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Construction of hpaA gene from a clinical isolate of *Helicobacter pylori* and identification of fusion protein

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Abstract

AIM: To clone hpaA gene from a clinical strain of *Helicobacter pylori* and to construct the expression vector of the gene and to identify immunity of the fusion protein.

METHODS: The hpaA gene from a clinical isolate Y06 of *H. pylori* was amplified by high fidelity PCR. The nucleotide sequence of the target DNA amplification fragment was sequenced after T-A cloning. The recombinant expression vector inserted with hpaA gene was constructed. The expression of HpaA fusion protein in *E.coli* BL21DE3 induced by IPTG at different dosages was examined by SDS-PAGE. Western blot with commercial antibody against whole cell of *H.pylori* as well as immunodiffusion assay with self-prepared rabbit antiserum against HpaA fusion protein were applied to determine immunity of the fusion protein. ELISA was used to detect the antibody against HpaA in sera of 125 patients infected with *H.pylori* and to examine HpaA expression of 109 clinical isolates of *H.pylori*.

RESULTS: In comparison with the reported corresponding sequences, the homologies of nucleotide and putative amino acid sequences of the cloned hpaA gene were from 94.25-97.32 % and 95.38-98.46 %, respectively. The output of HpaA fusion protein in its expression system of pET32a-hpaA-BL21DE3 was approximately 40 % of the total bacterial proteins. HpaA fusion protein was able to combine with the commercial antibody against whole cell of *H.pylori* and to induce rabbit producing specific antiserum with 1:4 immunodiffusion titer after the animal was immunized with the fusion protein. 81.6 % of the serum samples from 125 patients infected with *H.pylori* (102/125) were positive for HpaA antibody and all of the tested isolates of *H.pylori* (109/109) were detectable for HpaA.

CONCLUSION: A prokaryotic expression system with high efficiency of *H.pylori* hpaA gene was successfully established. The HpaA expressing fusion protein showed satisfactory immunoreactivity and antigenicity. High frequencies of HpaA expression in different *H.pylori* clinical strains and specific antibody production in *H.pylori* infected patients indicate that HpaA is an excellent and ideal antigen for developing *H.pylori* vaccine.

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INTRODUCTION

In China, chronic gastritis and peptic ulcer are the most prevalent gastric diseases and gastric cancer is one of the malignant tumors with high morbidities[1-34]. *Helicobacter pylori*, a microaerophilic, spiral and gram-negative bacillus, is recognized as a human-specific gastric pathogen that colonizes the stomaches of at least half of the world's populations[35]. Most infected individuals are asymptomatic. However, in some subjects, the infection causes acute, chronic gastritis or peptic ulceration, and plays an important role 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 organism is categorized as a class I carcinogen pronounced by the World Health Organization[44], and direct evidence of carcinogenesis was recently demonstrated in an animal model[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 cancers[47]. The selection of antigenic targets is critical in the design of *H.pylori* vaccine. To date, this field is scarcely touched upon. The majority of studies focused on urease enzyme, heat shock protein, vacuolating cytotoxin, and so on[35,48-50], but rarely on *H. pylori* adhesin (HpaA) which is a flagellar sheath protein with approximately 29KDa located in the bacterial outer membrane[51]. So, in this study, a recombinant plasmid inserted with the gene (hpaA) responsible for encoding HpaA was constructed and the immunogenicity and immunoreactivity of its expression product were examined. Furthermore, the fusion protein of HpaA and its rabbit antiserum were also used to detect serum samples from *H.pylori* infected patients and *H. pylori* isolates, respectively. The results of this study will be helpful for determining whether the recombinant HpaA (rHpaA) becomes one of the good candidates as an antigen in *H.pylori* vaccine.

