Construction of prokaryotic expression system of \(ltB-ureB\) fusion gene and identification of the recombinant protein immunity and adjuvanticity

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Abstract
AIM: To construct \(ltB-ureB\) fusion gene and its prokaryotic expression system and identify immunity and adjuvanticity of the expressed recombinant protein.

METHODS: The \(ureB\) gene from a clinical Helicobacter pylori (\(H. pylori\)) strain Y06 and the \(ltB\) gene from Escherichia coli (\(E. coli\)) strain 44851 were linked into \(ltB-ureB\) fusion gene by PCR. The fusion gene sequence was analyzed after T-A cloning. A prokaryotic recombinant expression gene from a clinical \(H. pylori\) strain was inserted with \(ltB-ureB\) insertion (\(pET32a-ltB-ureB\)) was constructed. Expression of the recombinant LTB-UreB protein (rLTB-UreB) in \(E. coli\) BL21DE3 induced by isopropylthio-\(\beta\)-D-galactoside (IPTG) at different concentrations was detected by SDS-PAGE. Western blot assays were used to examine the immunoreaction of rLTB-UreB by a commercial antibody against whole cell of \(H. pylori\) and a self-prepared rabbit anti-rUreB serum, respectively, and determine the antigenicity of the recombinant protein on inducing specific antibody in rabbits. GM\(_1\)-ELISA was used to demonstrate the adjuvanticity of rLTB-UreB. Immunoreaction of rLTB-UreB to the UreB antibody positive sera from 125 gastric patients was determined by using ELISA.

RESULTS: In comparison with the corresponding sequences of original genes, the nucleotide sequence homologies of the cloned \(ltB-ureB\) fusion gene were 100%. IPTG with different dosages of 0.1-1.0 mmol/L could efficiently induce rLTB-UreB. The output of the target recombinant protein expressed by \(pET32a-ureB\) in \(E. coli\) BL21DE3 was approximately 35% of the total bacterial proteins, rLTB-UreB mainly presented in the form of inclusion body. Western blotting results demonstrated that rLTB-UreB could combine with the commercial antibody against whole cell of \(H. pylori\) and anti-rUreB serum as well as induce rabbit to produce specific antibody. The strong ability of rLTB-UreB binding bovine GM\(_1\) indicated the existence of adjuvanticity of the recombinant protein. All the UreB antibody positive sera from the patients (125/125) were positive for rLTB-UreB.

CONCLUSION: A recombinant prokaryotic expression system with high expression efficiency of the target fusion gene \(ltB-ureB\) was successfully established. The expressed rLTB-UreB showed qualified immunogenicity, antigenicity and adjuvanticity. All the results mentioned above laid a firm foundation for further development of \(H. pylori\) genetically engineered vaccine.

INTRODUCTION
In China, gastritis and peptic ulcer are the most prevalent gastric diseases and gastric cancer is one of the malignant tumors with high morbidities\(^{[1]}\). Helicobacter pylori (\(H. pylori\)), a microaerophilic, spiral and Gram-negative bacterium, is recognized as a human-specific gastric pathogen that colonizes the stomachs of at least half of the world’s populations\(^{[2]}\). Most infected individuals are asymptomatic. However, in some subjects, \(H. pylori\) infection causes acute, chronic gastritis or peptic ulceration. Furthermore, the infection is also a high risk factor for the development of peptic ulcer and gastric adenocarcinoma, mucosa-associated lymphoid tissue (MALT) lymphoma and primary gastric non-Hodgkin’s lymphoma\(^{[3,4]}\).

Recently, direct evidence of carcinogenesis of the microbe in an animal model has been presented\(^{[5,6]}\). Immunization against the bacterium represents a cost-effective strategy to prevent \(H. pylori\)-associated common peptic ulcer diseases and to reduce the incidence of global gastric cancers\(^{[7]}\). However, no vaccines preventing \(H. pylori\) infection have been commercially available so far.

