

• *H pylori* •

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

Jie Yan, Yuan Wang, Shi-He Shao, Ya-Fei Mao, Hua-Wen Li, Yi-Hui Luo

Jie Yan, Ya-Fei Mao, Hua-Wen Li, Yi-Hui Luo, Department of Medical Microbiology and Parasitology, Medical College, Zhejiang University, Hangzhou 310031, Zhejiang Province, China

Yuan Wang, Shi-He Shao, Faculty of Laboratory Medicine, Northern University, Jilin 132001, Jilin Province, China

Supported by the Foundation of Ministry of Education of China for Outstanding Young Teachers

Correspondence to: Jie Yan, Department of Medical Microbiology and Parasitology, Medical College, Zhejiang University, 353 Yan An Road, Hangzhou 310031, Zhejiang Province, China. yanchen@mail.hz.zj.cn

Telephone: +86-571-87217385 **Fax:** +86-571-87217044

Received: 2003-12-28 **Accepted:** 2004-01-12

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 vector *pET32a* inserted with *ltB-ureB* fusion gene (*pET32a-ltB-ureB*) was constructed. Expression of the recombinant LTb-UreB protein (rLTb-UreB) in *E. coli* BL21DE3 induced by isopropylthio- β -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. GM₁-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 *pET32a-ltB-ureB-E.coli* BL21DE3 to express the rLTb-UreB. The output of the target recombinant protein expressed by *pET32a-ureB-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 GM₁ 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.

Yan J, Wang Y, Shao SH, Mao YF, Li HW, Luo YH. Construction of prokaryotic expression system of *ltB-ureB* fusion gene and identification of the recombinant protein immunity and adjuvanticity. *World J Gastroenterol* 2004; 10(18): 2675-2679

<http://www.wjgnet.com/1007-9327/10/2675.asp>

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^[3-8]. Recently, direct evidence of carcinogenesis of the microbe in an animal model has been presented^[9-11]. 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^[12]. 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*^[13-18]. 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 adjuvants^[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 adjuvant activity of the expressed target recombinant protein (rLTB-UreB) were 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 expression vector and host cell, respectively. Primers for PCR amplification were synthesized by BioAsia (Shanghai, China). *Taq*-plus high fidelity PCR kit and restriction endonucleases used 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* DH5 α strains respectively containing plasmid *pUCm-T-ureB*, *pUCm-T-ltb* were cultured in LB medium. The two plasmids were extracted by alkaline-denature 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 *EcoR* V and *Xho* I 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'-CCGGATATCATGAATAAAGTAA AATGTTA-3' (*EcoR* V). The sequence of antisense primer linking the 3'-end of *ltb* gene and the 5'-end of *ureB* gene was: 5'-AGAAACATATTCTTTCTGCTAATGTTTCCATA CTGATTGCCGC-3'. Total volume per PCR was 100 μ L containing 2.5 mol/L each dNTP, 250 nmol/L each of the two primers, 15 mol/L $MgCl_2$, 2.5 U *Taq*-plus polymerase, 100 ng *pUCm-T-ltb* DNA template and 1 \times PCR buffer (pH8.3). Parameters for PCR of *ltb* gene were: at 94 °C for 5 min, \times 1; at 94 °C for 30 s, at 48 °C for 30 s, at 72 °C for 45 s, \times 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), \times 20; finally at 72 °C for 7 min, \times 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 μ L 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, \times 1; at 94 °C for 30 s, at 45 °C for 30 s, at 72 °C for 150 s, \times 10; at 72 °C for 10 min, \times 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'-CGACTCGAGGAA AAT GCTAAAGAGTTGTGC-3' (*Xho* I). The 250 nmol/each of the two primers was added into each of the tubes. Parameters for *ltb-ureB* amplification were: at 94 °C for 3 min, \times 1; at 94 °C for 30 s, at 50 °C for 30 s, at 72 °C for 180 s, \times 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), \times 15; at 72 °C for 12 min, \times 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* DH5 α and then extracted 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* DH5 α after amplified in LB medium were extracted and then digested with *EcoR* V and *Xho* I, respectively^[32]. The fragment *ltb-ureB* and *pET32a* were recovered and then ligated. The recombinant expression vector *pET32a-ltb-ureB* was transformed into *E. coli* BL21DE3, and the expression system was named as *pET32a-ltb-ureB-E. coli* BL21DE3. The *ltb-ureB* fragment inserted in *pET32a* was sequenced again.

