The Role of a Dipeptide Transporter in the Virulence of Human Pathogen, *Helicobacter pylori*

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INTRODUCTION

*Helicobacter pylori* is a microaerophilic, Gram-negative bacterium that is closely related to chronic gastritis, peptic ulcers, and gastric cancer (Kusters et al., 2006; Chmiela and Kupcinskas, 2019). To colonize the human stomach, *H. pylori* has to pass through the mucous layer to the surface of gastric mucosal epithelial cells via the movement of its flagella, and then colonizes the epithelial cells with the aid of adhesins (Sgouros et al., 2015). The pathogenesis of *H. pylori* is driven by several virulence factors that facilitate bacterial colonization, induce inflammation, and damage host cells. Among the virulence factors confirmed to function in *H. pylori* infection, Type 4 secretion system (T4SS) and its effector protein CagA encoded by *cag* pathogenicity island (*cag*PAI) are one of the most extensively studied *H. pylori* virulence factors (Guillemin et al., 2002; Sanchezzauco et al., 2013). *cag*PAI is about 40 kb in size and comprises 26 genes that encode the components of the...
T4SS. Relying on the T4SS, which binds to the α5β1 integrin expressed on the surface of gastric epithelial cells (Kwok et al., 2007), *H. pylori* delivers CagA, ADP-heptose (Fünnkuch et al., 2019), and peptidoglycan into host cells (Viala et al., 2004). *H. pylori* infection activates nuclear factor-kappa B (NF-κB) (Holberg et al., 2007), and peptidoglycan into host cells (Viala et al., 2004).

Successful colonization requires adaptation of the bacterium to the gastric environment. Environmental factors such as pH, reactive oxygen species, temperature, or nutrients can affect the expression of *H. pylori* virulence factors (Merrell et al., 2003a; Pflock et al., 2006; Augusto et al., 2007; Noto et al., 2015). Acidic pH highly stimulates the expression of antioxidant proteins, flagellar structural proteins, and T4SS component proteins in *H. pylori* (Marcus et al., 2018). Upregulation of vacA and downregulation of genes related to motility were observed under iron-restricted conditions (Merrell et al., 2003b). Iron deficiency enhances *H. pylori* virulence; thus, *H. pylori* isolated from iron-depleted gerbils expressed significantly higher levels of CagA, which induced more robust proinflammatory responses (Noto et al., 2012).

Considering that nutrients are important for the growth of bacteria, genes involved in the metabolism serve as targets for antimicrobial therapies. The peptide transporter systems have been extensively investigated in bacteria such as *Escherichia coli* and *Lactococcus lactis* (Sanz et al., 2001; Harder et al., 2008). Peptide transporters play an important role in nutritional supply by providing carbon sources or nitrogen sources for bacterial growth (Gilvarg, 1972). Three types of peptide transporters in bacteria have been found to date: oligopeptide (Opp) transporters, dipeptide (Dpp) transporters, and dip/tripeptide (Dtp) transporters (Garai et al., 2017). The Opp and Dpp transporters belong to the ABC superfamily, while the Dtp transporters are responsible for the import of oligopeptides. The Dpp transporter in *H. pylori* is composed of five proteins encoded by *dppA*, *B*, *C*, *D*, and *F* (Davis and Mobley, 2005; Weinberg and Maier, 2007). DppA is a periplasmic peptide-binding protein, DppB and DppC are integral membrane proteins that form permeases for substrates, while DppD and DppF are cytoplasmic proteins responsible for ATP hydrolysis.

Apart from being involved in the transport of nutrients, peptide transporters play a role in the virulence of various bacterial pathogens (Samen et al., 2004; Moraes et al., 2014). In *E. coli*, the Dpp transporter acts as a primary chemoreceptor, and its interaction with the membrane components for dipeptide chemotaxis initiates flagellar motion (Manson et al., 1986). In *Borrelia burgdorferi*, an opp mutant strain promotes the expression of the virulence factor OspC by regulating the Rrp2-RpoN-RpoS pathway (Zhou et al., 2018). In group A *Streptococci*, Dpp mutation results in a decreased expression of SpeB, a major cysteine protease (Podbielski and Leonard, 1998). In *Pseudalteromonas*, DppA plays an important role in cold adaptation (Zhang et al., 2010). However, hitherto the role of Dpp transporters in the growth and pathogenesis of *H. pylori* remains unknown. A study has shown that expression of DppA in *H. pylori* was stimulated by gastric epithelial cells, suggesting that DppA might play an important role in the pathogenesis of *H. pylori* (Sharma et al., 2010).

