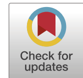
Heliyon 9 (2023) e17881

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Hp0521 inhibited the virulence of *H. pylori* 26,695 strain via regulating CagA expression

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ARTICLE INFO

Keywords:
Helicobacter pylori
cagPAI
hp0521
cagA

ABSTRACT

Hp0521 is the number of cag pathogenicity island (cagPAI) family in *Helicobacter pylori* (*H. pylori, Hp*), which encoded Cag2 protein. The aim of this study was to investigate the role of *hp0521* on the *H. pylori 26,695* strain. We constructed the recombinant prokaryotic expression plasmid pET-32a-*hp0521* and pET-32a-*hpc0521*. Then, we co-cultured the *H. pylori* wild strain 26,695 and *Δhp0521* strain with GES-1 cells to detect CagA protein transport and IL-8 secretion. We found that *Δhp0521* mutation increased the expression of *cagA, rpoB* and promoted the transportation of CagA protein in GES-1 cells. In addition, we also observed that *Δhp0521* mutation had no effect on other cagPAI protein stability and the expression of IL-8. Our findings suggested that *hp0521* may down-regulated the expression of cagA, rpoB and inhibited the transportation of CagA protein in GES-1 cells and had no effect on growth.

1. Introduction

H. pylori infection affects approximately 50% of the global population. Many studies showed that individuals infected with *H. pylori* had significantly increased risk of developing gastric cancer, ulcer disease, and gastric lymphoma [1–3]. Persistent infection with *H. pylori* leads to immune activation and chronic inflammatory response, which is the crucial step in the initiation and development in gastric cancer [4–6]. There are large differences between the type I *H. pylori* strain and the type II *H. pylori* strain, mainly due to the characteristic about 40 kb cagPAI DNA region and the type I *H. pylori* strain is present approximately 95% of East Asian isolates, compared to 60% of low-risk Western isolates [4]. The cagPAI DNA region contained more than 30 open reading frames (ORF), named cag1 to cag26 or cagA to cagZ and cagα to cagζ, which encoded multiple structural components of bacterial type IV secretion system (TFSS), translocated CagA to host gastric epithelial cells, led to activate a variety of cellular signal transduction pathways such as NF-κB, β-catenin, phosphatidylinositol-3-kinase/AKT, Src/MEK/extracellular signal-regulated kinase pathway [6–8] and caused a

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https://doi.org/10.1016/j.heliyon.2023.e17881
Received 5 May 2023; Received in revised form 21 June 2023; Accepted 29 June 2023
Available online 1 July 2023
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series of diseases such as cytoskeleton rearrangement, hummingbird sample extension form change, change of abnormal cell proliferation, cell connections pre-cancerous lesions [9–11]. Meanwhile, CagA activated NF-κB through the intracellular signaling pathway by phosphorylation of key players via inducing IL-8 expression [12–14]. It has been demonstrated that malignant growth is promoted by IL-8 and other related chemokines [12,14]. The vacuolating cytotoxin (VacA) is another effective toxin produced by *H. pylori* strains and encoded by the vacA gene. It is a pore-inducing toxin that triggers apoptosis by inducing epithelial cellular vacuolation of the stomach. VacA also exerts other effects on the host cells, including membrane channel formation, apoptosis and specific locus linked with gastric malignancy [15].

It is known that the type IV secretory system device encoded by *H. pylori* cagPAI mainly includes pilus components (CagC, H, I, L, Y), core complex proteins (CagH, M, N, T, U, V, W, X, Y, cagδ), Energy supply part (CagE, α,β), Transport-associated factors (CagF, Z, β), Specific glycosyl transferase (Cag γ) and substrate (CagA) [16].

The components of the pilus structure are essential for the process of binding, including CagL, CagY and CagI. CagL, a putative VirB5 ortholog, which is a specialized adhesin that plays an important role during CagA injection. CagL targets the pilus structure surface and activates integrin α5β1 receptor on gastric epithelial cells through the Arg-Gly-Asp (RGD) motif. Previous study showed that CagL promoted the interaction of cag-T4SS with integrin α5β1 to activate FAK and Src family kinases in the host cells [12,17,18]. As the component of cag-T4SS in *H. pylori*, CagI could form functional complex with CagL at the late stage during secretion apparatus assembly [19]. CagY serves as molecular switch or rheostat that alters the function of the cag-T4SS and 'tunes' the host inflammatory response to maximize persistent infection [20,21].

