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• *H pylori* •

Construction of expression systems for *flaA* and *flaB* genes of *Helicobacter pylori* and determination of immunoreactivity and antigenicity of recombinant proteins

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Abstract

AIM: To clone flagellin genes A (*flaA*) and B (*flaB*) from a clinical strain of *Helicobacter pylori* (*H pylori*) and to construct prokaryotic expression systems of the genes and identify immunity of the fusion proteins.

METHODS: The *flaA* and *flaB* genes from a clinical *H pylori* isolate Y06 were amplified by high fidelity PCR. The nucleotide sequences of target DNA amplification fragments from the two genes were sequenced after T-A cloning. The recombinant expression vector *pET32a* inserted with *flaA* and *flaB* genes was constructed, respectively. The expressions of FlaA and FlaB fusion proteins in *E. coli* BL21DE3 induced by isopropylthio-β-D-galactoside (IPTG) at different concentrations were examined by SDS-PAGE. Western blot using commercial antibodies against whole cell of *H pylori* and immunodiffusion assay using self-prepared rabbit antiserum against FlaA (rFlaA) or FlaB (rFlaB) recombinant proteins were applied to the determination of the fusion proteins immunity. ELISA was used to detect the antibodies against rFlaA and rFlaB in sera of 125 *H pylori* infected patients and to examine rFlaA and rFlaB expression in 98 clinical isolates of *H pylori*, respectively.

RESULTS: In comparison with the reported corresponding sequences, the nucleotide sequence homologies of the cloned *flaA* and *flaB* genes were from 96.28-97.13 % and 96.31-97.73 %, and their putative amino acid sequence homologies were 99.61-99.80 % and 99.41-100 % for the two genes, respectively. The output of rFlaA and rFlaB expressed by *pET32a-flaA*-BL21DE3 and *pET32a-flaB*-BL21DE3 systems was as high as 40-50 % of the total bacterial proteins. Both rFlaA and rFlaB were able to combine with the commercial antibodies against whole cell of *H pylori* and to induce rabbits to produce specific antibodies with the same 1:2 immunodiffusion titers after the animals were immunized with the two recombinant proteins. Ninety-eight and zero point 4 and 92.80 % of the serum samples from 125 patients infected with *H pylori* were positive for rFlaA and rFlaB antibodies, respectively. One hundred percent and 98.98 % of the 98 tested isolates of *H pylori* were detectable for rFlaA and rFlaB epitopes, respectively.

CONCLUSION: Two prokaryotic expression systems with high efficiency of *H pylori flaA* and *flaB* genes were successfully established. The expressed rFlaA and rFlaB showed satisfactory immunoreactivity and antigenicity. High frequencies of FlaA and FlaB expression in different *H pylori* clinical strains and the general existence of specific antibodies against FlaA and FlaB in *H pylori* infected patients strongly indicate that FlaA and FlaB are excellent antigen candidates for developing *H pylori* vaccine.

Yan J, Liang SH, Mao YF, Li LW, Li SP. Construction of expression systems for *flaA* and *flaB* genes of *Helicobacter pylori* and determination of immunoreactivity and antigenicity of recombinant proteins. *World J Gastroenterol* 2003; 9(10): 2240-2250
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INTRODUCTION

In China, chronic gastritis and peptic ulceration are two most common gastric diseases, and gastric cancer is one of the malignant tumors with high mortalities and morbidities[1-34]. *Helicobacter pylori* (*H pylori*), a microaerophilic, spiral and Gram-negative bacterium, is considered as a human-specific gastric pathogen that colonizes the stomach of at least half of the world population[35]. Most infected individuals are asymptomatic. However, in some subjects, the infection causes acute, chronic gastritis and peptic ulceration, and plays important roles in the development of peptic ulcer and gastric adenocarcinoma, mucosa-associated lymphoid tissue (MALT) lymphoma and primary gastric non-Hodgkin' s lymphoma[36-43]. This microorganism has been categorized as class I carcinogen by the World Health Organization[44], and direct evidence of carcinogenesis has been recently demonstrated in animal models[45, 46]. Immunization against the bacterium represents a cost-effective strategy to prevent *H pylori*-associated peptic ulcer diseases and to reduce the incidence of global gastric cancer[47]. Selection of antigenic targets is critical in design of *H pylori* vaccine. So far, no vaccine preventing *H pylori* infection has been commercially available. The majority of studies attempting to produce a vaccine have focused on urease enzyme, heat shock protein, and vacuolating cytotoxin[35, 48-50], but rarely on *H pylori* flagellin. *H pylori* flagellin is composed of two subunits, named as FlaA with 53KDa and FlaB with 54 KDa respectively. The flagellin plays a main role in motility and is necessary for colonization or persistence of *H pylori* infection[51]. The motility of *H pylori* is a virulent factor in the pathogenesis of gastric mucosal injury[52]. The data mentioned

above indicate that FlaA as well as FlaB may be used as antigen candidates for *H pylori* vaccine. Therefore, in this study, two prokaryotic vectors responsible for expressing recombinant FlaA (rFlaA) and FlaB (rFlaB) were constructed. Immunoreactivity and antigenicity of rFlaA and rFlaB were further examined. Furthermore, these two recombinant proteins were used for detecting specific antibodies in sera from *H pylori* infected patients, and rabbit anti-rFlaA and anti-rFlaB sera were prepared for examining the corresponding epitopes of *H pylori* clinical isolates. The results of this study may contribute to the development of *H pylori* vaccines.