Expression of the target recombinant protein *pET32a-ltb-ureB-E. coli* 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 \times 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 antigenicity of rLTB-UreB.

GM₁-ELISA GM₁-ELISA was used to demonstrate the adjuvant activity 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_{490} 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 μ g/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* genes with the expected sizes are shown in Figure 1.

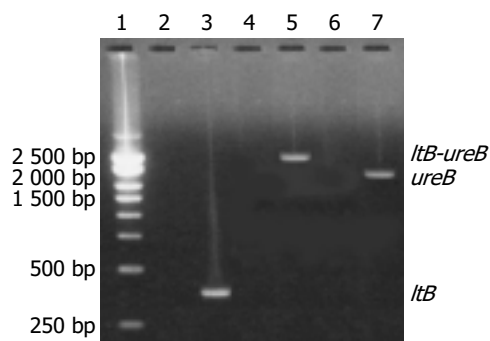


Figure 1 Target amplification fragments of *ltB* and *ureB* genes and *ltB-ureB* fusion gene. Lane 1: 250 bp DNA marker (BBST); Lanes 2, 4 and 6: Blank controls; Lanes 3 and 5: Target amplification fragments of *ltB* gene and *ltB-ureB* fusion gene, respectively; Lane 7: Target recovered fragment of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

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 *pET32a-ltB-ureB-E.coli* BL21DE3 system. The product of rLTB-UreB was mainly presented in the ultrasonic precipitate and the output was approximately 35% of the total bacterial proteins (Figure 3).