Despite the role of *H. pylori* in etiology has been determined, its actual distribution and association with related diseases are still controversial, especially among Asian countries. Compared with East Asian countries, Southeast and South Asian countries such as India, Pakistan, Thailand and Bangladesh have higher infection rates of *H. pylori*. However, the incidence rate of gastric cancer is relatively low [22–24].

The *H. pylori* genome is highly dynamic, which generates a huge diversity of strains. *Hp0521* is the second gene in the series of cagPAI. In our pervious bioinformatics analysis found that the random coil and alpha helix were the main structural component of the polypeptide chain of Cag2 protein. Cag2 protein was high homology with the DNA topoisomerase I, with the mediated cell wall extension tag protein and so forth [25]. In addition to their role in DNA replication, DNA topoisomerases have been implicated in the pathogenesis of bacteria such as *Shigella flexneri* and *Agrobacterium tumefaciens.* Expression of DNA Topoisomerase I (TopoI) in *S. flexneri* has been shown to up-regulate virulence gene transcription [26,27], down-regulation of *topA* gene in strain *Mycobacterium tuberculosis* (TB) resulted in loss of bacterial viability which correlated with a concomitant depletion of intracellular Topo I levels. The topA knockdown strain of *M. tuberculosis* failed to establish infection in a murine model of TB and was cleared from lungs in two months post infection [28] Schmidt H M et al. found that the *hp0521* deletion of *H. pylori* strains isolated from patients with functional dyspepsia (FD) in China was 88.5%, and there was significant difference between *H. pylori* strains isolated from Indian and Malay FD patients (20.4% and 25%, respectively) [26]. Takeshi Azuma et al. showed that among clinical isolates from patients with peptic ulcer or gastric cancer in Japan, the 0521 deletion was more common (8/11), and the 0521 deletion often occurred in the more virulent *H. pylori* East Asia type (EPIYA-D and. vacA s1c), which suggested that patients infected with strains in the Japanese cluster tended to have high-grade inflammation and high level of gastritis [29].

We conducted this study to profile the *Hp0521* alleles, their role and interaction with other cag PAI genes. And, we analyzed the expression and function of *hp0521* in *H. pylori* 26,695 strain. In addtion, we also constructed *Δhp0521* strain to identify the function of *hp0521*gene in *H. pylori 26,695* strain, including the growth situation, the expression and transport of protein CagA.

2. Materials and methods

2.1. Bacterial strain, cell lines and growth condition

H. pylori 26,695 strain were obtained from college of medicine, Jiangsu University, and cultured Columbia agar plates (HuanKai Microbial, Guangdong, China) containing 5% fetal bovine serum (FBS, hyclone) at 37 °C under microaerobic environment. The liquid medium consisted of brucella broth (Oxoid) containing 7% fetal calf serum Kanamycin (50 μg/ml) added on the Columbia agar plates, which were used to culture the defective strain *Δhp0521*. Normal gastric epithelial cell (GES-1) were obtained from our laboratory and cultured in RPMI1640 (Gibco) supplemented with 10% FBS and growth in 5% carbon dioxide (CO_2) at 37 °C condition.

Table 1
PCR Primers used for hp0521, hpc0521 and construction of hp0521 gene mutant.

Name	Product size (bp)	Sequence (5′ → 3′)	Site
hp0521F	240	GGATCCATGATACAAAGAGGATTGAGTAG	BamH I
hp0521R		CTCGAGATCCATTGCATTTGGGATATTTAG	XhoI
hpc0521F	348	CGCGGATCCATGATACAAAGAGGATTG	BamH I
hpc0521R		CCGCTCGAGGTTTCCTTTTTTTTCAAAAT	XhoI
F1 P1	804	GGGGTACCCATCCCAATCATCACTTATGCT	KpnI
P2		CCCTCGAGATGAGACACTACTCAATCCTCT	XhoI
F2 P3	783	CGGAATTCATCCTAAATATCCCAAATGCAA	EcoR I
P4		CGGGATCCATTTTCTTTTGTTGTTCAAAAA	BamH I