MATERIALS AND METHODS

Materials

A well-characterized clinical strain of *H.pylori*, provisionally named Y06, was provided by the Department of Medical Microbiology and Parasitology, College of Medical Sciences, Zhejing University. Plasmid pET32a (Promega) and *E.coli* BL21 DE3 (Promega) were used as expression vector and host cell, respectively. The primers for amplification were synthesized by BioAsia (Shanghai, China). EX Taq high fidelity PCR kit and restriction endonucleases were purchased from TaKaRa (Dalian, China). T-A cloning kit and sequencing service were offered by BBST (Shanghai, China). Rabbit antibody against the whole cell of *H.pylori* and HRP-labeling sheep antibodies against rabbit IgG and human IgG were

purchased from DACO and Jackson ImmunoResearch, respectively. The agents used in isolation and identification of *H.pylori* were purchased from Sigma and bioMérieux, etc. 126 biopsy specimens from patients with positive *H.pylori* (86 males and 40 females; age range: 6-78 years; mean age: 40.5 years) for gastroduodenoscopy in four different hospitals in Hangzhou were collected during the period of the end of 2001 to the midyear of 2002. Each of the patients consented to be enrolled in this study and all of them agreed to offer their biopsy samples. Among the 126 patients, 68 suffered from chronic gastritis (CG) in cluding 48 with chronic superficial gastritis, 10 with chronic active gastritis and 10 with chronic atrophic gastritis, and 58 patients suffered from peptic ulcer (PU) in cluding 12 with gastric ulcer, 40 with duodenal ulcer and 6 with gastric and duodenal ulcer. None of the patients had received nonsteroidal anti-inflammatory drugs or antacid drugs and antibiotics within the previous two weeks before the study. At the same time, 126 serum specimens from these patients were also collected.

Methods

Isolation and identification of clinical *H.pylori* strains Each of the biopsy specimens was homogenized with a tissue grinder and then inoculated onto Columbia agar plates supplemented with 8.0 % (V/V) sheep blood, 0.2 % (W/V) cyclodextrin, 5 mg/L trimethoprim, 10 mg/L vancomycin, 2.5 mg/L amphotericin B and 2 500 U/L polymyxin 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. Isolates were identified as *H.pylori* according to typical Gram stain morphology, positive biochemical tests for urease and oxidase, and agglutination with 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 routine phenol-chloroform method, DNase-free RNase digestion and phenol-chloroform extraction. The DNA template was solved 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 hpaA gene from *H.pylori* strain Y06 based on the published corresponding genome sequence[51,53]. The sequence of sense primer with an endonuclease site of *BamH* I was: 5' -CCGGGATCCATGA GAGCAAATAATC-3' . The sequence of antisense primer with an endonuclease site of *EcoR* I was: 5' -CCGGAATTCTTCT TATGCGTTATTTG-3' . The total volume of PCR reaction mixture was 100 μL containing 2.5 mol· L^{-1} each dNTP, 250 nmol· L^{-1} each primer, 15 mol· L^{-1} $MgCl_2$, 3.0 U EX Taq polymerase, 200 ng DNA template and 1×PCR buffer (pH8.3). The parameters for PCR were as follows: at 94 ℃ for 5 min, one cycle; at 94 ℃ for 30 sec, at 50 ℃ for 30 sec, at 72 ℃ for 60 sec, 10 cycle; at 94 ℃ for 30 sec, at 50 ℃ for 30 sec, at 72 ℃ for 70 sec (additional 10 sec for each of the following cycles), 20 cycle, at 72 ℃ for 10 min, 1 cycle. The results of PCR were observed under UV light after electrophoresis in 15 g· L^{-1} agarose pre-stained with ethidium bromide. The expected size of target amplification fragment was 802 bp.

Cloning and sequencing The target amplification DNA fragment of hpaA gene was cloned into pUCm-T vector (pUCm-T-hpaA) by using the T-A cloning kit according to the manufacturer' s instructions. The recombinant plasmid was amplified in *E.coli* strain DH5α and then extracted by Sambrook' s method[52]. A professional company (BBST) was responsible for nucleotide sequence analysis of the inserted fragments. Two different strains of *E.coli* DH5α containing pUCm-T-hpaA and expression vector pET32a were amplified respectively and the two plasmids were extracted[52]. The plasmids were digested with *BamH* I and *EcoR* I. The target fragments of hpaA and pET32a were recovered and then ligased. The recombinant expression vector pET32a-hpaA was transformed into *E.coli* BL21DE3. pET32a-hpaA was prepared for sequencing again after the amplification in its host cell.