MATERIALS AND METHODS

Materials

A clinical strain of *H pylori* was used in this study, which was provisionally named Y06, and well-characterized by the Department of Medical Microbiology and Parasitology, College of Medical Sciences, Zhejing University. A plasmid *pET32a* (Novagen) and an *E. coli* strain BL21DE3 (Novagen) were used as the expression vector and host cell, respectively. Primers for PCR amplification, the Pfu-Taq high fidelity PCR kit and restriction endonucleases were purchased from BioAsia (Shanghai, China). The T-A cloning kit and sequencing service were provided by BBST (Shanghai, China). Rabbit antiserum against the whole cell of *H pylori*, HRP-labeling sheep antisera against rabbit IgG and against human IgG were purchased from DAKO and Jackson ImmunoResearch, respectively. Agents used in isolation and identification of *H pylori* were purchased from Sigma and bioMérieux. Gastric biopsy specimens with positive *H pylori* isolation from 126 patients (86 males and 40 females, age range: from 6-78 years old, mean age: 40.5 years old) referred for gastroduodenoscopic examination in four different hospitals in Hangzhou were collected during the period between December 2001 and June 2002. Each of the patients gave a written informed content for this study. Of the 126 patients, 68 had chronic gastritis (CG, 48 superficial, 10 active and 10 atrophic), and the other 58 had peptic ulcer disease (PUD, 12 gastric ulcer, 40 duodenal ulcer and 6 gastric and duodenal ulcer). None of the patients had taken nonsteroidal anti-inflammatory drugs, antacids and antibiotics during the two weeks before seeking medical advice. At the same time, serum specimens were also collected from these patients.

Methods

Isolation and identification of *H pylori* Each gastric biopsy specimen was homogenized with a tissue grinder and then inoculated on Columbia agar plates supplemented with 8.0 % (V/V) sheep blood, 0.5 % (W/V) cyclodextrin, 5 mg/L trimethoprim, 10 mg/L vancomycin, 2 500 U/L cefsoludin and 2.5 mg/L amphotericin B. The plates were incubated at 37 ℃ under microaerobic conditions (5 % O_2, 10 % CO_2 and 85 % N_2) for 3 to 5 days. A bacterial isolate was identified as *H pylori* according to typical Gram staining morphology, biochemical tests positive for urease and oxidase, and agglutination with the commercial rabbit antibody against whole cell of the microbe. All of *H pylori* isolates were stored at -70 ℃ for ELISA.

Preparation of DNA template Genomic DNA of *H pylori* strain Y06 was extracted by the conventional phenol-chloroform method and DNase-free RNase treatment[52]. The obtained DNA was dissolved in TE buffer, and its concentration and purity were determined by ultraviolet spectrophotometry[52].

Polymerase chain reaction Oligonucleotide primers were designed to amplify the whole sequence of *flaA* and *flaB* genes from *H pylori* strain Y06 based on the published corresponding genomic sequences[53-56]. The sequence of *flaA* sense primer with an endonuclease site of *Eco*RV was 5' -CCG GATATCATGGCTTTTCAGGTCAA-3' . The sequence of *flaA* antisense primer with an endonuclease site of *Xho*I was 5' -CCGCTCGAGAAACTAAGTTAAAAGCC-3' . The sequence of *flaB* sense primer with an endonuclease site of *Eco*RI was 5' -CCGGAATTCATGAGTTTTAGGATAAA-3' . The sequence of *flaB* antisense primer with an endonuclease site of *Xho*I was 5' -CCGCTCGAGCTGTTATTGTAAAA GCC-3' . The total volume per PCR was 100 μl containing 2.5 mol· L^{-1} each dNTP, 500 nmol· L^{-1} each of the two primers, 15 mol· L^{-1} $MgCl_2$, 3.0 U Pfu-Taq polymerase, 100 ng DNA template and 1×PCR buffer (pH8.8). The parameters for PCR were at 94 ℃ for 5 min, ×1; at 94 ℃ for 30 s, at 52 ℃ for 30 s, at 72 ℃ for 90 s, ×10; at 94 ℃ for 30 s, at 52 ℃ for 30 s, at 72 ℃ for 100 s (10 s addition for the each of the following cycles), ×15; then at 72 ℃ for 10 min, ×1. The results of PCR were observed under UV light after electrophoresis in 15 g· L^{-1} agarose pre-stained with ethidium bromide. The expected sizes of target amplification fragments were 1 530 bp for *flaA* gene and 1 542 bp for *flaB* gene.