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 A_{490} 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 A_{490} 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 A_{490} 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 A_{490} value ranging from 0.37-1.98.

```

1: ATGAATAAAGTAAATGTTATGTTTATTACGGCGTTACTATCCTCTCTATGTCACAC
1: M N K V K C Y V L F T A L L S S L C A Y
61: GGAGCTCCCCAGTCTATTACAGAAGTATGTTTCGGAATATCGCAACACAAATATATACG
21: G A P Q S I T E L C S E Y R N T Q I Y T
121: ATAAATGACAAGATACTATCATATACGGAATCGATGGCAGGCAAAAGAGAAATGGTTATC
41: I N D K I L S Y T E S M A G K R E M V I
181: ATTACATTTAAGAGCGGCGCAACATTTTCAGGTGCAAGTCCCGGGCAGTCAACATATAGAC
61: I T F K S G A T F Q V E V P G S Q H I D
241: TCCCAAAAAAAGCCATTGAAAGGATGAAGGACACATTAAGAATCAGATCTGACCGGAG
81: S Q K K A I E R M K D T L R I T Y L T E
301: ACCAAAAATGATAAATTATGTGTATGGAATAATAAACCCCAATTCATTAATGCGGCAATC
101: T K I D K L C V W N N K T P N S I A A I
        ←ltB sequence | ureB sequence→
361: AGTATGAAAAACATTAGCAGAAAAAGAAATATGTTTCTATGTATGGTCTACTACAGCGCAT
121: S M E N I S R K E Y V S M Y G P T T G D
421: AAAGTGAGATTGGGCGATACAGACTTGATCGTGAAGTAGAACATGACTACACCTTTAT
141: K V R L G D T D L I A E V E H D Y T I Y
481: GCGGAAGAGCTTAAATTCGGTGCCGGAAGACTTTGAGGGAAGGCATGAGCCAATCCAAC
161: G E L K F G R G K T L R E G M S Q I Y T
541: AACCTAGCAAGAAGAAGTGGATTAAATCATCACTAACGCTTTAATCGTGGATTACACC
181: N P S K E E L D L I I T N A L I V D Y T
601: GGTATTATAAGCGGATATTGGTATTAAAGATGGCAAAATCGCTGGCATGGCAAAAGGC
201: G I Y K A D I G I K D G K I A G I G K G
661: GGTAACAAGACATGCAAGATGGCGTTAAAAACAATCTTACCGTGGTCTGCTACTGAA
221: G N K D M Q D G V K N N L S V G F A D T E
721: GCCTTAGCTGTGAAGGTTGATCGTAACTGCTGGTGGTATTGACACACATCCACTTC
241: A L A G E G L I V T A G G I D T H I H F
781: ATCTCCCCCAACAAATCCCTACAGCTTTTGAAGCGGTGAACAACCATGATTGGTGGC
261: I S P Q Q I P T A F A S G V T T M I G G
841: GGAAGTGGTCTGCTGATGGCACTAACGCAACCACTATCACTCCAGGCAAGAACTTA
281: G T G P A D G G T N A T I T P G R R N L
901: AAATGGATGCTCAGAGCGGTGAAGAATATTCATGAAGTATAGGTTTCTAGCTAAAGGT
301: K W M L R A A E E Y S M N L G F L A K G
961: AACACTTCTACAGATGCGAGCTTAGCCGATCAATTAAGCCGGTGGCATGGTTTTAA
321: N T S N D A S L A K I E A G A T A L S Q
1021: ATCCAGAAGACTGGGAACAACCTCTCTGCAATCAATCGTTAGATGTTGGCGAC
341: I H E D W G T T P S A I N H A L D V A D
1081: AAATACGATGTGCAAGTGCCTATCCACACAGACACTTTGAATGAAGCCGGTGTGTAGAA
361: K Y D V Q V A I H T D T L N E A G C V E
1141: GACACTATGGCAGCCATTGCGGAGCAGCACTATGCACACTTTCCACACTGAAGCGCGTGT
381: D T M A A I A G R T M H T E F H T E G A G
1201: GCGGACACGCTCTGATATTATTAAGTGGCGCGCAACACAACTCTGCCCGCTTCC
401: G G H A P D I I K V A G E H N I L P A S
1261: ACTAACCCCACTATCCCTTCTACTGTGAATACAGAAGCAGAACACATGGACATGCTTATG
421: T N P T I P F T V N T E A E H M D M L M
1321: GTGTGCCCACTTGGATAAAGCATTAAAGAAGATGTTCAAGTTCGCTGATTCAAGGATC
441: V C H L D K S I K E D V Q F A D L S Q
1381: CGCCCTCAAATATTGCGGCTGAAGACACTTTGCATGACATGGGGATTCTCCATCACT
461: R P Q T I A A E D T L H D M G I F S I T
1441: AGTTCTGACTCTCAAGCTATGGGTGCTGGTGAAGTTATCACTAGAAGTGGCAAAAC
481: S S D S Q A M G R V G E V I T R T W Q T
1501: GCTGACAAAAAAGAAATTTGGCGCGTGAAGAAGAAAAAGGCGATAACGACAA
501: A D K N K K E F G R L K E E K G D N A C
1561: TTCAGGATCAACGCTACTTGTCTAAATACACCATTAACCCAGCGATCGCTATGGGATT
521: F R I K R Y L S K Y T I N P A I A H G I
1621: AGCGAGTATGTAGTTCTGTAGAAGTGGCAAGTGGCTGACTTGGTATTGTGGAGTCCA
541: S E Y V G S V E V G K V A D L V L W S P
1681: GCATCTTTGGCGTGAAACCAACATGATCATCAAGGGCGGTTTCATTCGGTTAAGTCAA
561: A F F G V K P N M I I K G G V F I A L S Q
1741: ATGGGCGATGCGAAGCTTCTATCCCTACCCCAACACAGTTTATTACAGAGAAATGTTTC
581: M G D A N A S I P T P Q P V Y Y R E M F
1801: GCTCATCATGTTAAAGCCAAATACGATGCAAAATCACTTTTGTGTCTCAAGCGGCTTAT
601: A H H G K A K Y D A N I T F V S Q A A Y
1861: GACAAAGGCATTAAGAAGAAATTAGGCTTGAAGAGCAAGTGTTCGGGTAATAAATTCG
621: D K G I K E E L G L E R G V T L P V K N C
1921: AGAAACATCACTAAAAAGACATGCAATTAACGACACTACCGCTCACATTGAAGTCAAT
641: R N I T K K D M Q F N D T T A H I E V N
1981: CCTGAAACTTACCATGTGTTCTGGTGGTGGCAAGAAGTAAGTCTTAACACGACCACTAA
661: P E T Y H V F V D G K A G E V T S K P A K
2041: GTGAGCTTGGCACAACCTTTAGCATTTTCTCGAGACCAACCAACCACTGA
681: V S L A Q L F S I F L E H H H H H H *

```

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.