Expression and identification of the fusion protein The hpaA prokaryotic expression system pET32a-hpaA-BL21DE3 was rotationally cultured in LB medium at 37 ℃ under induction of isopropylthio-β-D-galactoside (IPTG) at different dosages of 1, 0.5 and 0.1 mmol· L^{-1}. The supernatant and precipitate were isolated by centrifugation after the engineering bacterium pellet was ultrasonically broken (300V, 5sec once for three times). The molecular weight, output and location of HpaA fusion protein were examined by SDS-PAGE. HpaA fusion protein was collected by Ni-NTA affinity chromatography. The immunoreactivity of HpaA fusion protein was determined by Western blot with commercial rabbit antibody against whole cell of *H.pylori* and HRP-labeling sheep antibody against rabbit IgG, respectively. Rabbits were immunized with HpaA fusion protein to obtain the antiserum. Immunodiffusion assay was applied to determine antigenicity of the fusion protein.

ELISA The antibodies against HpaA in sera of the patients infected with *H.pylori* were detected by ELISA with HpaA fusion protein at the concentration of 20 μg/mL as coated antigen, the patients' sera (1:200 dilution) as the first antibody, HRP-labeling sheep antibody against human IgG (1:4 000 dilution) as the second antibody and ortho-phenylene diamine as a substrate. The result of ELISA for a patient' s serum sample was considered as positive if its OD_{490} value was over the mean plus 3 SD of 6 cases of negative serum samples[54]. HpaA expression of clinical isolates of *H.pylori* was detected by ELISA using ultrasonic supernatant at the protein concentration of 50 μg/mL of each *H.pylori* isolate as coated antigen, the self-prepared rabbit antiserum against HpaA fusion protein (1:2 000 dilution) as the first antibody, HRP-labeling sheep antibody against rabbit IgG (1:3 000 dilution) as the second antibody and ortho-phenylene diamine as a substrate. The result of ELISA for an *H.pylori* ultrasonic supernatant was considered as positive if its OD_{490} value was over the mean plus 3 SD of 6 cases of ultrasonic supernatant at the same protein concentration of *E.coli* ATCC 25922 played as negative control[54].

Data analysis The nucleotide sequences of the cloned hpaA gene inserted in the two recombinant plasmid vectors were compared for homologies with 6 published hpaA gene sequences (X92502, NC000915, X61574, NC000921, AF479028 and U35455)[51,53,55-58] with a special molecular biological analysis soft ware.

RESULTS

PCR

Target fragment with expected size amplified from DNA template of *H.pylori* strain Y06 is shown in Figure 1.

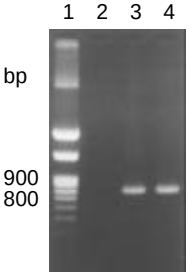

Figure 1 The target fragment of hpaA gene amplified from *H.pylori* strain Y06 (1: 100 bp DNA marker; 2: blank control; 3 and 4: the target fragment amplified from *H.pylori* strain Y06 DNA).