Previous studies revealed many protective protein antigens of the microbe such as UreB, HpaA, FlaA, CagA, VacA etc\(^{[8-18]}\). Among these protein antigens, UreB, one of the four subunits of an urease produced by almost all the isolated strains of \(H. pylori\), has been demonstrated to have the strongest antigenicity and protection in all known proteins of \(H. pylori\)\(^{[13,19,20]}\), \( ureB\) gene, responsible for encoding UreB with 569 amino acid residues, is a highly conserved nucleotide sequence with a similarity of approximately 95% in different \(H. pylori\) isolates\(^{[21-23]}\).

These data strongly indicate that UreB can be used as an excellent antigen candidate for \(H. pylori\) vaccine.

Since a genetically engineered vaccine composed of a single protein antigen usually showed a low immunization effect, it is necessary to increase immunogenicity of the antigen by co-administration with an appropriate adjuvant. Escherichia coli (\(E. coli\)) heat-labile toxin B subunit (LTB) and cholera toxin B subunit (CTB) were well-confirmed mucosal adjuvant\(^{[24-28]}\). However, some of the previous studies demonstrated that the mucosal adjuvanticity of LTB was stronger than that of CTB\(^{[26,29]}\). Furthermore, CTB activates Th2 pathway, and induces IL-4, a...
cytokine closely related to IgE-mediated allergic reaction, but LTB mainly stimulates Th1 pathway[26,30].

In order to simplify the procedure steps and further reduce cost in *H. pylori* vaccine production, we constructed ltb-ureB fusion gene and its recombinant prokaryotic expression system. The immunogenicity, antigenicity and adjuvanticity of the expressed target recombinant protein (rLTB-UreB) was examined. The results of this study would benefit the mass production of *H. pylori* UreB-associated genetically engineered vaccine at a lower cost.

### MATERIALS AND METHODS

#### Materials

Both the *ureB* gene from a clinical *H. pylori* strain Y06 and the *ltB* gene from *E. coli* strain 44851 (offered by National Institute for the Control of Pharmaceuticals and Biological Products of China) was cloned by our laboratory[31]. A plasmid pET32a (Novagen, Madison, USA) and *E. coli* BL21DE3 (Novagen, Madison, USA) were used as the vector and host cell, respectively. Primers for PCR amplification were synthesized by BioAsia (Shanghai, China). *Taq*-plus high fidelity PCR kit and restriction endonucleases were purchased from TaKaRa (Dalian, China). The T-A Cloning Kit, DNAagarose Gel Purification Kit and sequencing service were purchased from TaKaRa (Dalian, China). The T-A Cloning Kit, DNA Agarose Gel Purification Kit and sequencing service were provided by BBST (Shanghai, China). DAKO (Glostrup, Denmark) and Jackson ImmunoResearch (West Grove, USA) supplied rabbit antiserum against whole cell of *H. pylori*, HRP-labeling sheep anti-rabbit IgG and anti-human IgG antibodies, respectively. The UreB antibody positive serum samples from 125 *H. pylori* infected patients with gastritis or ulcer were stored at -70 °C in our laboratory[31].

#### Methods

**Extraction of DNA templates** *E. coli* DH5α strains respectively containing plasmid *pUCm-T*-ureB, *pUCm-T-ltB* were cultured in LB medium. The two plasmids were extracted by alkaline- denatured method and then purified by DNase-free RNase treatment and routine phenol-chloroform method described by Sambrook et al.[32]. The obtained DNA extracts were dissolved in TE buffer and their concentrations as well as purity were measured by ultraviolet spectrophotometry[32]. The *pUCm-T*-ureB DNA was further digested with restriction endo-nucleases EcoRV and XhoI at 37 °C for 3 h. The target fragment of *ureB* gene was separated by agarose gel electrophoresis and then recovered by DNA Agarose Gel Purification Kit.