Cloning and sequencing The target amplification DNA fragments from *flaA* and *flaB* genes were respectively cloned into *pUCm*-T vectors (*pUCm*-T-*flaA* and *pUCm*-T-*flaB*) by using the T-A cloning kit according to the manufacturer' s instructions. The recombinant plasmids were amplified in an *E. coli* strain DH5α and then extracted by the Sambrook' s method[57]. A professional company (BBST) was responsible for nucleotide sequence analysis of the inserted fragments. Three strains of *E. coli* DH5α containing *pUCm*-T-*flaA*, *pUCm*-T-*flaB* and expression vector *pET32a* were amplified in BL medium, and the three plasmids were extracted, respectively[57]. These plasmids were digested with *Eco*RV and *Xho*I, *Eco*RI and *Xho*I, respectively. The *flaA* target fragment and *pET32a*, and the *flaB* fragment and *pET32a* were recovered and then ligased. The recombinant expression vectors *pET32a-flaA* and *pET32a-flaB* were respectively transformed into *E.coli* BL21DE3, and the expression systems were named as *pET32a-flaA*-BL21DE3 and pET32a-*flaB*-BL21DE3. The target fragments of *flaA* and *flaB* genes inserted in *pET32a* plasmid were sequenced again.

Expression and identification of fusion proteins *pET32a-flaA*-BL21DE3 and *pET32a-flaB*-BL21DE3 were rotatively cultured in LB medium at 37 ℃ induced by isopropylthio-β-D-galactoside (IPTG) at different concentrations of 1.0, 0.5 and 0.1 mmol· L^{-1}. The supernatant and precipitate were separated through centrifugation after the bacterial pallet was ultrasonically broken (300V, 5 s×3). The molecular weight and output of rFlaA and rFlaB were examined by SDS-PAGE. The two recombinant proteins were collected by Ni-NTA affinity chromatography. The commercial rabbit antiserum against whole cell of *H pylori* and HRP-labeling sheep antiserum against rabbit IgG were used as the first and second antibodies to determine the immunoreactivity of rFlaA and rFlaB by Western blot. Rabbits were immunized with rFlaA and rFlaB, respectively, for preparation of antisera. Immunodiffusion assay was applied to the determination of the antigenicity of rFlaA and rFlaB.

ELISA The specific antibodies against FlaA and FlaB in sera of the 126 patients infected with *H pylori* were detected by ELISA, by using rFlaA and rFlaB as antigens at the coated concentration of 20 μg/ml and a patient serum sample (1:400 dilution) as the first antibody and HRP-labeling sheep antibody against human IgG (1:4 000 dilution) as the second antibody. The result of ELISA for a patient' s serum sample was considered as positive if the optical density at 490 nm (OD_{490}) was over the mean plus 3 SD of five negative serum samples[58]. FlaA and FlaB expression in clinical isolates of *H pylori* was detected by ELISA using the ultrasonic supernatant of each *H pylori*

isolate (50 μg/ml) as a coated antigen, the self-prepared rabbit antisera against rFlaA and rFlaB (1:800 dilution in both) as the first antibody and HRP-labeling sheep antibody against rabbit IgG (1:3 000 dilution) as the second antibody. The result of ELISA for a *H pylori* ultrasonic supernatant sample was considered as positive if its OD_{490} value was over the mean plus 3 SD of five ultrasonic supernatant samples at the same protein concentration of *E. coli* ATCC 25922[58].

Date analysis The nucleotide sequences of the cloned *flaA* and *flaB* genes were compared for homology with the 3 published *flaA* gene sequences (NC000915, NC000921, X60746)[53-55] and the 4 published *flaB* gene sequences (NC000915, NC000921, L08907, AF479024)[53,54,56,59] by using a molecular biological analysis software.

RESULTS

PCR

Target fragments of *flaA* and *flaB* genes with expected sizes amplified from DNA template of *H pylori* stain Y06 are shown in Figure 1.

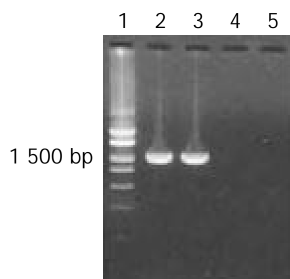

Figure 1 Target fragments of *flaA* and *flaB* genes amplified from *H pylori* strain Y06 DNA.

Nucleotide sequence analysis

The nucleotide sequences of *flaA* gene in *pUCm*-T-*flaA* and *pET32a-flaA* were completely the same and so as for *flaB* gene. The homologies of nucleotide and putative amino acid sequences of the cloned *flaA* gene compared with the published *flaA* sequences[53-55] were from 96.28 % to 97.13 % and from 99.61 % to 99.80 %, respectively (Figures 2 and 3). The homologies of nucleotide and putative amino acid sequences of the cloned *flaB* gene were 96.31-97.73 % and 99.41-100 %, compared with the published *flaB* sequences (Figures 4 and 5)[53,54,56,59].

Expression of target fusion proteins

IPTG at concentrations of 1.0, 0.5 and 0.1 mmol· L^{-1} efficiently induced the expression of rFlaA and rFlaB in *pET32a-flaA*-BL21DE3 and *pET32a-flaB*-BL21DE3 systems. The products of rFlaA and rFlaB were mainly presented in ultrasonic precipitates, and the output was 40-50 % of the total bacterial proteins (Figures 6 and 7).

Immunoreactivity and antigenicity of rFlaA and rFlaB

The commercial rabbit antibodies against the whole cell of *H pylori* combined with rFlaA and rFlaB as confirmed by Western blot (Figures 8 and 9). Both the titer of immunodiffusion assay between rFlaA and its rabbit antiserum, rFlaB and its rabbit antiserum was 1:2.

