protein on the *B. subtilis* spore coat using the spore coat protein CotC as a fusion partner. Western blot analyses were used to verify urease B surface expression on spores. Recombinant spores displaying the urease B antigen were used for oral immunization and were shown to generate humoral response in mice. Urease B-specific secretory IgA in faeces and IgG in serum reached significant levels 2 weeks after oral dosing. In addition, oral immunization of recombinant urease B spores induced a significant reduction (84 %) in the stomach bacterial load ($0.25 \pm 0.13 \times 10^6$ c.f.u.) compared to that in the non-recombinant spores treated group ($1.56 \pm 0.3 \times 10^6$ c.f.u.; $P<0.01$). This report shows that urease B expressed on *B. subtilis* spores was immunogenic, and oral administration of urease B spores can provide protection against *H. pylori* infection.

INTRODUCTION

*Helicobacter pylori* is a Gram-negative bacterium that colonizes the stomach in approximately half the world’s population. Infection may progress to pathological states, such as peptic ulcer disease and gastric adenocarcinoma, resulting in significant morbidity and mortality worldwide (de Martel et al., 2012; Nakamura et al., 2012; Toller et al., 2011; Uemura et al., 2001; You et al., 2012). Current regimens for treatment of *H. pylori* infection consist of a proton pump inhibitor (PPI) and antibiotics including amoxicillin, clarithromycin and metronidazole. Despite an eradication rate greater than 80 %, there are some limitations to the PPI-based triple therapy, such as poor patient compliance, emerging antibiotic resistance, frequent reinfection and high cost (Lee et al., 2013; Su et al., 2013). Vaccination would therefore be a suitable alternative or complement to antibiotic treatment to eradicate the bacterium and prevent reinfection (Czinn & Blanchard, 2011).

Identification of antigens that can induce effective immune responses is a crucial step in vaccine development. Many protein molecules expressed by *H. pylori* have been shown to possess immunogenicity, including urease, cytotoxin-associated antigen, neutrophil-activating protein A, *H. pylori* adhesin A, vacuolating toxin A, catalase and outer-membrane protein (Czinn & Blanchard, 2011; Liu et al., 2011).

Among these antigens the B subunit of the urease protein (urease B), a 61 kDa protein encoded by a 1.7 kbp gene, is the most promising one. *H. pylori* produces a large amount of urease, which is essential for the survival and pathogenesis of the bacterium. Ammonia generated by the hydrolysis of urea neutralizes gastric acidity and forms a neutral microenvironment surrounding the bacterium within the gastric lumen. Mice immunized with whole-cell lysate or
purified urease B protein were protected against experimental H. pylori SS1 infection (Hatzifoti et al., 2006; Li et al., 2010; Zhao et al., 2007). Despite these encouraging findings, an effective H. pylori vaccine remains elusive because immunization of mice reduces but rarely eliminates Helicobacter organisms in the stomach (Czinn & Blanchard, 2011). Therefore, the immunogenicity of urease B has to be improved. To achieve this goal, researchers have experimented with the use of adjuvants such as Freund’s, cholera toxin and Escherichia coli labile toxin. However, these adjuvants have no human application due to their toxicity (Summerton et al., 2010; Vermooten et al., 2012).

On the other hand, spore-forming bacilli can grow rapidly and secrete large quantities of proteins and other metabolites, making them an attractive candidate for industrial fermentation. Among the different types of spore-forming bacilli, Bacillus subtilis can secrete recombinant proteins directly into the culture medium (Zhang et al., 2005) and produce an endospore in the absence of nutrients. The mature spore can survive under extreme conditions such as excessive temperature and low pH (Driks, 1999; Nicholson et al., 2000). Therefore, spores can provide an ideal vehicle for the delivery of heterologous antigens to the acidic gastrointestinal tract (Duc et al., 2003, 2007). B. subtilis has been used as a probiotic for treating diarrhea in clinical practice. Given the safety profile and natural ability to survive in acidic environments, B. subtilis spores have been used as vehicle for developing mucosal vaccines for tetanus and anthrax in animal models (Duc et al., 2003, 2007). However, there are, to our knowledge, no previous reports on using B. subtilis spores as a mucosal vaccine vehicle for H. pylori.

In this study, we used probiotic B. subtilis as a vehicle and H. pylori urease B as a candidate antigen to develop an oral vaccine against H. pylori infection.

METHODS

Isolation of H. pylori genomic DNA and PCR amplification of the urease B gene. H. pylori Sydney strain 1 was grown on Columbia blood agar plates plus antibiotics at 37 °C for 4–5 days under microaerophilic conditions as previously described (DeJonghe et al., 2009).