Nucleotide sequence analysis

The hpaA gene nucleotide sequences in the recombinant plasmid vectors of pUCm-T-hpaA and pET32a-hpaA were completely the same. The homologies of the nucleotide and putative amino acid sequences in the pUCm-T-hpaA and pET32a-hpaA compared with the published hpaA gene sequences[51,53,55-58] were from 94.25-97.32 % and 95.38-98.46 %, respectively (Figures 2, 3).

```
[1]1   ATGAGAGCAAATAATCATTTTAAAGATTTTGCATGGAAAAAATGCCTTTTAGGCGCGAGC
[2]1   ....A.....................................................T......
[3]1   ....A.A.....GG.........G.............................A.....
[4]1   ....A.A.....GG.........G.....................T.............
[5]1   ....A.A.....GG.........G.....................................T
[6]1   ....A.A.....GG.........G.....................................
[7]1   ................................................................

[1]61  GTGGTGGCTTTATTAGTGGGATGCAGCCCGCATATTATTGAAACCAATGAAGTCGCTTTG
[2]61  ...........G.....................................................
[3]61  ....................G..............................G.........
[4]61  ....................G..T..........................T......
[5]61  ................................................................
[6]61  ....G...........................................................
[7]61  ...........G..G..................................................

[1]121 AAATTGAATTACCATCCAGCTAGCGAGAAAGTTCAAGCGTTAGATGAAAAGATTTTGCTT
[2]121 ................................................................
[3]121 ............................G...................................
[4]121 ...........................................................A...
[5]121 ................................................................
[6]121 ..G.............................................................
[7]121 ...........................................................C......

[1]181 TTAAGGCCAGCTTTCCAATATAGCGATAATATCGCTAAAGAGTATGAAAACAAATTCAAG
[2]181 ..............T.....C...........T.........................
[3]181 ....A.............C........C..T.................T.........
[4]181 ....................C...........T.........................
[5]181 ....................C...........T.........................
[6]181 ..............T.....C...........T.........................
[7]181 ..............T.....C...........T.........................

[1]241 AATCAAACCGCGCTCAAGGTTGAACAGATTTTGCAAAATCAAGGCTATAAGGTTATTAGC
[2]241 ...........................................G................
[3]241 .........A....T..A......G....C...........G.....C........C.AT
[4]241 .........A....T..A......G....C...........G................AT
[5]241 ...........................................G.....C........C.AT
[6]241 ..........T..............................G................AT
[7]241 .........A....T.........G....C............................

[1]301 GTAGATAGCAGCGATAAAGACGATTTTTCTTTTGCACAAAAAAAAAGAAGGGTATTTGGCG
[2]301 ........................C........T.G......................T
[3]301 ..G..............................G.......................
[4]301 ..G..............................G......................T
[5]301 ..G.....T........................G.......................
[6]301 ..G..............................G......................T
[7]301 ........................C........T.G......................C

[1]361 GTTGCTATGAATGGCGAAATTGTTTTACGCCCCGATCCTAAAAGGACCATACAGAAAAAA
[2]361 ................................................................
[3]361 ..........T..............................A.................
[4]361 ..C.............................................................
[5]361 ................................................................
[6]361 ..C...........T..................................................
[7]361 ..C.............................................................
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[1]421 TCAGAACCCGGGTTATTATTCTCCACCGGTTTGGACAAAATGGAAGGGGTTTTAATCCCG
[2]421 ..............G...........T........T.....................A
[3]421 ........................T........T.............C.........
[4]421 ........................T........T.........................
[5]421 ......................T...........T.........................
[6]421 ........T................T.................................
[7]421 ........................T.................................

[1]481 GCTGGGTTTATTAAGGTTACCATACTAGAGCCTATGAGTGGGGAATCTTTGGATTCTTTT
[2]481 ..C......G.C.....................................................
[3]481 ..C..T...G.C.....................................A.....C...
[4]481 .........G.C.....................................................
[5]481 ......................T.......................A.........
[6]481 .........G.C...........T.....................................
[7]481 .........G.C.....................................A.........

[1]541 ACGATGGATTTGAGCGAGTTGGACATTCAAGAAAAATTCTTAAAAACCACCCATTCAAGC
[2]541 .....................................................................
[3]541 ..............T.....A.....................G.....................
[4]541 .....................................................................
[5]541 ........................C.....................................
[6]541 .....................................................................
[7]541 .....................................................................

[1]601 CATAGCGGGGGGTTAGTTAGCACTATGGTTAAGGGAACGGATAATTCTAATGACGCGATC
[2]601 .....................................................................
[3]601 ........A.....................................................T......
[4]601 ........A.........................G...................A..T
[5]601 .....................................................................
[6]601 .....................................................................
[7]601 .....................................................................

[1]661 AAGAGCGCTTTGAATAAGATTTTTGCAAATATCATGCAAGAAATAGACAAAAAACTCACT
[2]661 .....................................................................
[3]661 ............................G..............G..T..G.........
[4]661 ............................G..............G..T..G.........
[5]661 ..............C.....................................................
[6]661 ........................G..G.....................................
[7]661 .....................................................G......

[1]721 CAAAAGAATTTAGAATCTTATCAAAAAGACGCCAAAGAATTAAAAGGCAAAAGAAACCGA
[2]721 ...................................G.........................
[3]721 ....G..............................G.....G...AA...G.........
[4]721 ....G..............................G.........AA...G.........
[5]721 ...................................G.....G...AA...G.........
[6]721 .....................................................................
[7]721 ...................................G.....G...AA...G.........

[1]781 TAAAAACAAATAACGCATAA
[2]781 ...
[3]781 ....G..............
[4]781 ...................
[5]781 ...
[6]781 ...................
[7]781 TAA.G..............GAA
```