2.2. Construction and identification of recombinant prokaryotic expression plasmid

Based on the full length sequence of the *0521* gene of *H. pylori* 26,695 recorded on PubMed, Primer Premier 6 was used to design primers with similar annealing temperature (Tm) for *hp0521* and *hpc0521* genes. Specific restriction enzyme sites BamH I and XhoI were added to the end of primer 5' and the expected PCR product lengths were 240 bp and 348 bp, respectively. The specific information of primers is shown in Table 1. The reaction parameters are as follows: pre denaturation at 94 °C for 5 min, 94 °C 5min, 50 °C 40s, 72 °C 45s, 40 cycles, 72 °C 10min. The amplified target DNA fragment from *hp0521* and *hpc0521* were cloned into pET-32a-T vector (pET-32a-*hp0521* and pET-32a-*hpc0521*) by using T-A Cloning Kit according to the manufacturer's instruction. The recombinant expression vector pET-32a-*hp0521* and pET-32a-*hpc0521* was transformed into *E. coli* Rossetta (DE3). The target *hp0521* and *hpc0521* fragment inserted in pET-32a plasmid was sequenced again.

2.3. Expression and identification of fusion protein and antibody production

pET-32a-*hp0521*, pET-32a-*hpc0521* and pET-32a, which obtained from our laboratory, were transferred into Rosetta through thermal shock, activated them at 37 °C for 12 h; and then cultured them in LB medium contain 50 μg/ml Ampicilin at 37 °C and induced by Isopropyl b-D-1-thiogalactopyranoside (IPTG), radioimmunoprecipitation assay (RIPA) lysis buffer containing phenylmethanesulfonyl fluoride (PMSF) were used to extract the whole protein of the bacteria. The mouse anti-His and HRP-labeling sheep anti-mouse IgG were used as the first and second antibodies, respectively, to identify the Cag2 fusion protein by Western blot.

Rabbit antiserum was prepared against the Cag2 purified protein in New Zealand rabbit (Laboratory animal center of Jiangsu University). Immunized it with purified protein (0.5 mg/ml) emulsified in Freund's complete adjuvant and followed immunization with purified protein (0.5 mg/ml) emulsified in Freund's complete adjuvant at an interval of 7 days. After that, protein solution was then given intravenously at weekly intervals for a further 3 weeks. The rabbit was bled 1 week after the last immunization and the serum was collected. ELISA assay was used to detect the titer of the antibody. Antibody was also identified by Western blot.

2.4. ELISA

GES-1 cells were seeded at a density of 4×10^5 cells per well in 6-well plate for 24 h and co-culture with *H. pylori* for 6 h at a multiplicity of infection (MOI) of 1:200 before IL-8 cytokine analysis. We analyzed the difference of secretion of IL-8 in GES-1 uninfected with *H. pylori*, wild strain 26,695 and *Δhp0521* infected with GES-1. and tested the OD values at 450 nm and obtained the amount of IL-8 by each treatment group.

2.5. Construction of H. pylori hp0521 gene deletion strain

The corresponding primers were used to amplify upstream and downstream homologous arms F1 and F2 (Table 1), then, pBlueKM40 suicide plasmid linear vector was obtained from Korea National Gyeongsang University Laboratory of Microbiology, and it was used to construct the suicide plasmid pBlueKM40-*Δhp0521*:Kan and apply electric transfer, resistance screening, PCR, sequencing, to obtain the defective strain *Δhp0521*.

2.6. Growth curve drawing

H. pylori strains were pre-incubated under microaerobic conditions for 48 h and suspended in liquid medium mentioned above. Then 142 μl cell suspension (OD600 = 1.4) was added into 2 ml liquid medium. The cultures were incubated under microaerobic conditions at 37 °C for 120 h, and the OD absorbance at OD600 was measured every 24 h.

2.7. Bacterial RNA isolation and RT-qPCR

Bacterial total RNA was extracted using TransZol Up reagent (TransGen Biotech, Beijing, China) followed by the manufacturer's specification. cDNA was synthesized using HiScript QRT SuperMix from qPCR Kit (TransGen Biotech). Then RT-qPCR was performed using TransStart Top Green qPCR Super Mix (TransGen Biotech) with target gene primers and 16S rRNA. Amplification cycles were carried out about 40 cycles of 95 °C for 15 s, 60 °C for 35s. Fold change of target gene mRNA expression was calculated via the $2^{-\Delta\Delta Ct}$ method.

