Recombinant Bacillus subtilis spores expressing cholera toxin B subunit and Helicobacter pylori urease B confer protection against H. pylori in mice

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Abstract

Helicobacter pylori infection is associated with chronic gastritis, peptic ulcers, gastric cancer and mucosa-associated lymphoid tissue lymphoma. The limitations of current therapies for H. pylori infection include poor compliance and antibiotic resistance. Therefore, an effective anti-H. pylori vaccine would be an alternative or complement to antibiotic treatment. Urease B (UreB) is considered an ideal vaccine antigen against H. pylori infection. In this study, cholera toxin B subunit (CTB), a mucosal adjuvant, was used to enhance the immunogenicity of a novel Bacillus subtilis spore vaccine expressing CTB-UreB, along with the B. subtilis spore coat protein CotC as a fusion protein. Oral administration of B. subtilis spores expressing CotC-UreB or CotC-CTB-UreB led to increased levels of UreB-specific IgG in serum and UreB-specific IgA in faeces, as well as elevated levels of IL-10 and IFN-γ in splenocytes. In addition, oral administration of CotC-UreB or CotC-CTB-UreB spores induced significant reductions (80.0 and 90.5 %, respectively) in gastric H. pylori bacterial load (1.11±0.36×10⁶ and 0.53±0.21×10⁶ c.f.u., respectively) compared to that of the CotC control group (5.56±1.64×10⁵ c.f.u., P<0.01). Moreover, CotC-CTB-UreB spores were significantly more effective at reducing the bacterial load than CotC-UreB spores (P<0.05). These results indicate that CotC-CTB-UreB-expressing B. subtilis spores are a potential vaccine candidate for the control of H. pylori infection.

INTRODUCTION

Helicobacter pylori is a microaerophilic, pathogenic spiral bacterium that causes chronic infection in humans with a high infection rate. H. pylori infection is closely correlated with certain diseases such as chronic gastritis, digestive ulcers, gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma [1–3]. Although it can be eradicated by antibiotic treatment coupled with a proton-pump inhibitor, current therapies have several limitations, including poor compliance, antibiotic resistance and frequent reinfection, underlining the need for alternative treatment [4–6].

An effective anti-H. pylori vaccine would represent an attractive alternative or complement to antibiotic treatment, with the goal of eradicating the bacteria and immunologically preventing reinfection [7]. Many H. pylori protein molecules have been shown to promote immunogenicity, including the vacuolating toxin A, heat shock protein A, neutrophil-activating protein A, catalase, adhesin A, outer membrane protein and urease [7–9]. Among these, the urease B subunit, a 569-amino acid protein encoded by the ureB gene, is the most promising for vaccine development [10].

Bacillus subtilis is a spore-forming bacillus that grows rapidly and can form an endospore when nutrients are exhausted. B. subtilis spores are resistant to extreme environments, such as desiccation, exposure to solvents and acidic or alkaline conditions [11]. These render B. subtilis spores an ideal oral vaccine vehicle that can present antigens to extreme gastrointestinal tract (GIT) environments [12–14].

The vaccination adjuvants and routes used are important for the induction of protective immunity against GIT pathogens. Cholera toxin (CT) is a potent mucosal adjuvant, but it can cause diarrhoea when administered orally [15]. However, the cholera toxin B subunit (CTB) is non-toxic, comprises an important component of the oral cholera vaccine and has been proven to be safe [16]. Furthermore, CTB acts as a strong adjuvant to coupled antigens when administered orally [17].
In a previous study, we showed that recombinant *B. subtilis* spores displaying the *H. pylori* UreB protein on the spore coat generated a humoral response and reduced the *H. pylori* bacterial load in the murine stomach [18]. In the present study, we used the *B. subtilis* spore coat protein CotC as a fusion partner to develop a novel *B. subtilis* spore vaccine in which the surface-displaying mucosal adjuvant CTB and *H. pylori* UreB were used to protect against *H. pylori* infection.