**Amplification of *ureB* and *ltB* gene** The sequence of *ltB* sense primer was: 5'-CCGGATATCTGAAATAAGTTAA AATTGTA-3' (*EcoR* V). The sequence of antisense primer linking the 5′-end of *ltB* gene and the 5′-end of *ureB* gene was: 5′-AGAAACATATTCTTTTCTGCTAATGTTTTCCATA CGTATTTGCCCGC-3′. Total volume per PCR was 100 µL containing 2.5 mol/L each dNTP, 250 nmol/L each of the two primers, 15 mol/L MgCl2, 2.5 U *Taq*-plus polymerase, 100 ng *pUCm-T-ltB* DNA template and 1×PCR buffer (pH8.3). Parameters for PCR of *ltB* gene were: at 94 °C for 5 min, ×1; at 94 °C for 30 s, at 48 °C for 30 s, at 72 °C for 45 s, ×10; at 94 °C for 30 s, at 48 °C for 30 s, at 72 °C for 50 s (an addition of 5 s for each of the following cycles), ×20; finally at 72 °C for 7 min, ×1. The results of PCR were observed under UV light after electrophoresis in 15 g/L agarose pre-stained with ethidium bromide. The expected size of target amplification fragment from *ltB* gene was 375 bp. The target fragment in the gel was recovered by using DNA Agarose Gel Purification Kit.

**Construction of *ltB*-ureB fusion gene by PCR** Total volume per tube was 90 µL containing all the PCR reagents mentioned above but not the primers, 100 ng of the recovered *ltB* DNA fragment with a cohesive end and 400 ng of the recovered *ureB* DNA fragment were added. Parameters for the following PCR were : at 94 °C for 5 min, ×1; at 94 °C for 30 s, at 45 °C for 30 s, at 72 °C for 150 s, ×10; at 72 °C for 10 min, ×1. After this PCR, the two fragments of *ureB* and *ltB* produced a complex fragment of *ureB*-ltB dependent on the cohesive end in the *ltB* fragment, which would be used as a template for the next PCR. The sense primer for *ureB*-ltB amplification was as previously mentioned. The sequence of antisense primer was: 5′-CGACCTCAGAGGA AAT GCTTATAGTGGTGTC-3' (*Xho* I). The 250 nmol/L each of the two primers was added into each of the tubes. Parameters for *ltB*-ureB amplification were: at 94 °C for 3 min, ×1; at 94 °C for 30 s, at 50 °C for 30 s, at 72 °C for 180 s, ×10; at 94 °C for 30 s, at 50 °C for 30 s, at 72 °C for 195 s (an addition of 15 s for each of the following cycles), ×15; at 72 °C for 12 min, ×1. Examination of the results of this PCR and recovery of the target fragment were the same as described above. The expected size of target amplification fragment from *ureB*-ltB fusion gene was 2 070 bp.

**T-A cloning, sequencing and subcloning of *ureB*-ltB fusion gene** The *ltB*-ureB amplification DNA fragment was cloned into plasmid vector *pUCm-T* (*pUCm-T-ltB*-ureB) by using T-A Cloning Kit according to the manufacturer’s instruction. The recombinant plasmid was amplified in *E. coli* DH5α and then recovered by Sambrook’s method[32]. A professional company (BBST) was responsible for nucleotide sequence analysis of the inserted fragment. Two plasmids *pUCm-T-ltB*-ureB and pET32a in two different strains of *E. coli* DH5α were amplified in LB medium were extracted and then digested with EcoRV and *Xho*I, respectively[32]. The fragment *ltB*-ureB were recovered and then ligased. The recombinant expression vector pET32a-*ltB*-ureB was transformed into *E. coli* BL21DE3, and the expression system was named as pET32a-*ltB*-ureB-Ecoli BL21DE3. The *ltB*-ureB fragment inserted in pET32a was sequenced again.