Bacteria were transferred to Brucella broth containing 10% FBS with antibiotics and grown at 37 °C and 5% CO2. H. pylori genomic DNA was isolated and purified from freshly grown stocks using a commercial DNA isolation kit, following the manufacturer’s instructions (TaKaRa). DNA was reconstituted in Tris/EDTA buffer (10 mM Tris/HCl, 1 mM EDTA, pH 7.6). Aliquots of genomic DNA were analysed for purity by agarose gel electrophoresis in a 1% (w/v) gel. The DNA preparation was stored at −20 °C until use.

H. pylori urease B DNA was amplified by PCR. Custom primers for amplification of urease B were synthesized commercially (Invitrogen). The designed primers included a forward primer: 5’GCGGAAATTT-CATGAAAAGATTAGCGAGAAAAG3’ with an EcoRI site (underlined), and a reverse primer: 5’AAAGGGGGCGCTAGAAAAATGCTAAAGGTTG3’ with a NotI site. The PCR conditions were as follows: 94°C for 4 min in followed by 35 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 90 s; the reaction continued for 10 min at 72°C after the last cycle.

Recombinant urease B protein and antisera production. The amplified product obtained was digested with EcoRI and NotI. The digested product was subcloned into the EcoRI and NotI double-digested pGEX-4T-1 vector (Pharmacia). The recombinant plasmid was sequenced and transformed into E. coli strain BL21 as described previously (Zhou et al., 2008a). Urease B fused with glutathione S-transferase (GST) was extracted from E. coli and purified using glutathione-Sepharose 4B resin (Pharmacia), followed by cleavage with thrombin (Pharmacia) as previously described (Zhou et al., 2008a). Purified proteins were separated by 12% SDS PAGE and stained with Cooammassie brilliant blue G-250.

The purified protein (100 µg) was resuspended in Freund’s complete adjuvant and then injected subcutaneously in to BALB/c mice, which received two additional doses at 2 week intervals. One week after the last injection, blood was collected from each animal and the antiserum titre of the recombinant protein was determined by ELISA to be 1:51200.

Construction of gene fusion. The urease B coding region was PCR amplified from the pGEX–urease B plasmid using the approach described in our previous work (Zhou et al., 2008a). The urease B fragment was ligated to the 3’ end of the cotC gene in the pUS186–CotC vector (Zhou et al., 2008a, b) and transformed into B. subtilis strain WB600 (Leighton & Doi, 1971; Spizizen, 1958). The sequence of the fusion gene was confirmed.

Preparation of spores. Spores were prepared as previously described (Zhou et al., 2008a). Briefly, sporulation was induced in Difco sporulation medium (DSM) and then cultured for 24 h. Lysozyme was used to dissociate the remaining sporangial cells and PMSF (1 mM) was used to inhibit proteolysis. Spores were purified and counted. Approximately 1011 spores were obtained from 1 DSM medium.

Spore coat protein extraction and Western blot analysis. Spore coat proteins were extracted from suspensions at high density (>1 × 1010 spores ml−1) as described previously (Nicholson & Setlow, 1990). Extracted proteins were separated on a 12% SDS-PAGE gel and then transferred onto a nitrocellulose membrane. Proteins were incubated with mouse anti-urease B serum, followed by HRP-conjugated rabbit anti-mouse IgG (Sigma). Membrane was developed with diamobenzidine substrate solution to visualize the proteins. Purified recombinant urease B was tested as control.

Immunization of mice and sample collection. Eight-week-old male BALB/c mice purchased from Sun Yat-sen University were maintained under specific pathogen-free conditions according to standard guidelines for the care and use of animals. Mice (10 mice per group) were orally immunized with suspensions of spores expressing CotC–urease B and control CotC spores. Naive mice served as controls. Mice were orally administered 1.0 × 1010 spores in a volume of 0.15 ml by intragastric lavage on days 0, 1, 2, 16, 17, 18, 33, 34 and 35. Serum and faecal samples were collected on days 1, 15, 32 and 50. The faecal samples were treated following the procedure of Robinson et al. (1997). The faecal pellet (0.1 g) was suspended in PBS with BSA (1%) and PMSF (1 mM), incubated at 4°C overnight and centrifuged the following day. Supernatants were stored at −20°C prior to ELISA.

Detection of urease B-specific antibodies by indirect ELISA. Urease B-specific IgG and faecal IgA were detected by indirect ELISA as described previously (Zhou et al., 2008a). Fifty microlitres of the
purified recombinant urease B antigen (5 µg ml⁻¹) was used to coat ELISA plates.

**H. pylori challenge.** Mice were orally infected with approximately 3 × 10⁸ c.f.u. of *H. pylori* SS1 bacteria in Brucella broth 2 weeks after the final immunization. All mice were sacrificed 4 weeks after *H. pylori* challenge. One-half of the stomach was homogenized in 2 ml Brucella broth with a tissue homogenizer. Serial dilutions of the homogenate were thereafter plated on *H. pylori* selective agar plates. After 7 days of incubation at 37 °C under microaerophilic conditions, visible colonies with typical *H. pylori* morphology were counted, and the urease test was performed for any uncertain colonies (Raghavan *et al.*, 2010).