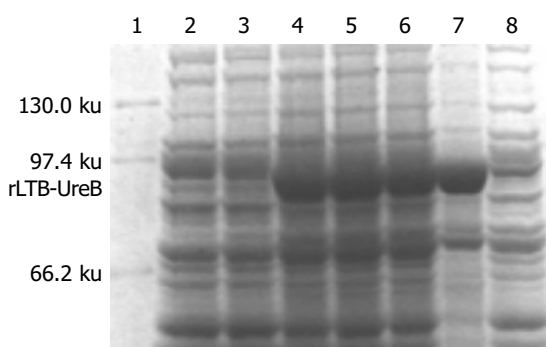


Figure 3 rLTB-UreB expression induced with different dosages of IPTG. Lane 1: Protein marker (Shanghai Shisheng); Lane 2: Blank control; Lane 3: Non-induced with IPTG; Lanes 4-6: Induced with 0.1, 0.5 and 1.0 mmol/L IPTG, respectively; Lanes 7 and 8: Bacterial precipitate and supernatant induced with 0.5 mmol/L IPTG, respectively.

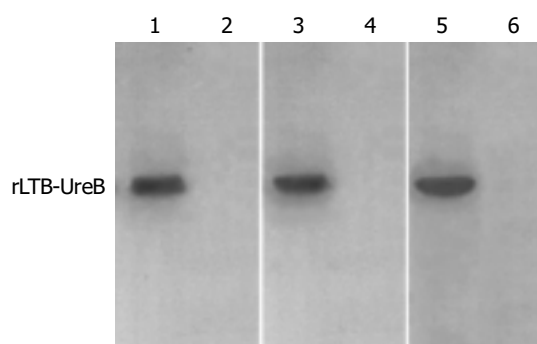


Figure 4 Western blotting of rLTB-UreB with rabbit antibody against whole cell of *H. pylori* and rUreB- and rLTB-UreB-immunized rabbit antisera. Lanes 1, 3 and 5: rLTB-UreB with rabbit antibody against whole cell of *H. pylori*, and rUreB- and rLTB-UreB-immunized rabbit antisera, respectively; Lanes 2, 4 and 6: Corresponding blank controls.

DISCUSSION

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^[13,19-23]. On the other hand, LTB is found to be the most efficient mucosal adjuvant with few possibility of inducing allergic reaction^[24-30]. So UreB and LTB should be the optimal antigen and adjuvant for developing orally taken *H. pylori* vaccine, respectively.

In the present study, *ltB-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.

SDS-PAGE performed in this study confirmed that the constructed prokaryotic expression system *pET32a-ltB-ureB-E.coli* BL21DE3 could produce rLTB-UreB with high efficiency even when the concentration of IPTG was as low as 0.1 mmol/L. The inclusion body as a major form of rLTB-UreB and higher output (35% of the total bacterial proteins) of the recombinant protein was beneficial to industrial production.

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 GM₁ receptor on the surface of cell^[27-33]. In this study, the strong binding to GM₁ receptor of rLTB-UreB was confirmed by GM₁-ELISA. Therefore, rLTB-UreB with qualified immunoreactivity, antigenicity and adjuvanticity could be used to develop *H. pylori* genetically engineered vaccine at lower costs.