In this work, we constructed Dpp transporter mutants in *H. pylori* and evaluated the effects of the Dpp system on growth, expression of virulence factors, and inflammatory responses of AGS cells stimulated by *H. pylori*.

### MATERIALS AND METHODS

#### Bacterial Strains and Cultivation Conditions

*Helicobacter pylori* 26695, NCTC11637 and Dpp transporter mutant strains were cultured in a microaerobic environment (5% O2, 10% CO2, and 85% N2) at 37°C on Columbia agar plates (Oxoid, Cambridge, United Kingdom) containing 7% sheep blood. For liquid cultivation of *H. pylori*, Brucella broth supplemented with 10% fetal bovine serum (FBS) was used, and the strains were incubated in a shaker at 120 rpm and 37°C. A total of 5 µg/ml kanamycin (MP Biomedicals, CA, United States) was supplied when necessary.

#### Construction of Isogenic ΔdppA, ΔdppB, ΔdppC, ΔdppD, ΔdppF Mutants of *H. pylori* 26695 and Isogenic ΔdppA Mutant of NCTC11637

To construct a *dppA* knockout mutant of *H. pylori* 26695 (*ΔdppA*), a DNA fragment containing an upstream sequence of *dppA* was amplified with the primers DppA-up-F and DppA-up-R, a DNA fragment containing a downstream sequence of *dppA* was amplified with the primers DppA-down-F and DppA-down-R, and a DNA fragment containing AphA, which confers kanamycin resistance, was amplified with primers DppA-Kana-F and DppA-Kana-R. The *dppA* upstream sequence, *dppA* downstream sequence, and kanamycin resistance DNA fragments were ligated into a pBluescript II SK (−) vector (Novagen, Madison, WI, United States) using the ClonExpress MultiS One Step Cloning Kit (Vazyme, Nanjing, China), resulting in pBluescript-DppAKO, which was further transformed into *E. coli* DH5α. The plasmid sequence was then confirmed using colony PCR and Sanger sequencing. The pBluescript-DppAKO was then purified and subsequently transformed to *H. pylori* 26,695 by electroporation, and bacteria were then cultivated on agar plates containing kanamycin. *dppA* knockout mutants were further confirmed by colony PCR and Sanger sequencing. The construction of isogenic *H. pylori* 26695 mutants of Δ*dppB*, Δ*dppC*, Δ*dppD*, Δ*dppF*, and NCTC11637Δ*dppA* was conducted in a similar manner, and the primers used are listed in Table 1.
TABLE 1 | Primers used in this study.