**METHODS**

**Bacterial strains**

*Escherichia coli* DH5-α and *B. subtilis* WB600 [19] were cultured at 35°C in lysogeny broth (LB) medium. *E. coli* strain DH5-α with plasmid pET28 was cultured in LB medium with 50 µg ml⁻¹ kanamycin. *B. subtilis* WB600 with plasmid pUS186-CotC [20] was cultured in LB medium with 20 µg ml⁻¹ kanamycin. *H. pylori* Sydney strain 1 (SS1) was cultured for 4 to 5 days at 37°C on Columbia blood agar supplemented with Dent selective supplement (Oxoid) in a microaerophilic atmosphere [18]. Translucent colonies from the Columbia blood agar with curved Gram-negative rods resembling *Helicobacter* spp. and positive in catalase, oxidase and urease enzyme tests were identified as *H. pylori* [21].

**Animals**

The animal experiments were carried out in strict accordance with the Guide for Chinese Association for Laboratory Animal Sciences. Eight-week-old female BALB/c mice were obtained from Guangdong Medical Laboratory Animal Center (Guangzhou, China). The mice were housed in a pathogen-free environment with food and water *ad libitum*. All experiments involving animals were approved by the Animal Experiments Committee of Guangzhou Children and Women’s Medical Center, Guangzhou Medical University (Ethics approval number: 2015020912).

**Gene cloning strategies**

The urease B coding gene was amplified by PCR using constructed pGEX-urease B plasmid as template [18]. The designed primers included a forward primer (5’-CCGCCTGACATGAAAAAGATTAGCAAGAAAGGA-3’) and a reverse primer (5’-AAAGCGGCCCCGCTGAAATGCTGAAAGATGTG-3’), with restriction sites for Sall and NotI (underlined) allowing amplified DNA to be cloned into the pET28a expression plasmid (Merck). The CTB coding region was cloned into the recombinant plasmid pET28a UreB to produce recombinant plasmid pET28a CTB-UreB, with forward (5’-CCGCATTTACACCTCTAAAATAATTCTGATT-TG-3’) and reverse (5’-CGAGTGCAGATTGCTTACATAAA TTAGGCAA-3’) primers with restriction sites EcoRl and Sall (underlined), respectively. All recombinant plasmids were identified by restriction endonuclease digestion analysis and DNA sequencing.

CTB-UreB DNA was amplified using the constructed pET28-CTB-UreB plasmid as template. The PCR primers included a forward primer (5’-CGCTCCTAGACACACCTC-3’) with an XbaI restriction site and a reverse primer (5’-CACCTGACAGCTAGAAATGCTAAGAGTT GT-3’) with a PstI restriction site. The purified CTB-UreB PCR product was double-digested by XbaI and PstI restriction enzymes, and cloned into the 3' terminal of the CotC in the recombinant pUS186-CotC plasmid. This recombinant plasmid was then transformed into *B. subtilis* WB600 cells by the competent-cell method [22] and confirmed by XbaI/PstI double-enzyme digestion and DNA sequencing.

**Preparation of spores and spore coat protein extraction**

Sporulation of recombinant *B. subtilis* strains was induced by Sporulation Medium (Difco) as previously described [18]. The collected spores were incubated with 4 mg ml⁻¹ lysozyme followed by washing twice in 1 M NaCl, 1 M KCl, and water respectively. After the final suspension in distilled water, the spores were incubated at 65°C for 1 h in a water bath to kill any residual vegetative cells [20]. Spore numbers were counted using a haemocytometer.

Spore coat proteins were extracted in SDS-DTT extraction buffer as described previously [20]. Recombinant coat proteins were analysed by SDS-PAGE.

**Western blot analysis**

After 12% SDS-PAGE, the extracted coat protein was then transferred onto a PVDF membrane in a Trans-Blot transfer cell (Bio-Rad). The membrane was incubated with rat immune sera for 1 h at 37°C; after washing the membranes with PBS, they were incubated with horseradish peroxidase (HRP)-coupled anti-rat IgG (Boster) at 37°C for 1 h, and the colour was visualized by diaminobenzidine substrate solution (Boster). All procedures were performed according to the manufacturer’s instructions.