2.8. Cell infection experiments and western blot

GES-1 cells were seeded at a density of 4×10^5 cells per well in 6-well plate for 24 h and co-culture with *H. pylori* for 6 h at a multiplicity of infection (MOI) of 1:200. Briefly, the infected GES-1 cells were harvested in lysis buffer (RIPA and PMSF) and separated by SDS-PAGE, transferred onto PVDF membranes. To analyze translocation of CagA, a monoclonal CagA antibody and Horseradish peroxidase-conjugated anti-mouse (Amersham, Germany) antibody were used as the first and second antibodies by Western blot and detected with the kit system for ECL (Amersham, Germany). The anti-GAPDH antibody was used as control.

2.9. Statistical analysis

All statistical analyses were performed using SPSS21. Significant differences among groups were measured by Student's t-test or one-way ANOVA. $p < 0.05$ was defined as statistically significant.

3. Results

3.1. The prokaryotic expression vector was constructed

To investigate the function of *hp0521* in the strain 26,695, we constructed the prokaryotic expression vector pET-32a-*hp0521*and pET-32a-*hpc0521*. The template was extracted from the standard strain 26,695 genome and the primer of specific *hp0521* and *hpc0521* were synthesized. As shown in Fig. 1A, we performed to obtain the expected sized of *hp0521* and *hpc0521*. Also, the pET-32a-*hp0521* and pET-32a-*hpc0521* recombinant expression plasmids were verified by BamH I and XhoII double enzyme digestion (Fig. 1B). The results indicated that we successfully constructed the prokaryotic expression vector of pET-32a-*hp0521*and pET-32a-*hpc0521*.

3.2. The expression of Cag2 protein was in H. pylori strain

To detect the expression of Cag2 fusion protein, the recombinant pET-32a-*hp0521* and pET-32a-*hpc0521* were transformed to host *Escherichia coli* Rossetta (DE3), and induced expression by adding IPTG to a concentration of 1 mmol/l. After cultured 12 h in LB medium at 37 °C, the bacteria were harvested by centrifugation. The whole bacterial protein of Rosetta expressing engineering strain pET-32a-*hp0521* or pET-32a-*hpc0521* were analyzed by Western blot. The data showed that there was no expression of Cag2 fusion protein in pET-32a-*hp0521* but Cag2 fusion protein expression was in the recombinant prokaryotic plasmid pET-32a-*hpc0521,* the specific reaction strip of Cag2 fusion protein appeared at 32 KDa (Fig. 1C). In addition, to detect the expression of Cag2 protein, we extracted the proteins of *H. pylori* strains NCTC11637 and 26,695. We found that Cag2 protein specific bands were observed at 13 KDa in *H. pylori* strain NCTC11637, while the *H. pylori* strain 26,695 showed no bands at less than 13 KDa via Western blot assays, which indicated that *hp0521* gene was not translated into Cag2 protein in the *H. pylori* 26,695 (Fig. 2) the cagA was used as a control. The results suggested that the coding ability of *hp0521* gene was restored after completing the missing two bases in *Hp26695* strain.

3.3. hp0521 had no effect on H. pylori growth

To further investigate the function of *hp0521* in *H. pylori 26,695* strain, we generated *hp0521* gene-deleted mutant of *H. pylori*