**Expression of the target recombinant protein** pET32a-*ltB*-ureB-Ecoli BL21DE3 was rotatively cultured in LB medium at 37 °C induced by isopropylthio-β-D-galactoside (IPTG) at different concentrations of 1.0, 0.5 and 0.1 mmol/L. The supernatant and precipitate were separated through centrifugation after the bacterial pellet was ultrasonically broken (300 V, 3×5 s). The molecular mass and output of the target recombinant protein (rLTB-UreB) were measured by SDS-PAGE.

**Identification of immunoreactivity and antigenicity of rLTB-UreB** The expressed rLTB-UreB was collected by Ni-NTA affinity chromatography. The commercial rabbit antiserum against whole cell of *H. pylori* or rabbit anti-rUreB serum prepared in our previous study and HRP-labeling sheep anti-rabbit IgG were used as the first and second antibodies, respectively, to determine the immunoreactivity of rLTB-UreB by Western blot. Rabbits were immunized with rUreB to prepare the antiserum and Western blot was applied again to determine the antigenity of rLTB-UreB.

GM1-ELISA GM1-ELISA GM1-ELISA was used to demonstrate the adjuvanticity of rLTB-UreB. Briefly, 40-well plates were coated by bovine GM1 (Sigma) and then added with rLTB-UreB. The rabbit anti-rLTB-UreB serum was used as the first antibody (1:100 dilution) and the commercial HRP-labeling sheep anti-human IgG (1:4 000 dilution) was applied as the second antibody. Each of the first antibody dilutions contained four wells. Negative controls without addition of rLTB-UreB with four repeated wells were set up and their mean A<sub>490</sub> value plus 3-fold SD values were used as the positive standard for each of the tested wells[33].

**ELISA** By using rLTB-UreB as coated antigen at the concentration of 20 µg/mL, each of the UreB antibody positive serum samples from the 125 patients (1:400 dilution) as the first antibody and HRP-labeling sheep anti-human IgG (1:4 000 dilution) as the second antibody, the immunoreaction of rLTB-UreB to the specific antibody in the sera were detected by
ELISA. In this assay, six UreB antibody negative serum samples were used as the control and the positive standard was similar to that in the GM1-ELISA.

**Statistical analysis**

The nucleotide sequence of the cloned ltB-ureB fusion gene was compared for homologies with the original sequences[31] by using a molecular biological analysis software.

**RESULTS**

**PCR**

The target fragments of ureB, ltB and ltB-ureB fusion gene with the expected sizes are shown in Figure 1.

**Nucleotide sequence analysis**

The homologies of the nucleotide sequences of the cloned ltB-ureB fusion gene compared with the original ltB and ureB gene sequences were 100%[31]. The nucleotide and putative amino acid sequences of the ltB-ureB fusion gene are shown in Figure 2.

**Expression of target fusion protein**

IPTG at concentrations of 1.0, 0.5 and 0.1 mmol/L could efficiently induce the expression of rLTB-UreB in E.coli system. The product of rLTB-UreB was generated and antisense primers, respectively. The framed area is the sequence from plasmid pUCm-T-ureB after digestion with both EcoR V and Xho I.

**Immunoreactivity and antigenicity of rLTB-UreB**

Commercial rabbit antibody against the whole cell of H pylori could combine with rLTB-UreB and induce rabbit to produce specific antibody as confirmed by Western blotting (Figure 4), respectively.

**GM1-ELISA**

Since the mean±SD of A490 of the negative control in the four repeated wells was 0.28±0.09, the positive reference value was 0.55. The mean±SD of A490 of the tested wells was 1.29±0.10, indicating that rLTB-UreB had the ability of binding to bovine GM1.

**ELISA**

Since the mean±SD of A490 values of the six UreB antibody negative serum samples was 0.17±0.03, the positive reference value for the specific antibody detection in patients’ sera was 0.26. According to the reference value, 100% (125/125) of the tested patients’ sera were positive for the antibodies against rLTB-UreB with an A490 value ranging from 0.37-1.98.