| Primers          | Sequence (5′–3′) |
|------------------|-----------------|
| **For construction of isogenic mutants in H. pylori 26,695** |                      |
| DppA-up-F        | AGGGCCGCAATTGGGTACCG |                      |
| DppA-up-R        | AACGCGCGCAGTCTCAGGATGATGATGGATGATG |                      |
| DppA-down-F      | CATTATTATTACCTCGAAGCTCACAACACCTTATTCTTTCGAC |                      |
| DppA-down-R      | GTGCAAGCGACCTCTAGGATAATTGAAGCTGAGGAC |                      |
| DppA-Kana-F      | CTGCGAACAAATTAATGAAGCTGATGACGATG |                      |
| DppA-Kana-R      | TATAGGGCGCAAATTGGGTACCGATCGTAAATAGCTGAACTG |                      |
| **For construction of isogenic mutants in NCTC11637** |                      |
| DppA-up-F        | AGGGCCGCAATTGGGTACCG |                      |
| DppA-up-R        | AACGCGCGCAGTCTCAGGATGATGATGGATGATG |                      |
| DppA-down-F      | CATTATTATTACCTCGAAGCTCACAACCTTATTCTTTCGAC |                      |
| DppA-down-R      | GTGCAAGCGACCTCTAGGATAATTGAAGCTGAGGAC |                      |
| DppA-Kana-F      | CTGCGAACAAATTAATGAAGCTGATGACGATG |                      |
| DppA-Kana-R      | TATAGGGCGCAAATTGGGTACCGATCGTAAATAGCTGAACTG |                      |
| **For qPCR**     |                      |
| CagAδ-F          | GTGCTATGGGGATTGTTGGGATA |                      |
| CagAδ-R          | TTGCTTGAGATTTTTGAGTTTCG |                      |
| CagV-F           | GGCTTTTCTCTCTCTGCAAGCTGAC |                      |
| CagV-R           | CAATTTTAAATTCTCCTGTGATG |                      |
| CagS-F           | CAAGGGAGCGTTAGATAAGGTTCT |                      |
| CagS-R           | AATTAGGATTCTCTGCAATGGCAT |                      |
| CagQ-F           | CCGTAAAGCGTATCGTCTGAGATG |                      |
| CagQ-R           | TATAGGGCGCAAATTGGGTACCGATCGTAAATAGCTGAACTG |                      |
| **For construction of isogenic mutants in H. pylori 26,6965** |                      |
| DppB-up-F        | AAAACCGCGCAGTAAACAGCTGGAAGGAGGATG |                      |
| DppB-up-R        | ATTATGATGATGATGATGATGATGATGATGATG |                      |
| DppB-down-F      | AGAAAATGCTAGAATGATGATGATGATGATGATG |                      |
| DppB-down-R      | GTGCAAGCGACCTCTAGGATAATTGAAGCTGAGGAC |                      |
| DppB-Kana-F      | CTGCGAACAAATTAATGAAGCTGATGACGATG |                      |
| DppB-Kana-R      | TATAGGGCGCAAATTGGGTACCGATCGTAAATAGCTGAACTG |                      |
| **For qPCR**     |                      |
| CagAδ-F          | GTGCTATGGGGATTGTTGGGATA |                      |
| CagAδ-R          | TTGCTTGAGATTTTTGAGTTTCG |                      |
| CagV-F           | GGCTTTTCTCTCTCTGCAAGCTGAC |                      |
| CagV-R           | CAATTTTAAATTCTCCTGTGATG |                      |
| CagS-F           | CAAGGGAGCGTTAGATAAGGTTCT |                      |
| CagS-R           | AATTAGGATTCTCTGCAATGGCAT |                      |
| CagQ-F           | CCGTAAAGCGTATCGTCTGAGATG |                      |
| CagQ-R           | TATAGGGCGCAAATTGGGTACCGATCGTAAATAGCTGAACTG |                      |

Underlined part indicates overlapping DNA sequences.

**Cell Lines, Cultivation, and Co-culture of AGS Cells and H. pylori Strains**

The human gastric epithelial AGS cell line (derived from a human gastric adenocarcinoma) was cultured in a DMEM/F12 medium (HyClone Laboratories Inc., Logan, UT, United States), with supplementation of 10% FBS (PANS, Aidenbach, Bayern,
Germany) at 37°C in a 5% CO₂ humidified atmosphere. For *H. pylori* infection assays, AGS cells were grown in 6-well plates (NUNC, Thermo, DE, United States) until the confluence reached 75% in DMEM/F12 medium containing 10% FBS. Before infection, the supernatant was removed, and cells were washed twice with phosphate-buffered saline (PBS), followed by culture in FBS-free DMEM/F12 for 4 h. *H. pylori* strains were first cultivated on agar plates; then, the bacteria were collected and resuspended in Brucella broth at an initial OD₆₀₀ of 0.1, followed by culture for 24 h. Bacterial cells were then pelleted and washed twice with DMEM/F12 medium, resuspended in DMEM/F12 medium, and added to the AGS cell culture at a multiplicity of infection (MOI) of 100.

**Determination of Bacterial Growth Rates**

To monitor the growth of *H. pylori* strains, bacteria were first cultivated on Columbia agar plates for 3 days, followed by collection of bacterial cells and resuspension in Brucella broth at an initial OD₆₀₀ = 0.1. Next, the bacteria were cultured at 37°C with agitation. The OD₆₀₀ values of the bacterial culture were recorded every 8 h. Each experiment was repeated at least three times.