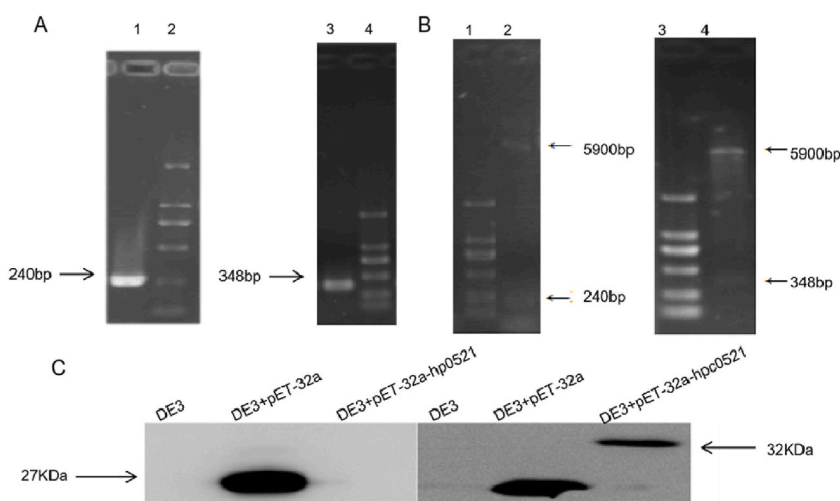

Fig. 1. The prokaryotic expression vector was constructed. The fragment of *hp0521* and *hpc0521* gene were cloned into pET32a (+) and then transformed to host *Escherichia coli* Rossetta (DE3). The recombinant pET-32a-*hp0521*-DE3 and pET-32a-*hpc0521*-DE3 were induced expression by adding IPTG to a concentration of 1 mmol/L After cultured 12 h in LB medium at 37 °C, the bacteria were harvested by centrifugation. The whole bacterial protein of Rosetta expressing engineering strain pET-32a-*hp0521* or pET-32a-*hpc0521* were analyzed by Western blot. (A) PCR products of *hp0521* and *hpc0521* gene, 1: *hp0521*, 2 and 4: DL-2000 DNA Marker, 3: *hpc0521*. (B) Constructed recombinant expression plasmids, 1 and 3: DL-2000 DNA Marker, 2: pET-32a-*hp0521* recombinant expression plasmid verified by BamH I and XhoII double enzyme digestion, 4: pET-32a-*hpc0521* recombinant expression plasmid verified by BamH I and XhoII double enzyme digestion. (C) Identification of recombinant fusion protein of *hp0521* and *hpc0*521 by Western blot. The target plasmid is induced by adding IPTG to express in engineered *Escherichia coli* DE3. DE3 is used as a negative control, DE3+pET-32a is used as a positive control, and DE3+pET-32a-*hp0521* and DE3+pET-32a-*hpc0521* are used as experimental groups. The mouse anti-His and HRP-labeling sheep anti-mouse IgG were used as the first and second antibodies, respectively.

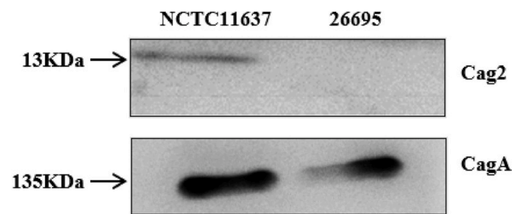

Fig. 2. The expression of Cag2 protein was in *H. pylori* strain NCTC11637. Western blot was used to detect Cag2 protein in *H. pylori* strain NCTC 11637 and *H. pylori* 26,695 strain. The cagA was used as control.

26,695 (*Δhp0521*). The *hp0521* gene deleted mutant was constructed following an allelic exchange mutagenesis strategy using the recombinant plasmid containing 1.4 kb kanamycin cassette (KMr) flanked by the up and downstream of the *hp0521* open reading frames (Fig. S1A). The suicide plasmid pBlueKM40-*Δhp0521* was transferred into *H. pylori 26,695* through electroporation. The suicide plasmid pBlueKM40-*Δhp0521* was identified by restriction enzyme digestion (Fig. S1B) and was transferred into *H. pylori 26,695*. The result of the gene-deleted mutant was confirmed through PCR (Fig. S1C) and sequencing.

As mentioned above,the 142 μl cell suspension (OD600 = 1.4) was added into 2 ml liquid medium. The cultures were incubated under microaerobic conditions at 37 °C for 120 h, and the OD absorbance at OD600 was measured every 24 h. As shown in Fig. 3A, the wild-type 26,695 and *Δhp0521* strain exhibited similar growth profiles under same culture conditions. ($p > 0.05$). Then, we isolated total RNA of *H. pylori* wild strain 26,695 and *Δhp0521* strain. We observed that there was no polar effect on two downstream genes (*hp0522 and hp0523*) in *Δhp0521* strain. (Fig. 3B). In addition, we examine 14 genes (*cagA, vacA, ureA, rpoB, flaB (hp0115), hp0116, hp0333, hp0440, hp0516, hp0520, hp0792, hp1293, hp1324*) that may interact with *hp0521* gene, including virulence factors *cagA, vacA, ureA* and *flaB* genes by RT-PCR. Our data showed that the deletion of *hp0521* gene led to the up-regulation of *cagA* and *rpoB* gene mRNA levels ($p < 0.05$) (Fig. 3C). However, the expressions of other related genes were no statistically significant ($p > 0.05$).