**Figure 1** Target amplification fragments of ltB and ureB genes and ltB-ureB fusion gene. Lane 1: 250 bp DNA marker (BBST); Lanes 2, 4 and 6: Blank controls; Lanes 3 and 5: Target amplification fragments of ltB gene and ltB-ureB fusion gene, respectively; Lane 7: Target recovered fragment of ureB gene from plUCm-T-ureB after digestion with both EcoR V and Xho I.

**Figure 2** Nucleotide and putative amino acid sequences of ltB-ureB fusion gene. Note: Underlined areas are sense, linking and antisense primers, respectively. The framed area is the sequence from plasmid pET32a. “*” means stop codon.

1: ATGAAATAGATTTTCTTATGATCTTCTCTCTCTCTATGACAC
2: N S K V K C Y V L T A L S L S L C A Y
3: GAGCTTTCCCGACTTATAGTACATCTTTCTCTCTATGACAC
4: 1 500 bp
5: 2 000 bp
6: 2 500 bp
7: 500 bp
8: 250 bp
9: 1801: GCTCATCATGGTAAAGCCAAATACGATGCAAACATCACTTTTGTGTCTCAAGCGGCTTAT
10: 1741: ATGGGCGATGCGAACGCTTCTATCCCTACCCCACAACCAGTTTATTACAGAGAAATGTTC
11: 1621: AGCGAGTATGTAGGTTCTGTAGAAGTGGGCAAAGTGGCTGACTTGGTATTGTGGAGTCCA
12: 1501: GCTGACAAAAACAAAAAAGAATTTGGCCGCTTGAAAGAAGAAAAAGGCGATAACGACAAC
13: 1381: CGCCCTCAAACTATTGCGGCTGAAGACACTTTGCATGACATGGGGATTTTCTCCATCACT
14: 1261: ACTAACCCCACTATCCCTTTCACTGTGAATACAGAAGCAGAACACATGGACATGCTTATG
15: 1201: GGCGGACACGCTCCTGATATTATTAAAGTGGCCGGCGAACACAACATTCTGCCCGCTTCC
16: 1141: GACACTATGGCAGCCATTGCCGGACGCACTATGCACACTTTCCACACTGAAGGCGCTGGT
17: 1081: AAATACGATGTGCAAGTCGCTATCCACACAGACACTTTGAATGAAGCCGGTTGTGTAGAA
18: 1021: ATCCACGAAGACTGGGGAACAACTCCTTCTGCAATCAATCATGCGTTAGATGTTGCGGAC
19: 961: AACCTACTACATGCGGGATGAGGATATATATAGTAGTCATCAACACTTTGCTCGTCTAAG
20: 921: GNMKRDMQDGVKNNLNLYSGPATE
21: 721: GCTTCCACGTGAAAGGTTTTATGTTAGCTACTCTGACGAGATTACCAACMCACMCHCCTC
22: 641: RNINTKDWTPSAINHLDYAV
23: 601: GYBYDYQVAIHTDTLDLINEAGCVC
24: 561: AFGVVKPPNMIKKGFLAIKLRG
25: 521: FRKRYLKSITYNPAPAHHGI
26: 481: SSSDSQAMGRGYGEYITRTWQT
27: 480: GGTAACAAAGACATGCAAGATGGCGTTAAAAACAATCTTAGCGTGGGTCCTGCTACTGAA
28: 461: TNPSTIPFTVNTENAEHMHMDML
29: 421: GTTGTCGACCATTCTGATAAATGATTTAAGTGCGCAGGACMACACMCACMCACGCTCGTC
30: 401: GBAPFIDIMVAGEHNIILPS
31: 381: BDMAAIAIAGRMWHMRITIVEAG
32: 361: AACACTTGCATTCCGAGACGAGCTACTACCTTTCTAATGACGAAAGCAGACATCCGACGCTT
33: 341: INHEDWGTTPSAINHLDYAV
34: 321: NTSNDASAQDGFLAIKLRG
35: 301: KWMRLAAEAYSMNLMFLKARG
36: 281: GTGGTCGACCATTCTGATAAATGATTTAAGTGCGCAGGACMACACMCACMCACGCTCGTC
37: 261: GCCTCTAATCAGTCTGCCGAGACCTTTGCTCTGACGAGATTACCAACMCACMCACMCHCCTC
38: 241: GCTCCATAACATGCGGGATGAGGATATATATAGTAGTCATCAACACTTTGCTCGTCTAAG
39: 221: GNMKRDMQDGVKNNLNLYSGPATE
40: 201: GICYKADIGIKGDGIAGIKGK
41: 181: NPSKSKEELDLILITNIALTYDV
42: 161: AAATATGACAAGATACTATCATATACGGAATCGATGGCAGGCAAAAGAGAAATGGTTATC
43: 141: KERVLGDSTDLVIAEVEHBDYT
44: 121: ATAAATGACAAGATACTATCATATACGGAATCGATGGCAGGCAAAAGAGAAATGGTTATC
45: 101: TKIIDKIVWNKNTPNISAI
46: 81: GM1-ELISA
47: 61: GAGCTTTCCCGACTTATAGTACATCTTTCTCTCTCTATGACAC
48: 41: AGTATGGAAAACATTAGCAGAAAAGAATATGTTTCTATGTATGGTCCTACTACAGGCGAT
49: 21: GIYKADIGIKGDGIAGIKGK
50: 2: YS0FQIPITAFASSGVTTMIGW
51: 1: MNNKVKCVYVLTTALLSSLCAY
52: 36: 490 value ranging from 0.37-1.98.
The most efficient mucosal adjuvant with few possibility of H pylori optimal antigen and adjuvant for developing orally taken vaccine, respectively. Corresponding blank controls. Lanes 2, rabbit antibody against whole cell of H pylori, respectively; Lanes 7 and 8: Bacterial precipitate and supernatant induced with 0.5 mmol/L IPTG, respectively. Lanes 1, 3 and 5: rLTB-UreB with rabbit antibody against whole cell of H pylori, and rabbit anti-rUreB and rLTB-UreB-immunized rabbit antisera. Lanes 2, 4 and 6: Corresponding blank controls.