ELISA

Since the mean ±SD of OD_{490} values of the five negative serum samples were 0.338±0.036 for rFlaA and 0.102±0.051 for rFlaB in the detection of specific antibodies in patients' sera, the positive reference value was 0.446 for FlaA and 0.255 for FlaB. According to the reference values, 98.4 % (123/125, one serum sample was contaminated) of the tested patients' serum samples were positive for antibodies against rFlaA with an OD_{490} value range of 0.52-1.76, and 92.8 % (116/125) were positive for antibodies against rFlaB with an OD_{490} value range of 0.26-1.50. Since the mean ±SD of OD_{490} of the five negative bacterial controls was 0.200±0.046 for FlaA and 0.170±0.044 for FlaB in the detection of clinical *H pylori* isolates, the positive reference value was 0.338 for FlaA and 0.302 for FlaB. According to the reference values, 100 % (98/98) of the tested *H pylori* isolates were detectable for the epitope of rFlaA with an OD_{490} value range of 0.36-2.01 and 99 % (97/98) of the isolates were detectable for the epitope of rFlaB with an OD_{490} value range of 0.31-1.78.

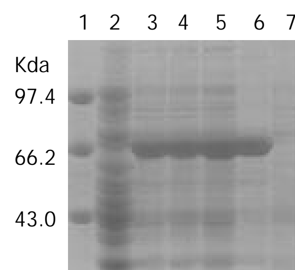

Figure 6 Expression of rFlaA induced by IPTG at different concentrations.

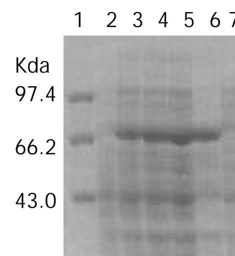

Figure 7 Expression of rFlaB induced by IPTG at different concentrations.

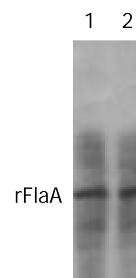

Figure 8 Western blot result of rabbit antibodies against whole cell of *H pylori* and rFlaA.

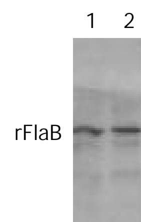

Figure 9 Western blot result of rabbit antibodies against whole cell of *H pylori* and rFlaB.

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(1)1     ATGGCTTTTCAGGTCAATACAAATATCAATGCGATGAATGCGCATGTGCAATCCGCACTC
(2)1     ............................................................
(3)1     .......................................................A....
(4)1     ............................................................

(1)61    ACTCAAAATGCGCTTAAAACTTCATTGGAGAGATTGAGTTCAGGTTTAAGGATTAATAAA
(2)61    ........C.....................C.............................
(3)61    ............................................................
(4)61    ........C..A..................C......................C......

(1)121   GCGGCTGATGATGCATCAGGCATGACGGTGGCAGATTCTTTGCGTTCACAAGCGAGCAGT
(2)121   ...........C....................G...........................
(3)121   ...........C....................G...........................
(4)121   ...........C....................G..............G............

(1)181   TTGGGTCAAGCGATTGCCAACACGAATGACGGCATGGGGATTATCCAAGTTGCGGATAAG
(2)181   ...............................................G............
(3)181   ...............................................G............
(4)181   ...............................................G............

(1)241   GCTATGGATGAGCAGTTAAAAATCTTAGACACCGTTAAGGTTAAAGCGACTCAAGCGGCT
(2)241   ............................................................
(3)241   ............................................................
(4)241   ..............A.............................................

(1)301   CAAGACGGGCAAACTACGGAATCTCGTAAAGCGATTCAATCTGACATCGTTCGTTTGATT
(2)301   .....T...........A..........................................
(3)301   .....T......................................................
(4)301   .....T......................................................

(1)361   CAAGGTTTAGATAATATCGGTAACACGACTACTTATAACGGGCAAGCGTTATTGTCTGGT
(2)361   ...........C...........T....................................
(3)361   ...........C...........T....................................
(4)361   ........G.................A..G..............................

(1)421   CAATTCACTAACAAAGAATTCCAAGTAGGGGCTTATTCTAACCAAAGCATTAAGGCTTCT
(2)421   ........C............................................A......
(3)421   ...............................................T............
(4)421   ............................................................

(1)481   ATCGGCTCTACCACTTCGGATAAAATCGGTCAGGTTCGTATCGCTACAGGCGCGTTAATC
(2)481   .................C................................T.........
(3)481   .................C..........................................
(4)481   .................C..........................................

(1)541   ACGGCTTCTGGGGATATTAGCTTGACTTTTAAACAAGTGGATGGCGTGAATGATGTAACT
(2)541   ..C........A................................................
(3)541   ............................................T...............
(4)541   ............................................................

(1)601   TTAGAGAGCGTAAAAGTTTCTAGTTCAGCAGGCACGGGGATCGGTGTGTTAGCGGAAGTG
(2)601   ...................................A.....T..C........A......
(3)601   .................C.................A.....T..C........A......
(4)601   ...................................A........C........A......