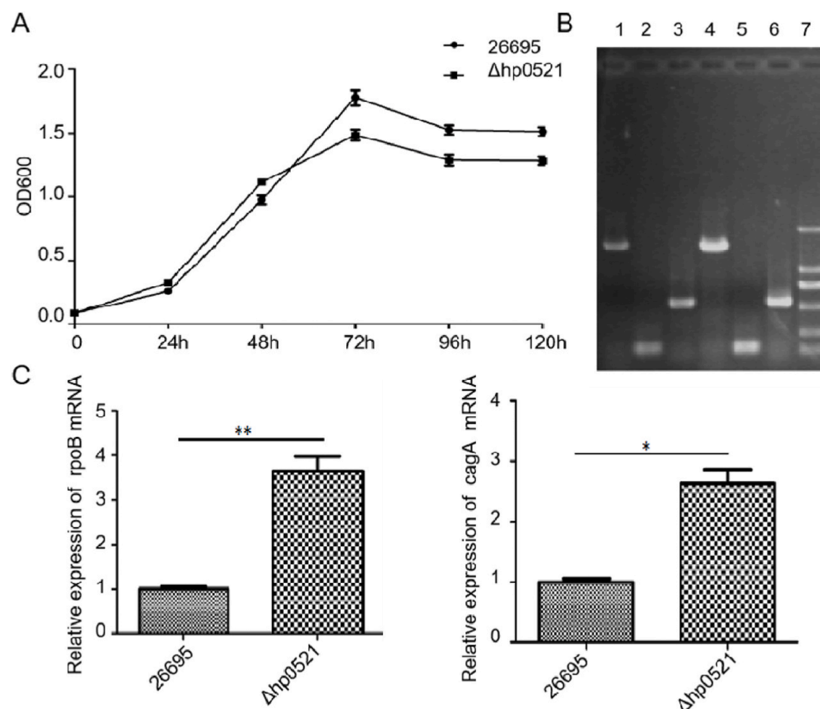

Fig. 3. The effect of *hp0521* gene on the *H. pylori* 26,695 strain. (A) Construction of growth curve was initiated by incubating 142 μl pre-cultured cell suspension (OD600 = 1.4) into 2 ml liquid medium. The cultures were incubated under microaerobic conditions at 37 °C for 120 h and OD values were determined every 24 h. The wild-type 26,695 and *Δhp0521* strain exhibited similar growth profiles under same culture conditions. The experiment was repeated three times independently. ($p > 0.05$). (B) The expression of two downstream genes was not affected with the loss of 0521 gene. PCR were used to detect the transcript levels for the *0522* and *0523* cistrons, downstream of *0521*, were not changed between*Δhp0521* mutant and wild-type 26,695 1–3 *hp0522*, 16sRNA (control) and *hp0523* genes were amplified from *H. pylori* 26,695.; 4–6: same genes were amplified from*Δhp0521* mutant. (C) qRT-PCR detect 14 genes that may interact with *hp0521* and found that the deletion of *hp0521* gene could up-regulate *cagA* and *rpoB* gene mRNA levels. *$p < 0.05$, **$p < 0.01$.

3.4. Δhp0521 strain influenced the expression of CagA protein and cagPAI encoded proteins in H. pylori 26,695

CagA was the critical virulence factor which was injected into host cells via cag PAI encoded T4SS by *H. pylori.* To research the role of*Δhp0521* strain, we extracted total proteins of *H. pylori* wild strain 26,695 and *Δhp0521* strain. According to the analysis of protein band gray scanning software (Image J), As is showed in the (Fig. 4A) histogram, the relative protein level of cagA/cagX was higher in *Δhp0521* strain than the *H. pylori* 26,695 strain, The experiment was repeated three times independently. $p < 0.01$And we further monitored whether *hp0521* gene affected the stability of other proteins encoded by cagPAI. The results suggested that the outer membrane structure proteins encoded by cagPAI, such as CagX, CagM, CagL, CagI and endophytin CagV as well as membrane-related CagS and Cagζall could be expressed in the *H. pylori* wild strain 26,695 and *Δhp0521* strain (Fig. 4B), which indicated that the absence of *hp0*521 had no effect on the stability of the seven cagPAI coding proteins.