**RNA Sequencing and Data Analysis**

To prepare total RNA for transcriptomic study, *H. pylori* 26,695 and ΔdppA cells were cultivated in Brucella broth containing 10% FBS for 20 h until reaching the exponential phase in a shaker at 120 rpm in a microaerobic environment (5% O₂, 10% CO₂, and 85% N₂) at 37°C. Total RNA was isolated using the RNeasy Mini Kit (QIAGEN, Valencia, CA, United States). RNA degradation and contamination were monitored on 1% agarose gels. RNA sequencing was carried out at Novogene (Beijing, China), and RNA purity was confirmed using a NanoPhotometer spectrophotometer (IMPLEN, CA, United States). RNA concentration was measured using the Qubit 2.0 Flurometer (Life Technologies, United States). RNA integrity was assessed using the RNA Nano 6000 Assay Kit with a Qubit 2.0 Flurometer (Life Technologies, Carlsbad, CA, United States). RNA concentration was measured using the NanoPhotometer spectrophotometer (IMPLEN, CA, United States). RNA degradation and contamination were monitored on 1% agarose gels. RNA sequencing was carried out at Novogene (Beijing, China), and RNA purity was confirmed using a NanoPhotometer spectrophotometer (IMPLEN, CA, United States). RNA concentration was measured using the Qubit 2.0 Flurometer (Life Technologies, United States). RNA integrity was assessed using the RNA Nano 6000 Assay Kit for the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, United States). Ribosomal RNA (rRNA) was then depleted using the Ribo-zero kit (Ambion, Thermo, DE, United States) in accordance with the manufacturer’s instructions. Sequencing libraries were generated using the NEBNext Ultra Directional RNA Library Prep Kit for Illumina (NEB, Ipswich, MA, United States) following the manufacturer’s recommendations, and index codes were added to attribute sequences to each sample. The clustering of the index-coded samples was performed on a cBot Cluster Generation System using the TruSeq PE Cluster Kit v3-cBot-HS (Illumina, San Diego, CA, United States). After cluster generation, the library preparations were sequenced on an Illumina Hiseq platform, and paired-end reads were generated. The resulting P-values were adjusted using the Benjamini and Hochberg’s approach for controlling the false discovery rate. Genes with an adjusted P-value < 0.05 found by DESeq were designated as differentially expressed. The data were deposited in the NCBI Gene Expression Omnibus database (GEO1) under accession number GSE164216.

**Bacterial Pulldown Assays**

*Helicobacter pylori* was grown in Brucella broth containing 10% FBS for 20 h, after which the bacterial cells were washed twice and resuspended in PBS. Approximately 3 × 10⁷ cells were incubated with α5β1 integrin (250 µg/mL) (R&D Systems, Minneapolis, MN, United States) for 30 min at 37°C with rotation. In order to measure the amount of α5β1 integrin bound by *H. pylori*, the samples were centrifuged at 6,000 rpm for 10 min. Bacterial cells were collected, washed twice with PBS, and then resuspended in 1 × SDS loading buffer (60 mM Tris-HCl [pH 6.8], 2% SDS 10 ml, 10% glycerol, 100 mM DTT, 0.01% bromophenol blue). After denaturation by boiling for 10 min, the samples were resolved on a 10% SDS-polyacrylamide gel (SDS-PAGE). Western blot with an anti-β1 Rabbit antibody (1:1000; Cell Signaling Technology, Danvers, MA, United States) was performed as described previously.

**Bacteria Protein Extraction and Western Blotting**

To determine the expression of CagA, *H. pylori* cells were harvested after 20 h culture in Brucella broth containing 10% FBS. The cells were then washed twice with PBS, and total lysates were obtained using RIPA lysis buffer (Beyotime, Shanghai, China). The concentration of proteins was determined using a BCA Protein Assay Kit (Beyotime, Shanghai, China). Protein samples were then subjected to electrophoresis using a 10% SDS-PAGE gel and subsequently transferred to PVDF membranes (Millipore, Darmstadt, Germany) for antibody

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1http://www.ncbi.nlm.nih.gov/geo
Adhesion Tests
AGS cells were seeded in 6-well plates at a density of $3.5 \times 10^5$ cells/well with 2 ml of DMEM/F12 to form a confluent monolayer, and then infected with \textit{H. pylori} at an MOI of 100 as described above. After 4 h of infection, the AGS cells were washed three times with PBS to remove any unattached bacteria. To determine the number of adherent \textit{H. pylori}, the AGS cells were lysed using 0.1% saponin for 20 min at room temperature. After a serial dilution, 50 $\mu$l of each diluted cell lysate containing bacteria was placed on a Columbia sheep blood plate. Subsequently, the bacteria were incubated under microaerobic condition (5% O$_2$, 15% CO$_2$, and 75% N$_2$) for 4 days, and colonies were counted.