REFERENCES

- Zhang Z, Yuan Y, Gao H, Dong M, Wang L, Gong YH. Apoptosis, proliferation and p53 gene expression of *H. pylori* associated gastric epithelial lesions. *World J Gastroenterol* 2001; 7: 779-782
- Michetti P, Kreiss C, Kotloff KL, Porta N, Blanco JL, Bachmann D, Herranz M, Saldinger PF, Cortesey-Theulaz I, Losonsky G, Nichols R, Simon J, Stolte M, Ackerman S, Monath TP, Blum AL. Oral immunization with urease and *Escherichia coli* heat-labile enterotoxin is safe and immunogenic in *Helicobacter pylori*-infected adults. *Gastroenterology* 1999; 116: 804-812
- Suganuma M, Kurusu M, Okabe S, Sueoka N, Yoshida M, Wakatsuki Y, Fujiki H. *Helicobacter pylori* membrane protein 1: a new carcinogenic factor of *Helicobacter pylori*. *Cancer Res* 2001; 61: 6356-6359
- Nakamura S, Matsumoto T, Suekane H, Takeshita M, Hizawa K, Kawasaki M, Yao T, Tsuneyoshi M, Iida M, Fujishima M. Predictive value of endoscopic ultrasonography for regression of gastric low grade and high grade MALT lymphomas after eradication of *Helicobacter pylori*. *Gut* 2001; 48: 454-460
- Uemura N, Okamoto S, Yamamoto S, Matsumura N, Yamaguchi S, Yamakido M, Taniyama K, Sasaki N, Schlemper RJ. *Helicobacter pylori* infection and the development of gastric cancer. *N Engl J Med* 2001; 345: 8298-8332
- Morgner A, Miehle S, Fischbach W, Schmitt W, Muller-Hermelink H, Greiner A, Thiede C, Schetelig J, Neubauer A, Stolte M, Ehninger G, Bayerdorffer E. Complete remission of primary high-grade B-cell gastric lymphoma after cure of *Helicobacter pylori* infection. *J Clin Oncol* 2001; 19: 2041-2048
- Kate V, Ananthakrishnan N, Badrinath S. Effect of *Helicobacter pylori* eradication on the ulcer recurrence rate after simple closure of perforated duodenal ulcer: retrospective and prospective randomized controlled studies. *Br J Surg* 2001; 88: 1054-1058
- Yao YL, Zhang WD. Relation between *Helicobacter pylori* and gastric cancer. *Shijie Huaren Xiaohua Zazhi* 2001; 9: 1045-1049
- Goto T, Nishizono A, Fujioka T, Ikewaki J, Mifune K, Nasu M. Local secretory immunoglobulin A and postimmunization gastritis correlate with protection against *Helicobacter pylori* infection after oral vaccination of mice. *Infect Immun* 1999; 67: 2531-2539
- Watanabe T, Tada M, Nagai H, Sasaki S, Nakao M. *Helicobacter pylori* infection induces gastric cancer in mongolian gerbils. *Gastroenterology* 1998; 115: 642-648
- Honda S, Fujioka T, Tokieda M, Satoh R, Nishizono A, Nasu M. Development of *Helicobacter pylori*-induced gastric carcinoma in mongolian gerbils. *Cancer Res* 1998; 58: 4255-4259
- Hatzifoti C, Wren BW, Morrow WJ. *Helicobacter pylori* vaccine strategies-triggering a gut reaction. *Immuno Today* 2000; 21: 615-619
- Cortesey-Theulaz I, Porta N, Glauser M, Saraga E, Vaney AC, Haas R, Kraehenbuhl JP, Blum AL, Michetti P. Oral immunization with *Helicobacter pylori* urease B subunit as a treatment against *Helicobacter* infection in mice. *Gastroenterology* 1995; 109: 115-121
- Opazo P, Muller I, Rollan A, Valenzuela P, Yudelevich A, Garcia-de la Guarda R, Urrea S, Venegas A. Serological response to *Helicobacter pylori* recombinant antigens in Chilean infected patients with duodenal ulcer, non-ulcer dyspepsia and gastric cancer. *APMIS* 1999; 107: 1069-1078
- Suerbaum S, Josenhans C, Labigne A. Cloning and genetic characterization of the *Helicobacter pylori* and *Helicobacter mustelae* *flaB* flagellin genes and construction of *H. pylori* *flaA*- and *flaB*-negative mutants by electroporation-mediated allelic exchange. *J Bacteriol* 1993; 175: 3278-3288
- Ghiara P, Rossi M, Marchetti M, Di Tommaso A, Vindigni C, Ciampolini F, Covacci A, Telford JL, De Magistris MT, Pizza