3.5. Δhp0521 induced CagA protein transport

The functionality of the CagPAI was examined by the determination of CagA delivered into GES-1 cells, and the induction of IL-8. To investigate the function of *hp0521* in GES-1 cells, GES-1 cells were infected with wild-type 26,695 strains and *Δhp0521*strains for 6 h at a multiplicity of infection of 1:200 respectively. And then, we extracted cell proteins and analyzed CagA protein level via Western blot. The data showed that CagA protein was transported in the GES-1 cells and was higher expressed in the cells infected with *Δhp0521* strain (Fig. 5A). In addition, *H. pylori* wild strain 26,695 and *Δhp0521* strain induced secretion of IL-8 by GES-1 cells had no significance (Fig. 5B), indicating that the *hp0521* gene may not be involved in the related inflammatory reaction induced by IL-8 secretion.

4. Discussion

Gastric cancer is the fifth most common malignancy and the third leading cause of cancer death. *H. pylori* has been confirmed as the grade I carcinogen and the only pathogen to be capable of being planted in the human stomach [30], which led to immune activation and chronic inflammatory reaction [31,32]. Epidemiological studies showed that the global infection rate of *H. pylori* was about 50% [32,33].10–15% of infected people develop ulcers, nearly 1% develop gastric cancer and almost 90% non-cardia gastric cancer are attributed to this bacterial infection [32,34–36]. Previous study found that after eradicated *H. pylori*, the average of incidence of gastric cancer decreased from 40.3/100,000 to 30.3/100,000 person years in Taiwan [32,37]. The data indicated that *H. pylori* was one of the main risk factor for the occurrence of gastric cancer and eradicating *H. pylori* was related to reducing the incidence rate of gastric cancer. Additionally, a large number of studies found that there was association between non-gastrointestinal vascular diseases and patients carrying *H. pylori*, which further revealed the universality of *H. pylori*'s pathogenic range [38–40].

It was known that the high pathogenic *H. pylori* always containing a functional cagPAI, which encoded and assembled TFSS injects cytotoxin CagA peptidoglycan and other pathogenic factors into the host cells, led to high secretion of IL-8 in gastric tissue, resulted the emergence of chronic superficial gastritis [30]. The C-terminal region of CagA protein, as the main virulent protein of *H. pylori*, contains different Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs that serve as phosphorylation sites for the protein [41]. *H. pylori* strains are mainly divided into two types: East Asia type (EPIYA-D) and Western type (EPIYA-C). The CagA oncogenic potential had been linked to the EPIYA motifs C and D [15,41] In vitro and in vivo studies demonstrated that East Asian type CagA was more carcinogenic than Western type CagA [12]. This could be related to the affinity of the SHP2 phosphatase protein to join the EPIYA C and D motifs, which affected the carcinogenic ability of *H. pylori* strains [41]. Studies indicated that the *hp0521* allele was also associated with EPIYA motif, EPIYA-C tends to appear with *hp0521*A, while EPIYA-D tends to appear with *hp0521* ES (restricted deletion of hp0521) [26].

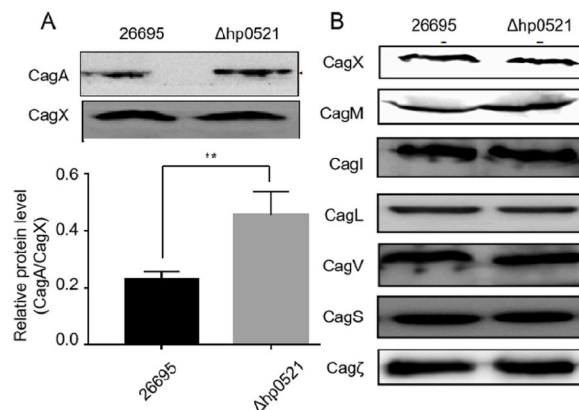

Fig. 4. The analysis of the expression of CagA and CagPAI proteins in *H. pylori 26,695* strain and *Δhp0521* strain. (A) According to the analysis of protein band gray scanning software, compared with the structural protein CagX stably expressed by *H. pylori*, the expression of CagA in the deletion strain △*hp0521* was higher than that in the wild strain 26,695 $^{**}p < 0.01$. (B) Western blot were used to detect other proteins encoded by cagPAI of*Δhp0521* strain and the *H. pylori* 26,695 strain.