**Statistical analysis.** Results were expressed as the mean ± SD. The statistical significance of the observed difference was assessed by ANOVA. Differences between groups were considered significant at an interval level of *P*, 0.05. Data were analysed using spss for Windows version 13.0.

## RESULTS

### Isolation of *H. pylori* genomic DNA and PCR amplification of the urease B gene

The target fragment of the urease B gene with the expected size (about 1.7 kb) amplified from DNA template of *H. pylori* SS1 strain is shown in Fig. 1. The urease B PCR product was verified by sequencing.

### Expression of urease B in *E. coli*

We constructed a recombinant pGEX-4T-1 plasmid (pGEX-4T-1–urease B) containing *H. pylori* urease B in which the ORF of urease B was in-frame to the tag protein of the vector. GST–urease B fusion proteins were expressed in *E. coli* upon induction of IPTG, and showed a molecular mass of 87 kDa as expected. Thrombin treatment of the fusion protein yielded a protein of 61 kDa, the molecular mass of urease B (Fig. 2). We were able to obtain an average of 1.9 mg urease B protein per 500 ml cell culture.

### Expression of urease B in the spore coat

An average of 0.4 mg of total coat proteins was reproducibly extracted from 10¹⁰ spores, indicating 4 fg total proteins from each spore. SDS-PAGE analysis of the coat protein fraction extracted from recombinant spores revealed a protein band of approximate 69.8 kDa in size (equal to 8.8 kDa CotC plus 61 kDa urease B; Fig. 3). Western blotting with urease B-specific antiserum also showed a positive band of approximate 69.8 kDa in spores of recombinant strains (Fig. 4).

### Humoral response of mice immunized with recombinant urease B spores

Fresh faecal pellets from orally immunized mice were tested for the presence of urease B-specific secretory IgA by ELISA (Fig. 5a). Oral immunization with spores expressing CotC–urease B elicited urease B-specific IgA responses. The levels of faecal urease B-specific IgA (absorbance value 0.25 ± 0.07) were significantly higher in the recombinant CotC–urease B spore treated group than in the CotC spore treated group (absorbance value 0.07 ± 0.03; *P*,< 0.05). There were no significant differences in urease B-specific IgA between the naive and non-recombinant spore treated groups (*P*,> 0.05). Serum levels of anti-urease B IgG (absorbance value 0.52 ± 0.37) were also significantly higher in the mice treated with recombinant spores than in the non-recombinant spore treated groups after 2 weeks (*P*,< 0.01; Fig. 5b). These findings suggested that oral administration of recombinant spores could induce not only mucosal immunity but also systemic immunity.

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**Fig. 1.** PCR product of urease B gene from *H. pylori*. Lane 1, DNA marker (bp); lane 2, PCR product of urease B.

**Fig. 2.** SDS-PAGE (12%) of recombinant urease B in *E. coli* BL21. Lane 1, protein molecular mass marker; lane 2, bacterial lysate with parent of plasmid pGEX-4T-1 induced with IPTG; lane 3, bacterial lysate with recombinant pGEX-4T-1–urease B fusion induced with IPTG; lane 4, fusion protein purified with glutathione-Sepharose column; lane 5, recombinant urease B cleaved by thrombin. Arrow indicates the fusion protein.
Fig. 3. SDS-PAGE (12%) of coat proteins extracted from spores of recombinant strain (CotC–urease B) and CotC strain. Lane 1, spore coat proteins extracted from CotC strain; lane 2, spore coat proteins extracted from CotC–urease B-expressing strain; lane 3, protein molecular mass markers. Arrow indicates the CotC–urease B fusion protein.

Efficacy of urease B-expressing spores in generating protective immunity against \textit{H. pylori}

Mice were treated orally by intragastric lavage with 1.0 x 10^8 recombinant spores for 3 days consecutively in weeks 1, 3 and 5. All mice were challenged by \textit{H. pylori} 2 weeks after final immunization and sacrificed 4 weeks after \textit{H. pylori} challenge. Oral immunization of recombinant urease B spores induced a significant reduction (84\%) in the stomach bacterial load (0.25 ± 0.13 x 10^6 c.f.u.) compared to that in the non-recombinant spore treated group (1.56 ± 0.3 x 10^6 c.f.u.; P<0.01; Fig. 6). The bacterial load in the urease B spores group was also significantly different from that in the naive group (1.69 ± 0.34 x 10^6 c.f.u.; P<0.01). The bacterial load in the non-recombinant spore treated group was not significantly different from that in the naive group (P=0.07).