**Oral immunization of mice and *H. pylori* challenge**

Four groups of female BALB/c mice (eight mice per group) were immunized orally with spore suspensions of CotC-UreB, CotC-CTB-UreB or control CotC. Mice were treated with 1.0×10⁸ spores by intragastric lavage on days 0, 1, 13, 27 and 41. Blood was collected by submandibular venipuncture, and harvested sera were stored at −80°C until use. Faecal samples were treated as described previously [20]. Mice were fed with 3×10⁸ c.f.u. of *H. pylori* SS1 bacteria by intragastric lavage 2 weeks after the final spore treatment. Mice were sacrificed and evaluated 4 weeks after *H. pylori* challenge.

**ELISA analysis of urease B-specific antibodies**

For urease B-specific IgG measurement, 5 µg ml⁻¹ recombinant urease B [23] in 0.1 ml of 0.05 M bicarbonate buffer (pH 9.6) was used to coat the wells at 4°C overnight. The wells were washed with PBS containing 0.05% Tween 20 (PBS-T) and blocked with 1% BSA in PBS-T for 2 h at...
25 °C, and then 50 µl of a 1:8000 dilution of serum sample was added to each coated well and incubated at 4 °C overnight. After washing the wells with PBS-T buffer, 50 µl of 1 µg ml⁻¹ biotinylated rat anti-mouse IgG antibody (BD Biosciences) was added to each well and incubated for 1 h at 25 °C, followed by the addition of avidin-HRP (Sigma) and incubation for 45 min at 25 °C. After washing the wells with PBS-T, colour was detected by TMB substrate reagent (BD Biosciences) at 25 °C for 30 min. Reactions were stopped using 2 M H₂SO₄. Urease B-specific faecal IgA was measured by a similar protocol, except that biotinylated rat anti-mouse IgA antibody was used as the detection antibody and 50 µl original supernatant was used as the sample.

**Splenocyte culture and cytokine analysis**

Mice were sacrificed 4 weeks after *H. pylori* challenge, and spleens were isolated as described previously [24]. Splenocytes (4 x 10⁶ well⁻¹ ml⁻¹) were cultured in RPMI1640 (supplemented with 100 µg ml⁻¹ streptomycin, 100 U ml⁻¹ penicillin and 10 % fetal bovine serum) in the presence or absence of urease B (2.5 µg ml⁻¹). Supernatants were collected after 72 h incubation at 35 °C and stored at −80 °C until use. IFN-γ, IL-4 and IL-10 levels in supernatants were measured by sandwich ELISA kits according to the manufacturer’s instructions (BD Biosciences).

**Quantification of *H. pylori* in the stomach**

The bacterial load in the murine stomach was measured by *H. pylori* quantitative culture, as described previously [18]. Weighed murine stomach samples were homogenized in LB. Serial dilutions of the stomach homogenate were plated on Columbia blood agar plates supplemented with antibiotics. After incubation at 37 °C for 5 days under microaerophilic conditions, *H. pylori* bacterial clones were counted and bacterial loads were expressed as c.f.u. per gram of gastric tissue.

**Statistical analysis**

Data were expressed as means±SD. All data were analysed using SPSS computer software version 13.0 (SPSS). Statistical significances among different groups were determined by one-way ANOVA. P values less than 0.05 were considered significant.

**RESULTS**

**Construction of gene fusion**

A recombinant pET-28a plasmid (pET-28a UreB) containing HP-UreB was constructed. The sequence analysis showed that the UreB ORF was in-frame to the His-tag of the recombinant plasmid. CTB was cloned into pET-28a upstream of UreB to generate the pET-28a-CTB-UreB plasmid. A target fragment of the expected size including the UreB (~1.9 kb) and CTB-UreB (~2.2 kb) fusion gene was obtained by double-enzyme digestion. Recombinant plasmids were verified by sequencing.

For transfer of the gene fusion into *B. subtilis*, the CTB-UreB fusion gene was amplified using the pET-28a-CTB-UreB plasmid as a template. Recombinant pUS186-CotC-CTB-UreB plasmid was constructed and transformed into *B. subtilis* WB600 cells. The target DNA band of approximately 2.2 kb was obtained by XbaI/PstI double-enzyme digestion. Sequence analysis revealed that CTB-UreB was in the C-terminal of CotC and in frame.