- M, Rappuoli R, Del Giudice G. Therapeutic intragastric vaccination against *Helicobacter pylori* in mice eradicates an otherwise chronic infection and confers protection against reinfection. *Infect Immun* 1997; **65**: 4996-5002
- 17 **Nilsson I**, Utt M. Separation and surveys of proteins of *Helicobacter pylori*. *J Chromatogr B Analyt Technol Biomed Life Sci* 2002; **771**: 251-260
- 18 **Marchetti M**, Arico B, Burrone D, Figura N, Rappuoli R, Ghiara P. Development of a mouse model of *Helicobacter pylori* infection that mimics human disease. *Science* 1995; **267**: 1655-1658
- 19 **Rupnow MF**, Owens DK, Shachter R, Parsonnet J. *Helicobacter pylori* vaccine development and use: a cost-effectiveness analysis using the Institute of Medicine Methodology. *Helicobacter* 1999; **4**: 272-280
- 20 **Pappo J**, Thomas WD Jr, Kabok Z, Taylor NS, Murphy JC, Fox JG. Effect of oral immunization with recombinant urease on murine *Helicobacter felis* gastritis. *Infect Immun* 1995; **63**: 1246-1252
- 21 **Tomb JF**, White O, Kerlavage AR, Clayton RA, Sutton GG, Fleischmann RD, Ketchum KA, Klenk HP, Gill S, Dougherty BA, Nelson K, Quackenbush J, Zhou L, Kirkness EF, Peterson S, Loftus B, Richardson D, Dodson R, Khalak HG, Glodek A, McKenney K, Fitzgerald LM, Lee N, Adams MD, Venter JC. The complete genome sequence of the gastric pathogen *Helicobacter pylori*. *Nature* 1997; **388**: 539-547
- 22 **Akada JK**, Shirai M, Takeuchi H, Tsuda M, Nakazawa T. Identification of the urease operon in *Helicobacter pylori* and its control by mRNA decay in response to pH. *Mol Microbiol* 2000; **36**: 1071-1084
- 23 **Labigne A**, Cussac V, Courcoux P. Shuttle cloning and nucleotide sequences of *Helicobacter pylori* genes responsible for urease activity. *J Bacteriol* 1991; **173**: 1920-1931
- 24 **Verweij WR**, de Haan L, Holtrop M, Agsteribbe E, Brands R, van Scharrenburg GJ, Wilschut J. Mucosal immunoadjuvant activity of recombinant *Escherichia coli* heat-labile enterotoxin and its B subunit: induction of systemic IgG and secretory IgA responses in mice by intranasal immunization with influenza virus surface antigen. *Vaccine* 1998; **16**: 2069-2076
- 25 **Tochikubo K**, Isaka M, Yasuda Y, Kozuka S, Matano K, Miura Y, Taniguchi T. Recombinant cholera toxin B subunit acts as an adjuvant for the mucosal and systemic responses of mice to mucosally co-administered bovine serum albumin. *Vaccine* 1998; **16**: 150-155
- 26 **Yamamoto M**, McGhee JR, Hagiwara Y, Otake S, Kiyono H. Genetically manipulated bacterial toxin as a new generation mucosal adjuvant. *Scand J Immunol* 2001; **53**: 211-217
- 27 **de Haan L**, Feil IK, Verweij WR, Holtrop M, Hol WG, Agsteribbe E, Wilschut J. Mutational analysis of the role of ADP-ribosylation activity and GM1-binding activity in the adjuvant properties of the *Escherichia coli* heat-labile enterotoxin towards intranasally administered keyhole limpet hemocyanin. *Eur J Immunol* 1998; **28**: 1243-1250
- 28 **Saito K**, Shoji J, Inada N, Iwasaki Y, Sawa M. Immunosuppressive effect of cholera toxin B on allergic conjunctivitis model in guinea pig. *Jpn J Ophthalmol* 2001; **45**: 332-338
- 29 **Tamura S**, Hatori E, Tsuruhara T, Aizawa C, Kurata T. Suppression of delayed-type hypersensitivity and IgE antibody responses to ovalbumin by intranasal administration of *Escherichia coli* heat-labile enterotoxin B subunit-conjugated ovalbumin. *Vaccine* 1997; **15**: 225-229
- 30 **Douce G**, Fontana M, Pizza M, Rappuoli R, Dougan G. Intranasal immunogenicity and adjuvanticity of site-directed mutant derivatives of cholera toxin. *Infect Immun* 1997; **65**: 2821-2828
- 31 **Xia XP**, Yan J, Zhao SF. Cloning, expression and identification of *Escherichia coli* *LTB* gene and *Vibrio cholerae* *CTB* gene. *Zhejiang Daxue Xuebao Yixueban* 2003; **32**: 17-20
- 32 **Sambrook J**, Fritsch EF, Maniatis T. Molecular Cloning, A Laboratory Manual [M]. 2nd edition. New York: Cold Spring Harbor Laboratory Press 1989: pp1.21-1.52, 2.60-2.80, 7.3-7.35, 9.14-9.22
- 33 **de Haan L**, Holtrop M, Verweij WR, Agsteribbe E, Wilschut J. Mucosal immunogenicity of the *Escherichia coli* heat-labile enterotoxin: role of the A subunit. *Vaccine* 1996; **14**: 260-266

Edited by Zhu LH Proofread by Chen WW and Xu FM