(1)661   ATTAACAAAAATTCTAACCGAACAGGGGTTAAAGCTTATGCGAGCGTTATCACCACGAGC
(2)661   ........G..C..............C........C........................
(3)661   ..C.........................................................
(4)661   ..C..T.....C................................................

(1)721   GATGTGGCGGTCCAATCAGGAAGTTTGAGTAATTTAACTTTAAATGGGATCCATTTGGGT
(2)721   ..............G.......................C...........T........G
(3)721   ..............G.......................C....................C
(4)721   ..............G.......................C...........T.........

(1)781   AATATCGCAGATATTAAGAAAAATGACTCAGACGGAAGGTTAGTCGCAGCGATCAATGCG
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(2)781  .......................C............C.A.....................
(3)781  ............................................................
(4)781  .......................C....................................

(1)841  GTTACTTCAGAAACCGGCGTGGAAGCTTATACGGATCAAAAAGGGCGCTTGAATTTGCGC
(2)841  ............................................................
(3)841  .................T..........................................
(4)841  ..C..............T..........................................

(1)901  AGTATAGATGGTCGTGGGATTGAAATCAAAACCGATAGCGTCAGTAATGGGCCTAGTGCT
(2)901  ......................................T.....................
(3)901  ...................................C........................
(4)901  ...................................C...........C........C...

(1)961  TTAACGATGGTCAATGGCGGTCAGGATTTAACAAAAGGTTCTACTAACTATGGGAGGCTT
(2)961  ...........T..........................C...........C..A......
(3)961  ......................................C...........C..A......
(4)961  ......................................C...........C..A......

(1)1021 TCTCTCACACGCTTAGACGCTAAAAGCATCAATGTCGTTTCGGCTTCTGATTCGCAACAT
(2)1021 .......................G..........................C.........
(3)1021 ...........A...........G...........A.....A........C..A..G...
(4)1021 ...........A...........G..........................C..A..G...

(1)1081 TTAGGTTTCACAGCGATTGGTTTTGGGGAATCTCAAGTGGCAGAAACCACGGTGAATTTG
(2)1081 .....C......................................................
(3)1081 ............................................................
(4)1081 .....C.....G................................................

(1)1141 CGCGATGTTACTGGGAATTTTAACGCTAATGTCAAATCAGCCAGTGGCGCGAACTATAAC
(2)1141 ...........C...................................T............
(3)1141 ...........C................................................
(4)1141 ............................................................

(1)1201 GCCGTGATCGCTAGCGGTAACCAAAGCTTGGGATCTGGGGTTACAACCTTGAGAGGCGCG
(2)1201 .....C........T..C..T.............................A.....T...
(3)1201 .....C........T..C..T.............................A.........
(4)1201 ..................................................A.........

(1)1261 ATGGTGGTGATTGATATTGCGGAATCGGCGATGAAAATGTTGGATAAAGTCCGCTCTGAT
(2)1261 ..............C.....C..G..T..........................A......
(3)1261 ....................C..G..T.................................
(4)1261 ..............C.....C.....A................................C

(1)1321 TTAGGTTCTGTGCAAAATCAAATGATTAGCACCGTGAATAACATCAGCATCACTCAAGTG
(2)1321 ............................................................
(3)1321 ............................................................
(4)1321 ................................T...........................

(1)1381 AATGTTAAAGCGGCTGAGTCTCAAATCAGGGATGTGGATTTTGCTGAAGAGAGCGCGAAT
(2)1381 .................A..........................................
(3)1381 .................A..........................................
(4)1381 .................A.......................C..................

(1)1441 TTCAATAAAAACAATATTTTGGTGCAATCAGGCAGCTATGCGATGAGTCAAGCTAACACC
(2)1441 ..............C.......CA........T....................C......
(3)1441 ..............C.......C..............................C......
(4)1441 .....C........C.......C..............................C..T...

(1)1501 GTCCAACAAAATATCTTAAGGCTTTTAACTTAG
(2)1501 ..T..............................
(3)1501 ..T..............................
(4)1501 ..T..............................
```

Figure 2 Homologies of nucleotide sequence of cloned *H pylori flaA* gene with reported sequences. (1): the sequencing result of *H pylori* strain Y06 *flaA* gene; (2)-(4): the reported sequences from GenBank (No. NC000915, strain 26695; No. NC_000921, strain J99; No. X60746, strain 898-1). Underlined areas indicate the positions of primer sequences.

```
(1)1   MAFQVNTNINAMNAHVQSALTQNALKTSLERLSSGLRINKAADDASGMTVADSLRSQASS
(2)1   ............................................................
(3)1   ............................................................
(4)1   ..................E.........................................

(1)61  LGQAIANTNDGMGIIQVADKAMDEQLKILDTVKVKATQAAQDGQTTESRKAIQSDIVRLI
(2)61  ............................................................
(3)61  ............................................................
(4)61  ............................................................

(1)121 QGLDNIGNTTTYNGQALLSGQFTNKEFQVGAYSNQSIKASIGSTTSDKIGQVRIATGALI
(2)121 ............................................................
(3)121 ............................................................
(4)121 ............................................................