**Expression of CTB-UreB in the spore coat**

The spore coat protein was extracted in SDS-DTT buffer by sonication, and 12 % SDS-PAGE analysis showed the recombinant CotC-CTB-UreB protein to be approximately 81.8 kDa (8.8 kDa CotC+12 kDa CTB+61 kDa UreB). The recombinant CotC-CTB and CotC-UreB proteins were approximately 20.8 and 69.8 kDa, respectively, as expected (Fig. 1a). Western blotting with specific antisera also revealed positive bands in recombinant strains (Fig. 1b).

**Oral administration of recombinant spores increased faecal IgA and serum IgG levels**

Oral administration of recombinant spores expressing CotC-UreB and CotC-CTB-UreB led to increased levels of UreB-specific secretory faecal IgA and serum UreB-specific IgG. Levels of faecal UreB-specific IgA (OD₄₅₀) in the CotC-UreB- (0.24±0.08)- and CotC-CTB-UreB (0.37±0.16)-treated mice were higher than that in the CotC-treated mice (0.042±0.02) after 2 weeks of treatment (P<0.01). IgA levels reached their peak after 4 weeks. Moreover, specific IgA levels in the CotC-CTB-UreB treatment group were higher than that in the CotC-UreB treatment group (P<0.05). There were no significant differences in the level of UreB-specific IgA between the naive, CotC and CotC-CTB treatment groups (P>0.05) (Fig. 2b).

Serum UreB-specific IgG levels (OD₄₅₀) in the CotC-UreB and CotC-CTB-UreB treatment groups were also significantly higher than those in the naive, CotC and CotC-CTB treatment groups after 2 weeks (P<0.01). There was also a significant difference between the CotC-UreB and CotC-CTB-UreB groups, with IgG levels being higher in the CotC-CTB-UreB group (0.58±0.16) than that in the CotC-UreB (0.44±0.13) group (P<0.05) (Fig. 2a). These findings indicate that oral administration of recombinant CotC-UreB or CotC-CTB-UreB spores can induce UreB-specific intestinal mucosal immunity and systemic immunity in mice. Moreover, CTB enhances UreB-specific immunity when fused with UreB in recombinant spores.

**Splenocytes from mice treated with recombinant spores secreted more IL-10 and IFN-γ**

We compared the levels of IL-4, IL-10 and IFN-γ in splenocytes isolated from mice following *in vitro* UreB protein stimulation. Splenocytes from mice treated with recombinant CotC-CTB, CotC-UreB or CotC-CTB-UreB produced significantly more IL-10 and IFN-γ than those from CotC-treated mice (CotC-CTB-UreB vs CotC, P<0.01; CotC-CTB, CotC-UreB vs CotC, P<0.05). Among these treatments, CotC-CTB-UreB stimulated splenocytes to produce significantly more IL-10 and IFN-γ than CotC-CTB or CotC-UreB (P<0.05). However, levels of IL-4 were not significantly different among the treatment groups after 2 weeks (P>0.05).
significantly different between any of the recombinant treatments groups and the CotC group (P>0.05) (Fig. 3).

**Protection efficacy of recombinant spores against H. pylori**

The prophylactic effects of recombinant spores were assessed by gastric *H. pylori* bacterial loads. Oral administration of recombinant spores expressing CotC-UreB or CotC-CTB-UreB significantly reduced bacterial loads in the stomach \[1.11\pm0.36 \times 10^5 \text{ c.f.u. (g tissue)}^{-1}\] compared to treatment with CotC spores \[5.56 \pm1.64 \times 10^5 \text{ c.f.u. (g tissue)}^{-1}, P<0.01\] (Fig. 4). There were no differences in gastric *H. pylori* bacterial load between mice treated with CotC or CotC-CTB spores and naive mice (P>0.05). Moreover, CotC-CTB-UreB spores were more effective at reducing bacterial load than CotC-UreB spores (90.5 % reduction vs 80.0 %, P<0.05).