Dual-Luciferase Reporter Assay
NF-$\kappa$B activation was determined using luciferase reporter assays. AGS cells were seeded in 12-well plates at a density of $5 \times 10^5$ cells/well in 1 ml DMEM/F12 with FBS and cultured overnight. Subsequently, 1 $\mu$g of pNLS2.NF-$\kappa$B-RE (Promega, Madison, WI, United States) and 0.1 $\mu$g of pRL-TK (Promega, Madison, WI, United States) were co-transfected into these AGS cells using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, United States) following the manufacturer’s instruction. After 48 h of culture, the cells were infected with \textit{H. pylori} strains at an MOI = 100. After infection for 4 h, the AGS cells were harvested, and luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, United States) in accordance with the manufacturer’s instruction. Each result represents the mean of three independent experiments.

IL-8 Secretion Assays
The AGS cells were seeded in 6-well culture plates at a density of $3.5 \times 10^5$ per well in 2 ml DMEM/F12 medium with FBS to form a confluent monolayer. After 20 h of culture, the supernatant was replaced with fresh DMEM/F12 without serum after washing with 1 $\times$ PBS to starve cells for 4 h. Next, the cells were infected with \textit{H. pylori} 26,695 at an MOI of 100 or infected with NCTC11637 at an MOI of 30. After 4 h of infection, the supernatant was harvested, and IL-8 concentration was measured using enzyme linked immunosorbent assay (ELISA) with a Human IL-8 ELISA kit (BD Biosciences, San Jose, CA, United States) in line with the manufacturer’s instructions.

RESULTS
Dpp Transporters Are Important for the Growth of \textit{H. pylori}
Five genes were studied, \textit{dppA}, \textit{B}, \textit{C}, \textit{D}, and \textit{F}, which encode a dipeptide (Dpp) transporter comprising two permeases, two ATPases, and a substrate-binding protein (Davis and Mobley, 2005). To study the role of Dpp transporters in the virulence of \textit{H. pylori}, we first constructed isogenic mutants of \textit{dppA}, \textit{dppB}, \textit{dppC}, \textit{dppD}, and \textit{dppF} in \textit{H. pylori} 26,695, and then assessed the impact of the Dpp transporter on bacterial growth. Growth curves showed that \textit{H. pylori} 26,695 \textit{ΔdppA}, \textit{ΔdppB}, \textit{ΔdppC}, \textit{ΔdppD}, and \textit{ΔdppF} strains proliferated slower than a wild-type strain (Figures 1A–E). Specifically, \textit{DppF} had the strongest effect on the growth of \textit{H. pylori}, suggesting its important role in the bacterial growth. We also constructed a \textit{ΔdppA} mutant in the \textit{H. pylori} strain NCTC11637 and conducted the same experiment. The \textit{ΔdppA} mutant grew slower compared with the NCTC11637 wild type (Figure 1F). The results indicated that the importance of the Dpp system for the growth of \textit{H. pylori}.

Transcriptomic Profiling of Gene Expression in \textit{H. pylori} Wild-Type and \textit{ΔdppA} Strains
To further examine the roles of DppA, we performed RNA-seq analysis and investigated the genes expressed differentially between \textit{H. pylori} 26695 and a \textit{ΔdppA} strain. We found that 253 genes were differentially expressed with a $| \log_2 \text{fold change} | > 1$, including 116 genes that were upregulated and 137 genes that were downregulated in \textit{ΔdppA} ($P < 0.05$) (Figure 2A). These genes are listed in Table 2. We also performed functional classification of the genes upregulated and downregulated in \textit{ΔdppA} (Figure 2B). We found that genes involved in energy metabolism, cellular processes, transportation, and translation were significantly downregulated in \textit{ΔdppA}, which might contribute to the decreased growth rate of \textit{H. pylori} in this genetic background. Genes involved in DNA metabolism, bacterial pathogenesis, and motility were upregulated in this strain, and they might contribute to the virulence of \textit{H. pylori}.