(1)181 TASGDISLTFKQVDGVNDVTLESVKVSSSAGTGIGVLAEVINKNSNRTGVKAYASVITTS
(2)181 ............................................................
(3)181 ............................................................
(4)181 ............................................................

(1)241 DVAVQSGSLSNLTLNGIHLGNIADIKKNDSDGRLVAAINAVTSETGVEAYTDQKGRLNLR
(2)241 ............................................................
(3)241 ............................................................
(4)241 ............................................................

(1)301 SIDGRGIEIKTDSVSNGPSALTMVNGGQDLTKGSTNYGRLSLTRLDAKSINVVSASDSQH
(2)301 ............................................................
(3)301 ............................................................
(4)301 ............................................................

(1)361 LGFTAIGFGESQVAETTVNLRDVTGNFNANVKSASGANYNAVIASGNQSLGSGVTTLRGA
(2)361 ............................................................
(3)361 ............................................................
(4)361 ............................................................

(1)421 MVVIDIAESAMKMLDKVRSDLGSVQNQMISTVNNISITQVNVKAAESQIRDVDFAEESAN
(2)421 ............................................................
(3)421 ............................................................
(4)421 ............................................................

(1)481 FNKNNILVQSGSYAMSQANTVQQNILRLLT      510aa
(2)481 .......A......................
(3)481 .......A......................
(4)481 .......A......................
```

Figure 3 Homologies of putative amino acid sequence of *H pylori flaA* gene with reported sequences. (1): the sequencing result of cloned *H pylori* strain Y06 *flaA* gene; (2)-(4): the reported sequences from GenBank (No. NC000915, strain 26695; No. NC_000921, strain J99; No. X60746, strain 898-1).

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(1)1    ATGAGTTTTAGGATAAATACCAATATCGCCGCTTTAACTTCTCATGCGGTAGGGGTTCAA
(2)1    ............................................................
(3)1    ..................................................G.........
(4)1    ............................................................
(5)1    ............................................................

(1)61   AACAACAGAGACCTTTCAAGCTCGCTTGAAAAGTTAAGCTCAGGGCTTAGGATCAATAAG
(2)61   ...........................................................A
(3)61   ............................................T..............A
(4)61   ...........................................................A
(5)61   ...........................................................A

(1)121  GCCGCTGACGATTCTAGTGGGATGGCGATCGCTGATAGCTTAAGGAGTCAAAGCGCGAAT
(2)121  ............................................................
(3)121  ............................................................
(4)121  ............................................................
(5)121  ............................................................
```

```
(1)181  TTAGGCCAGGCGATTCGCAACGCTAATGACGCTATTGGTATGGTTCAAACCGCTGATAAA
(2)181  ..G..T..A.....C........C.............................A......
(3)181  ............................................................
(4)181  ..G..T..A.....C.............................................
(5)181  ..G.........................................................

(1)241  GCGATGGATGAGCAAATCAAAATCTTAGACACCATTAAAACCAAAGCCGTTCAAGCCGCT
(2)241  ............................................................
(3)241  ............................................................
(4)241  ............................................................
(5)241  ............................................................

(1)301  CAAGATGGGCAAACTTTAGAAAGCCGAAGAGCACTCCAGAGCGATATTCAAAGGTTGTTA
(2)301  ................................G...........................
(3)301  ................................G...........................
(4)301  ................................G...........................
(5)301  ................................G...........................

(1)361  GAAGAACTGGACAATATCGCTAACACCACAAGCTTTAACGGCCAACAAATGCTTTCAGGA
(2)361  ............................................................
(3)361  ........A...................................................
(4)361  ........A...................................................
(5)361  ........A...........C.......................................

(1)421  AGTTTTTCTAACAAAGAATTTCAAATTGGCGCGTATTCTAACACCACGGTTAAAGCGTCT
(2)421  ..........................................G.................
(3)421  ............................................................
(4)421  ............................................................
(5)421  .....................................................G......

(1)481  ATTGGCTCAACAAGCTCAGATAAGATTGGGCATGTGCGCATGGAAACCTCTTCTTTTAGC
(2)481  ...........G...................................T............
(3)481  ...........G..T..G.....A.......................T..A.........
(4)481  ...........G...........A..C....................T............
(5)481  ...........G...........A....................................

(1)541  GGTGAAGGCATGCTCGCTAGCGCGGCGG CGCAAAACTTGACTGAAGTGGGATTGAATTT
(2)541  ....C....................... A..............................
(3)541  ............................ ...............................
(4)541  ..........................C.G...... ........................
(5)541  ..C....................A.... ...............................

(1)600  CAAACAAGTCAATGGCGTGAACGATTATAAGATTGAAACCGTGCGCATTTCTACGAGCGC
(2)600  .....................T...................................T..