Figure 2 Homology comparison of *H.pylori* hpaA gene nucleotide sequences ([1]-[6]: the reported sequence from GenBank (No. X92502, strain 11637; No. NC000915, strain 26695; No. X61574, strain 8826; No. NC_000921, strain J99; No. AF479028, strain CH-CTX1; No. U35455, strain CCUG 17874); [7]: the sequencing result of *H.pylori* strain Y06 hpaA gene. outline region: oligonucleotide primer sequences; square frame: start code and end code).

```
[1]1   MRANNHFKDFAWKKCLLGASVVALLVGCSPHIIETNEVALKLNYHPASEKVQALDEKILL
[2]1   .K..........................................................
[3]1   .KT.G............T.........................................
[4]1   .KT.G.........F.............................................
[5]1   .KT.G..........................................................
[6]1   .KT.G..............G.....................................
[7]1   ..........................................................

[1]61  LRPAFQYSDNIAKEYENKFKNQTALKVEQILQNQGYKVISVDSSDKDDFSFAQKKEGYLA
[2]61  ..............................................L..S........
[3]61  .K.....................T....E.........N...................
[4]61  ......................T....E.........N...................
[5]61  ........................................N...................
[6]61  ......................V..............N...................
[7]61  ......................T....E..................L..S........

[1]121 VAMNGEIVLRPDPKRTIQKKSEPGLLFSTGLDKMEGVLIPAGFIKVTILEPMSGESLDSF
[2]121 ........................................V................
[3]121 ...I....................................V................
[4]121 ........................................V................
[5]121 ..........................................................
[6]121 ........................................V................
[7]121 ........................................V................

[1]181 TMDLSELDIQEKFLKTTHSSHSGGLVSTMVKGTDNSNDAIKSALNKIFANIMQEIDKKLT
[2]181 ..........................................................
[3]181 ..................................................S....M.....
[4]181 ..................................................S....M.....
[5]181 ..........................................................
[6]181 ..................................................GS.........
[7]181 ..........................................................

[1]241 QKNLESYQKDAKELKGKRNR
[2]241 ....................
[3]241 ..............N....
[4]241 .R............N....
[5]241 ..............N....
[6]241 ....................
[7]241 ..............N....
```

Figure 3 Homology comparison of the putative amino acid sequences of hpaA gene ([1]-[6]: the reported sequence from GenBank (No. X92502, strain 11637; No. NC000915, strain 26695; No. X61574, strain 8826; No. NC_000921, strain J99; No. AF479028, strain CH-CTX1; No. U35455, strain CCUG 17874); [7]: the sequencing result of Hp strain Y06 hpaA gene).

Expression of target fusion protein

1, 0.5 and 0.1 mmol· L^{-1} of IPTG were able to efficiently induce the expression of HpaA fusion protein in pET32a-hpaA-BL21DE3 system. The product of HpaA fusion protein was mainly presented in ultrasonic precipitate and the output was approximately 40 % of the total bacterial proteins (Figure 4).

Immunoreactivity and antigenicity of HpaA fusion protein

The commercial rabbit antibody against the whole cell of *H.pylori* could combine with HpaA fusion protein confirmed by Western blot (Figure 5) and the titer demonstrated by immunodiffusion assay between HpaA fusion protein and rabbit antiserum against the fusion protein was 1:4.