DISCUSSION

\textit{H. pylori} is one of the most common chronic bacterial infections of humans, and affects at least half of the world’s population. Its eradication can prevent the recurrence of gastroduodenal ulceration. Moreover, it has been widely accepted that there has been a strong association between \textit{H. pylori}-associated gastritis and gastric cancer development (Lee et al., 2012). Thus, the development of vaccine strategies for \textit{H. pylori} is indispensable.

\textit{Bacillus} spores have previously been used as a vehicle for delivering recombinant proteins such as TP22.3 (Zhou et al., 2008a). Recombinant spores expressing bacterial antigens elicited specific immune responses and provided protection following mucosal immunization of mice (Mauriello et al., 2004; Negri et al., 2013; Zhou et al., 2008a). Here we reported the first application, we believe, of spores expressing the \textit{H. pylori} urease B antigen as a vaccine candidate.

The B subunit of the urease protein is a 61 kDa protein encoded in a 1.7 kbp gene and has the ability to counteract gastric acidity, which is crucial for the survival of \textit{H. pylori}. Urease-deficient \textit{H. pylori} mutants fail to colonize the gastric mucosa (Eaton et al., 1991; Tsuda et al., 1994). Mice immunized with purified urease B protein were protected against \textit{H. pylori} SS1 infection (Bégue & Sadowska-Krowicka, 2010; Kleanthous et al., 1998). In the present study we created urease B-expressing \textit{B. subtilis} spores. Similar to our previous study (Zhou et al., 2008a), \textit{B. subtilis} WB600 strain was used as the expression host to improve the stability of the recombinant protein.

The size of the \textit{B. subtilis} spores is approximately 1.2 μm in length, allowing for the uptake by M cells and transport into Peyer’s patches. Herein we showed that mice treated with urease B recombinant spores had significantly higher amounts of specific IgA in faeces and IgG in serum than those treated with non-recombinant spores. These results suggest that urease B recombinant spores induced not only mucosal immunity but also system immunity when delivered orally in mice. Consistent with our other study (Zhou et al., 2008a), the presence of spores did not affect the immunogenicity of urease B.

Our results also show that serum-specific IgG levels in mice with orally administrated recombinant spores were much higher than local mucosal IgA levels. In contrast, in our previous study we found that, although recombinant spores expressing tegumental protein 22.3 kDa of \textit{Clonorchis sinensis} induced a significant level of protection (44.7\%; P<0.05) in rats challenged with \textit{C. sinensis} metacercariae (Zhou et al., 2008a), they only induced local mucosal IgA responses (no obvious serum IgG responses). This difference suggests that rats might need a higher quantity of recombinant \textit{B. subtilis} spores to induce specific system IgG immunity than mice.

Secretory IgA is the most abundant class of antibody in the intestinal lumen of humans and in most other mammals; it has long been recognized as a first line of defence (Brandtzæg, 2010; He et al., 2007). Our work demonstrated that oral administration of spores expressing urease...
B can induce both local and systemic immunity. In addition, oral immunization with recombinant urease B spores significantly reduced the gastric bacterial load (84%; P<0.01). The role of antibodies in the protection against *H. pylori* infection is not clear. Studies using knockout mice showed that, in contrast to CD4+ T cells, B cells and antibodies are dispensable in the protection against *H. pylori* infection (Akhiani et al., 2004; Ermak et al., 1998). On the other hand, a recent human birth cohort study in Bangladesh found that infants fed on breast milk containing high titres of *H. pylori*-specific IgA antibodies showed a significant delay in acquisition of *H. pylori* infection compared to those on low-titre breast milk. These findings suggest that IgA antibodies may play a role in immune exclusion of bacteria at the site of infection (Bhuiyan et al., 2010).

The bacterium *B. subtilis* is currently consumed as a probiotic by both humans and animals. Although it is unclear how probiotics protect against bacteria, there have been some theories regarding their beneficial effects, such as competition for nutrients with the harmful bacteria and stimulation of mucosal and systemic host immunity (Kritas et al., 2006). *H. pylori* increases the production of reactive oxygen species and reactive nitrogen species in human stomachs, and this has been reported to impact upon gastric inflammation and carcinogenesis (Handa et al., 2011).

A recent randomized, double-blinded, placebo-controlled trial showed multi-species probiotics supplementation can improve redox haemostasis and intestinal barrier function (Lamprecht et al., 2012). It has been shown that CotC spore-immunized mice showed some, but not statistically significant, reduction in bacterial load (7% reduction) compared with a naive group, suggesting the probiotic effect of *B. subtilis*.

In conclusion, urease B expressed on *B. subtilis* spores was immunogenic, oral administration of recombinant spores could induce mucosal and systemic-specific humoral immunity and oral administration of urease B-expressing *B. subtilis* spores could provide protection against *H. pylori*.

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