(3)600  .....................T......................................
(4)600  .....................T......................................
(5)600  .....................T................................A.....

(1)660  TGGCACTGGGATCGGAGCGTTAAGCGAAATCATCAATCGTTTTTCTAACACTTTAGGCGT
(2)660  ............T...............................................
(3)660  C...........T..G.......................C............C.......
(4)660  ......A.....T...............................................
(5)660  ......A.....T..G...........G...........C............C.......

(1)720  TAGGGCGTCTTATAATGTCATGGCTACCGGCGGCACTCCCGTGCAATCAGGAACTGTTAG
(2)720  ......T.....................................................
(3)720  ...A..A................................T..............C..G..
(4)720  ..........................................A.................
(5)720  ......T........C..............T.....A..G.................G..

(1)780  GGAGCTTACCATTAATGGCGTAGAAATTGGGACCGTGAATGATGTGCATAAAAATGACGC
(2)780  ......................................................C.....
(3)780  A.............................A.......................C.....
(4)780  ......................................................C.....
(5)780  A..A..C.......................A................G......C.....

(1)840  TGATGGGAGGTTGACTAATGCGATCAACTCCGTCAAAGACAGGACCGGCGTGGAAGCGAG
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(2)840  .........A...........A.......................G..T............
(3)840  ...C...................................TC.C....T...........
(4)840  ...C........................................................
(5)840  ...C..C..A...................................G.....A........

(1)900  CTTGGATATTCAAGGGCGCATTAATTTGCACTCCATTGACGGGCGTGCGATTTCTGTGCA
(2)900  ...........................A.................C..............
(3)900  .......................................T.....C..............
(4)900  .............................................C..............
(5)900  .......................................T.....C..............

(1)960  TGCAGCGAGCGCGAGCGGTCAGGTTTTTGGGGGAGGGAATTTTGCAGGGATTTCTGGGAC
(2)960  ............................................................
(3)960  ................................................A...........
(4)960  .........T.................................A................
(5)960  ..................C.........................................

(1)1020 ACAACATGCGGTTATTGGGCGCTTAACCTTGACCAGGACCGACGCTAGAGACATCATTGT
(2)1020 ...G................................A.......................
(3)1020 ...G.............................T....................T..C..
(4)1020 ...G.....A..........................A.................T.....
(5)1020 ...G..........................A..T..A........C.....T........

(1)1080 GAGCGGTGTGAATTTTAGCCATGTGGGCTTTCATTCCGCTCAAGGGGTGGCAGAATACAC
(2)1080 ...............C............................................
(3)1080 ............................................................
(4)1080 ............................................................
(5)1080 ............C...............................................

(1)1140 CGTGAATTTGAGAGCGGTTAGGGGCATTTTTGATGCGAATGTGGCTTCAGCAGCCGGAGC
(2)1140 ............................................................
(3)1140 ...................................................G........
(4)1140 .........................................................G..
(5)1140 ............G...............................................

(1)1200 GAACGCTAATGGCGCACAAGCGGAGACCAATTCTCAAGGTATAGGGGCTGGGGTAACAAG
(2)1200 ...............G............................................
(3)1200 ...............G.............................A..C..A........
(4)1200 .....................T.................C....................
(5)1200 ...............G............................................

(1)1260 CCTTAAAGGAGCGATGATTGTGATGGATATGGCGGACTCAGCGCGCACGCAATTAGACAA
(2)1260 .........G.......................A..T.................G.....
(3)1260 .........G.......................A..T.......................
(4)1260 .........G.................C........T.......................
(5)1260 .........G..........................T..T....................

(1)1320 GATCCGCTCGGATATGGGTTCGGTGCAAATGGAATTGGTTACAACCATTAATAATATTTC
(2)1320 ............................................................
(3)1320 ..........................................C........C........
(4)1320 ......A.....................................................
(5)1320 ..........................................C.................

(1)1380 TGTAACCCAAGTGAATGTTAAAGCGGCTGAATCTCAAATCAGAGATGTGGATTTTGCTGA
(2)1380 ............................................................
(3)1380 ......T.......................G..............C..............
(4)1380 ............................................................
(5)1380 .............................................C..............

(1)1440 AGAAAGTGCGAACTTTTCTAAATACAATATTTTGGCGCAAAGCGGGAGTTTTGCTATGGC
(2)1440 ...G..C.....................................................
(3)1440 ...G..C.....................................................
(4)1440 ...G........................................................
(5)1440 ...G..C...................................T.................

(1)1500 ACAAGCGAATGCGGTGCAACAAAATGTCTTAAGGCTTTTACAATAA
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```
(2)1500 G.............................................
(3)1500 G................................A............
(4)1500 .....................G........................
(5)1500 G....................G........................
```