ELISA

The mean ±SD at OD_{490} of the negative serum samples was 0.11±0.03 and the positive reference value was 0.20. The mean ±SD at OD_{490} of the negative bacterial control was 0.04±0.04 and the positive reference value was 0.16. According to the reference values, 81.6 % (102/125, another serum sample was

contaminated) of the tested patients' serum samples were positive for antibodies against HpaA fusion protein with a range of 0.54-1.84 and 100 % (109/109, other 17 isolates could not be revived from -70 ℃) of the tested *H.pylori* isolates were detectable for HpaA with a range of 0.52-1.47.

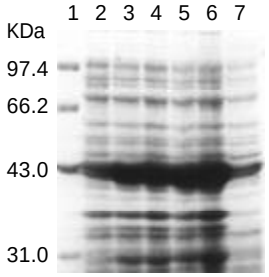

Figure 4 Expression of HpaA gene in pET32a-hpaA-BL21DE3 induced by IPTG with different dosages shown in SDS-PAGE (1: protein marker; 2: non-induced; 3-5: induced with 0.1, 0.5, 1 mmol·L^{-1} of IPTG, respectively; 6: the bacterial precipitate induced with 1 mmol·L^{-1} of IPTG; 7: the bacterial supernatant induced with 1 mmol·L^{-1} of IPTG).

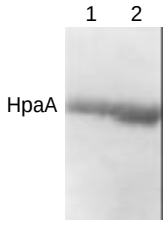

Figure 5 Western blot result of commercial rabbit antibody against whole cell of *H.pylori* and HpaA fusion protein (1 and 2: 20 μl and 40 μl of expressed HpaA product induced with 0.1 mmol·L^{-1} of IPTG, respectively).

DISCUSSION

The outer membrane is a continuous structure on the surface of gram-negative bacteria. For outer membrane proteins in the outer monolayer of bacterial membrane, they have bilateral particular significance as a potential target for protective immunity and bacterial pathogens. Outer membrane vaccines have been used with considerable success to induce protection against a number of organisms, including heat shock protein, vacuolating cytotoxin, ureases A, B of *H.pylori* and so on[59-65]. HpaA is one of the major structural outer membrane proteins of *H.pylori* and plays an important role in adhesion of the microbe[51,53]. HpaA gene is located in genome DNA of *H.pylori* and considerably conservative for its nucleotide and amino acid sequences[55,56]. Furthermore, antibody against HpaA could be found in approximately 86 % of *H.pylori* infected patients' sera and this proportion was obviously higher than that of heat shock protein (68 %) and vacuolating cytotoxin (68 %)[57] and was similar to that of urease B[64]. Therefore, HpaA is an ideal antigen candidate for *H.pylori* vaccine.

In this study, the homologies of the nucleotide and putative amino acid sequences in the cloned HpaA gene from *H.pylori* strain Y06 compared with the 6 published hpaA gene sequences[51, 53, 55-58] were as high as 94.25-97.32 % and 95.38-98.46 %, respectively, whereas the nucleotide and putative amino acid homologies among the 6 HpaA gene sequences were 93.72-98.21 % and 95.00-98.78 %. These data indicate that the mutation level of the HpaA gene of *H.pylori* strain Y06 is within the range reported by the literatures.

The results of SDS-PAGE demonstrated that the constructed expression system pET32a-hpaA-BL21DE3 was able to efficiently produce the target fusion protein presented with the form of inclusion body even if induced by IPTG at lower concentration of 0.1 mmol· L^{-1} and the output was approximately 40 % of the total bacterial proteins.

The rabbit antibody against the whole cell of *H.pylori* could recognize and combine with HpaA fusion protein confirmed by Western blot, indicating that the fusion protein has an active and satisfactory immunoreactivity. The immunodiffusion assay performed in this study demonstrated that HpaA fusion protein could efficiently induce rabbit to produce specific antibody with a higher titer against the fusion protein, which indicates that HpaA fusion protein has favorable antigenicity. The results of ELISA showed that all of the tested clinical isolates of *H.pylori* would express HpaA and the majority of *H.pylori* infected patients' sera (81.6 %) were present for the specific antibody against the microbe.

All the evidences mentioned above suggest that HpaA is an excellent and ideal antigen for developing *H.pylori* vaccine and an HpaA expression system with high efficiency was successfully constructed in this study.

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