**DISCUSSION**

*B. subtilis* is regarded as non-pathogenic, classified as a novel food which is currently used as a probiotic for both animal and human consumption [25]. *B. subtilis* can survive within the GIT; ingested *B. subtilis* spores are not only able to germinate in the small intestine but actually go on to re-sporulate as they move through the GIT [26]. Studies in animals have shown that mice given a fixed, oral dose of *B. subtilis* spores excreted more spores in their faeces than the dose administered [27]. These features of intestinal residency and safety make *B. subtilis* a suitable candidate for delivery of foreign antigens to the GIT [28].

Recent, studies in our laboratory have shown that recombinant *B. subtilis* that expressed *H. pylori* UreB on the surface inhibited *H. pylori* infection in a mouse model [18]. In the present study, we used CotC, a spore coat protein, as a fusion protein to develop a novel *B. subtilis* spore vaccine in which the surface-displaying mucosal adjuvant CTB was combined with the *H. pylori* vaccine candidate UreB. CTB is a strong mucosal adjuvant for uncoupled and coupled proteins when treated via the mucosal route [29]. Guo et al. [17] have shown that oral administration of rCTB-UreA could induce a humoral immune response to UreB and UreA, inhibiting *H. pylori* urease activity and protecting mice from *H. pylori* infection. Similarly, recombinant CTB-epitope vaccine consisting of T-cell and B-cell epitopes from both UreA and UreB induced protection against *H. pylori* infection in the Mongolian gerbil [30]. In the present study, we found that oral administration of spores expressing recombinant CotC-CTB-UreB significantly decreased the gastric bacterial load in mice (90.5 % reduction). This protective effect was better than that induced by spores expressing CotC-UreB alone (80 % reduction), indicating that CTB enhances the protective effect of UreB when fused with UreB on the surface of *B. subtilis* spores. Antibiotic resistance and patient compliance are currently regarded as the major limitations in eradicating *H. pylori* infection through proton-pump inhibitor-based triple therapy (see reference [31]); therefore, a safe, stable and easy-to-use vaccine could...
be an important supplement. In the present study, the average body weight (24.36±0.54 g) in CTB-UreB-treated mice (18 weeks) was significantly higher than that of the without-immunization control group (20.88±0.58 g, \( P < 0.01 \)) (data not shown), indicating that CTB-UreB spores could improve the health status of \( H. pylori \)-infected mice.

Secretory IgA aggregates bacterial pathogens, inhibits their motility and prevents their adherence to epithelial cells [32]. Studies have suggested that locally produced secretory IgA can inhibit \( H. pylori \) infection by blocking adhesion and colonization and allowing killing of the bacteria by monocytes or neutrophils through bacterial opsonization [33]. These findings indicate that specific secretory IgA may play a key role in immune exclusion of \( H. pylori \) in the stomach. In the present study, UreB-specific IgA levels in mice treated with CotC-CTB-UreB were significantly higher than those in mice treated with CotC-UreB, indicating that CTB enhanced specific mucosal immunity in mice orally administered recombinant CotC-CTB-UreB spores. Furthermore, a significant correlation was observed between decreasing bacterial loads and increasing mucosal UreB-specific sIgA in CotC-UreB and CotC-CTB-UreB treatment groups (data not shown), suggesting a protective role for mucosal sIgA during \( H. pylori \) infection.

**Fig. 2.** Urease B-specific serum IgG and faecal secretory IgA levels following treatment with \( B. subtilis \) spores. Groups of eight mice were treated orally with recombinant spores expressing CotC-CTB, CotC-UreB, CotC-CTB-UreB or CotC. Serum and faecal samples from mice were measured for serum urease B-specific IgG and faecal urease B-specific IgA by ELISA. (a) Serum urease B-specific IgG; (b) faecal urease B-specific secretory IgA. **\( P < 0.01 \) vs CotC group.