Figure 4 Homologies of nucleotide sequence of cloned *H pylori flaB* gene with reported sequences. (1) the sequencing result of *H pylori* strain Y06 *flaB* gene; (2)-(5): the reported sequences from GenBank (No. NC000915, strain 26695; No. NC_000921, strain J99; No. L08907, strain 85P; No. AF479024, strain CH-CTX1). Underlined areas indicate the positions of primer sequences.

```
(1)1    MSFRINTNIAALTSHAVGVQNNRDLSSSLEKLSSGLRINKAADDSSGMAIADSLRSQSAN
(2)1    ............................................................
(3)1    ............................................................
(4)1    ............................................................
(5)1    ............................................................

(1)61   LGQAIRNANDAIGMVQTADKAMDEQIKILDTIKTKAVQAAQDGQTLESRRALQSDIQRLL
(2)61   ............................................................
(3)61   ............................................................
(4)61   ............................................................
(5)61   ............................................................

(1)121  EELDNIANTTSFNGQQMLSGSFSNKEFQIGAYSNTTVKASIGSTSSDKIGHVRMETSSFS
(2)121  ..................................A.........................
(3)121  ............................................................
(4)121  ............................................................
(5)121  ............................................................

(1)181  GEGMLASAAAQNLTEVGLNFKQVNGVNDYKIETVRISTSAGTGIGALSEIINRFSNTLGV
(2)181  .A..........................................................
(3)181  ............................................................
(4)181  .........GA.................................................
(5)181  ............................................................

(1)241  RASYNVMATGGTPVQSGTVRELTINGVEIGTVNDVHKNDADGRLTNAINSVKDRTGVEAS
(2)241  ............................................................
(3)241  ............................................................
(4)241  ............................................................
(5)241  ...................................R........................

(1)301  LDIQGRINLHSIDGRAISVHAASASGQVFGGGNFAGISGTQHAVIGRLTLTRTDARDIIV
(2)301  ............................................................
(3)301  ............................................................
(4)301  ..................................T.........................
(5)301  ............................................................

(1)361  SGVNFSHVGFHSAQGVAEYTVNLRAVRGIFDANVASAAGANANGAQAETNSQGIGAGVTS
(2)361  ............................................................
(3)361  ............................................................
(4)361  ............................................................
(5)361  ............................................................

(1)421  LKGAMIVMDMADSARTQLDKIRSDMGSVQMELVTTINNISVTQVNVKAAESQIRDVDFAE
(2)421  ............................................................
(3)421  ............................................................
(4)421  ............................................................
(5)421  ............................................................

(1)481  ESANFSKYNILAQSGSFAMAQANAVQQNVLRLLQ    514aa
(2)481  ..................................
(3)481  ..................................
(4)481  ..................................
(5)481  ..................................
```

Figure 5 Homologies of putative amino acid sequences of *H pylori flaB* gene with reported sequences. (1): the sequencing result of *H pylori* strain Y06 *flaB* gene; (2)-(5): the reported sequences from GenBank (No. NC000915, strain 26695; No. NC_000921, strain J99; No. L08907, strain 85P; No. AF479024, strain CH-CTX1).

DISCUSSION

In the present study, *H pylori flaA* and *flaB* genes were detected in genomic DNA of almost all *H pylori* isolates, and their nucleotide and amino acid sequences were considerably conserved[53,54]. The FlaA and FlaB expressed by *H pylori* rendered the organism strong motility in mucous environment, induced IL-8 secretion and facilitated inflammation in gastric tissue[51,52]. Furthermore, we observed that serum antibodies against FlaA and FlaB were present in approximate 98.4 % and 92.8 % of *H pylori* infected patients, respectively, the rates were significantly higher than those of heat shock protein (68 %) and vacuolating cytotoxin (68 %)[60]. These data indicate that *flaA* and *flaB* genes express their products in majority of *H pylori* strains and efficiently induce specific antibodies, implying a brilliant potential for developing *H pylori* vaccine.

The *flaA* gene from *H pylori* strain Y06, cloned in this study, showed high homologies of nucleotide and putative amino acid sequences compared with the published corresponding sequences (Figures 2 and 3)[53-55]. Similarly, the homologies of nucleotide and putative amino acid sequences of the cloned *flaB* gene from *H pylori* strain Y06 were quite high when compared with the published corresponding sequences (Figures 4 and 5)[53,54,56-59]. The high conservation of nucleotide and putative amino acid sequences found in the cloned *flaA* and *flaB* genes were probably due to their expression products just as the structural peptides of *H pylori*.

In the present study, SDS-PAGE demonstrated that the constructed expression systems *pET32a-flaA*-BL21DE3 and *pET32a-flaB*-BL21DE3 were able to efficiently produce the target recombinant proteins. However, rFlaA and rFlaB were mainly presented with the form of inclusion body even if they were induced by IPTG at a lower concentration. The high output of rFlaA and rFlaB (40-50 %) was beneficial to the production of a possible *H pylori* vaccine.

The rabbit antiserum against the whole cell of *H pylori* recognizes and combined with rFlaA and rFlaB as confirmed by Western blot, indicated that the two recombinant proteins had a relatively high immunoreactivity. The immunodiffusion assay performed in this study demonstrated that rFlaA and rFlaB could efficiently induce rabbit to produce specific antibodies with a higher titer, which indicated that these two recombinant proteins exhibited favorable antigenicity.

All tested *H pylori* isolates (98/98) expressed FlaA while 99.0 % (97/98) of the tested isolates expressed FlaB, as detected by ELISA. Of the *H pylori* infected patients, 98.4 % (123/125) and 92.8 % (116/125) were seropositive for the specific antibodies against rFlaA and against rFlaB, respectively. The universal existence of FlaA and FlaB in *H pylori* strains and the efficient induction of specific antibodies against FlaA and FlaB in patients were the strong favorable evidences for using these two recombinant proteins as the potential antigens in the development of *H pylori* vaccine.

In conclusion, FlaA and FlaB are excellent and ideal antigens that can be potentially used for the development of *H pylori* vaccine, and the expression systems of FlaA and FlaB with a high efficiency has been successfully constructed.

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