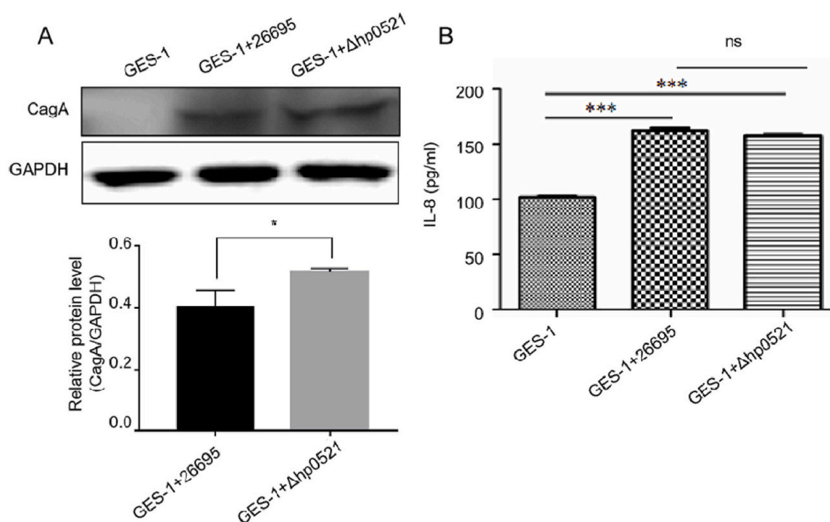

Fig. 5. The effect of *hp0521* gene on CagA protein translocation and secretion of IL-8 in GES-1 cells. GES-1 cells were infected with wild-type 26,695 strains and *Δhp0521* strains for 6 h at a multiplicity of infection of 1:200 before total protein extraction or cytokine analysis. (A) The change of CagA translocation was examined by Western blot. Compared with the wild strain 26,695, the *Δhp0521* strain promoted the CagA protein translocation in GES-1 cells. $*p < 0.05$ (B) ELISA suggested that GES-1 cells co-cultured with *H. pylori* promote the secretion of IL-8, and compared with the wild-type 26,695 strains, *Δhp0521* strain has no influence. *ns* no statistical significance, $***p < 0.001$.

Comparison with the tendency for selected virulence loci to occur together within the single strain, *hp0521* ES tends to appear in the more virulent group (oipA ON, vacA s1i1m1/m2, babA+) [26].

In our research, we focused on *hp0521* gene prokaryotic expression protein and protein function. We found that *H. pylori* 26,695 did not encode Cag2 protein due to that the *hp0521* gene had the premature termination of codon after the deletion of two bases. And the coding ability of *hp0521* gene was restored after completing the missing two bases in *H. pylori* 26,695 strain Compared the *H. pylori* wild 26,695 strain and *Δhp0521* strain, we discovered that the deletion of *hp0*521 had no effect on *H. pylori* growth, the stability of cagPAI coding proteins and the secretion of IL-8. However, we found that *hp0521* gene may interact with cagA and rpoB, and *Δhp0521* strain increased CagA expression. *Δhp0521* mutation seems to be a more virulent expression in the evolution of *H. pylori*, which is consistent with previous studies [26], Also, *Δhp0521* strain co-cultured with GES-1 cells promoted the CagA protein translocation, which suggested that *hp0521* may inhibited the generation of CagA protein.

In conclusion, this study based on *H. pylori* wild 26,695 strain *hp0521* gene function on systematic discussion, we found that *hp0521* may down-regulated the expression of cagA, rpoB and the transportion of CagA proteinin GES-1 cells. In addition, we also observed that *hp0*521 had not affect other cagPAI protein stability and expression of IL-8. Our findings suggested *Δhp0521* mutation was more virulent expression in the evolution of *H. pylori* and the absence of *hp0521* could be the new independent marker. Further studies are needed to establish this association in the large population.

Ethics approval and consent to participate

The animal experiments in this article are mainly conducted in the animal center of Jiangsu University, and the experimental animal license is obtained (license number: SYXK (su)2017-0063).

Funding

This study was supported by the Changzhou Health Commission Youth talent science and technology project (Grant No. QN202040), Changzhou High-Level Medical Talents Training Project (2022CZBJ077).

Author contribution statement

Min Yu and Min Xu: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Yixin Shen: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Yun Liu and Chi Xu: Performed the experiments, Analyzed and interpreted the data.

Tongbao Feng: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data. Analyzed and interpreted the data; Wrote the paper.

Ping Zhang: Conceived and designed the experiments, Analyzed and interpreted the data; Contributed reagents, materials, analysis

tools or data.

Data availability statement

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. Special thanks to Professor Shihe Shao of Jiangsu University for her guidance and help.

Additional information

No additional information is available for this paper.

Patient consent for publication

Not applicable.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e17881.

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