The selection of antigenic targets is critical in the design of H pylori vaccine. A large number of published data showed that UreB might be the most definitive antigen candidate for H pylori vaccine\(^{12,19-23}\). On the other hand, LTB is found to be the most efficient mucosal adjuvant with few possibility of inducing allergic reactions\(^{24-26}\). So UreB and LTB should be the optimal antigen and adjuvant for developing orally taken H pylori vaccine, respectively.

In the present study, lbB-ureB fusion gene was obtained by using three PCRs and the nucleotide sequence of the gene showed absolutely the same as the corresponding ones. This data indicated that the method used for constructing fusion gene was highly efficient and of high fidelity.

The results of Western blotting in this study demonstrated that the rLTB-UreB could combine with both the commercial antibody against whole cell of H pylori and rabbit anti-rUreB serum. And this recombinant protein was able to efficiently induce rabbit to produce specific antibody. Furthermore, all the UreB antibody positive serum samples from 125 patients confirmed by our previous studies could recognize rLTB-UreB.

In the reports, the adjuvanticity of LTB was based on the binding ability to GM, receptor on the surface of cell\(^{27-31}\). In this study, the strong binding to GM, receptor of rLTB-UreB was confirmed by GM-ELISA. Therefore, rLTB-UreB with qualified immunoreactivity, antigenicity and adjuvanticity could be used to develop H pylori genetically engineered vaccine at lower costs.

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Edited by Zhu LH Proofread by Chen WW and Xu FM