**Fig. 3.** Cytokine secretion by splenocyte cultures. Splenocyte cells were prepared and cultured for 72 h in the presence or absence of recombinant Urease B. Culture supernatant IL-4, IFN-\( \gamma \) and IL-10 levels were determined by ELISA. Data are expressed as means±SD of each group. **\( P < 0.01 \), *\( P < 0.05 \).
The Th1/Th2 gastric immune response profile is considered to be an important factor in reduction of gastric *H. pylori* bacterial load. Ikekawa *et al.* [34] showed that oral administration of CT and *H. pylori* whole-cell sonicate induced Th2 immunity and reduced gastric bacterial load in mice. However, Shi *et al.* [35] reported that oral administration of CpG-oligodeoxynucleotide and *H. pylori* whole-cell sonicate induced protection against *H. pylori* challenge, and this was related to high levels of IFN-γ and IgG2a and was abolished in IFN-γ-knockout mice. Taylor *et al.* [36] showed that *H. pylori* LPS−/CT vaccine induced Th2 immunity which induced no reduction in bacterial load, but an *H. pylori* LPS+/CpG vaccine, which promoted Th1 immunity, induced protection against *H. pylori* challenge. In our study, mice orally administered recombinant CotC-UreB or CotC-CTB-UreB *B. subtilis* spores displayed a bias towards Th1 immunity, with IFN-γ levels in splenic cultures significantly elevated compared to those in the control CotC group. Consistent with this, Huang *et al.* [37] showed that immunization of mice with recombinant *B. subtilis* spores that co-expressed *Bacillus anthracis* protective antigen with listeriolysin O increased specific IgG2a. This brought about an increase in toxin-neutralizing activity, coupled with specific IL-12 and IFN-γ responses of splenocytes, suggesting a Th1 bias response. Similarly, in a study by Mauriello *et al.* [38], oral administration of *B. subtilis* spores surface displaying the tetanus toxin fragment C induced splenic and mesenteric lymph node cell proliferation, as well as the production of IFN-γ in mice, indicating that the recombinant spores displaying the tetanus toxin fragment C induced a strong Th1 cell-mediated immune response.

IL-10 is an important anti-inflammatory cytokine, and IL-10−/− mice developed chronic lower bowel inflammation when infected with several *Helicobacter* spp. [39, 40]. Research has indicated that oral administration of attenuated *Salmonella typhimurium* expressing *H. pylori* UreB increases the secretion of IL-10 in splenic cell cultures [41]. In the present study, recombinant CotC-UreB, CotC-CTB and CotC-CTB-UreB spores significantly increased IL-10 levels in splenic cell cultures compared to those of non-recombinant CotC spores, and IL-10 levels in mice treated with CotC-CTB-UreB spores were higher than those in CotC-UreB spore-treated mice, suggesting that the adjuvant CTB promotes IL-10 secretion in splenic cell culture stimulated by UreB.

In conclusion, we have demonstrated that recombinant *B. subtilis* spores that express CotC-UreB or CotC-CTB-UreB on their surface induce Th1-predominant immunity and specific IgA secretion, which provides protection against *H. pylori* challenge. Moreover, CotC-CTB-UreB expression is more effective at reducing the *H. pylori* bacterial load than CotC-UreB, suggesting that recombinant CotC-CTB-UreB *B. subtilis* spores may represent a promising vaccine candidate for the control and prevention of *H. pylori* infection. However, recombinant CotC-CTB-UreB spores did not generate full protection (100 %) in mice. Ongoing studies by our group are constructing *B. subtilis* spores expressing CTB with other *H. pylori* vaccine candidates, such as cytotoxin vacA, heat shock proteins and neutrophil-activating protein A, and evaluating the prophylactic and therapeutic effects of individual and combined recombinant spore vaccines in mice. Also, further studies are needed for testing the efficacy of different vaccination strategies (two or three vaccinations), in order to simplify and optimize the present immunization strategies (nine vaccinations).

**Fig. 4.** *H. pylori* bacterial loads (c.f.u. (g stomach tissue)−1) in mice. Groups of eight mice were treated orally with spores surface expressing CotC-CTB-UreB, CotC-CTB, CotC-UreB or CotC, followed by oral challenge with 3×10^8^ c.f.u. of *H. pylori* SS1 and examination 4 weeks later. **P<0.01, *P<0.05.**

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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