The Dpp Transporter Activates the Expression of CagA
The transcriptomic study revealed differential expression of \textit{H. pylori} virulence genes, especially those involved with \textit{cagPAI}, between the wild-type and \textit{ΔdppA} strain. In this study, we focused on those virulence factors that are closely related to the cellular inflammatory response. We first investigated the expression of CagA at both the mRNA and protein levels. The qPCR results showed that CagA mRNA levels in \textit{ΔdppA}, \textit{ΔdppB}, \textit{ΔdppC}, \textit{ΔdppD}, and \textit{ΔdppF} strains were lower than in the \textit{H. pylori} 26,695 wild-type strain (Figure 3A). CagA protein expression was also investigated, and similar results were obtained, i.e., CagA expression was repressed in \textit{ΔdppA}, \textit{ΔdppB}, \textit{ΔdppC}, \textit{ΔdppD}, and \textit{ΔdppF} strains (Figures 3B,C). This was also shown in the NCTC11637 background, as
CagA expression was significantly lower in ΔdppA compared with the wild type (Figures 3D,E). This result demonstrated that the Dpp transporter is important for the expression of CagA, and that DppA, DppB, DppC, DppD, and DppF are all critical for the function of the Dpp transporter to regulate the expression of CagA.

The Dpp Transporter Causes the Inhibition of the Expression of Genes Encoding T4SS

Our RNA sequencing data suggested that components of T4SS, including Cag3, Cag5, Cag6, CagZ, Cag7, and Cag22, were upregulated in ΔdppA mutants (Table 2). Thus, we analyzed all 26 genes related to T4SS; surprisingly, most of these genes were expressed relatively highly in ΔdppA mutants (Figure 4A). Next, we performed qPCR to confirm this result. T4SS genes comprise nine operons, according to a previous study (Kabamba et al., 2018). To evaluate the effects of Dpp on the expression of T4SS genes, we compared the mRNA levels of Cagζ, CagV, CagU, CagS, CagQ, CagP, CagL, CagY, CagM, CagE, and CagC from each operon. Except Cagζ and CagC, whose expression showed no difference between wild type and ΔdppA, the expression of CagV, CagU, CagS, CagQ, CagP, CagM, and CagL was significantly higher in ΔdppA compared with H. pylori 26695 (Figure 4B). This was consistent with our transcriptomic data.
TABLE 2 | Differentially expressed genes identified by RNA-seq.

| Gene expression | Gene no. | Gene name or function | log<sub>2</sub> (fold change) |
|-----------------|----------|-----------------------|-----------------------------|
| Upregulated     | HP0059   | Predicted gene        | 2.4264                      |
|                 | HP0114   | Predicted gene        | 1.3287                      |
|                 | HP0115   | flaB                  | 1.7312                      |
|                 | HP0116   | topA                  | 1.378                       |
|                 | HP0117   | Predicted gene        | 1.3026                      |
|                 | HP0119   | Predicted gene        | 2.2661                      |
|                 | HP0131   | Predicted gene        | 1.7324                      |
|                 | HP0132   | sdaA                  | 1.2527                      |
|                 | HP0140   | lctP                  | 1.1428                      |
|                 | HP0142   | mutY                  | 1.0653                      |
|                 | HP0143   | Predicted gene        | 1.0722                      |
|                 | HP0230   | kdsB                  | 1.2419                      |
|                 | HP0260   | mod                   | 1.4971                      |
|                 | HP0261   | Predicted gene        | 1.1733                      |
|                 | HP0262   | Predicted gene        | 1.128                       |
|                 | HP0263   | hpaIm                 | 1.3069                      |
|                 | HP0328   | lipXK                 | 1.5594                      |
|                 | HP0329   | nadE                  | 1.03                        |
|                 | HP0342   | Predicted gene        | 1.4897                      |
|                 | HP0343   | Predicted gene        | 2.741                       |
|                 | HP0346   | Predicted gene        | 1.4341                      |
|                 | HP0366   | Spore coat polysaccharide biosynthesis protein C | 2.1667 |
|                 | HP0367   | Predicted gene        | 1.6527                      |
|                 | HP0373   | Predicted gene        | 2.8507                      |
|                 | HP0388   | rRNA methyltransferase | 1.2813                      |
|                 | HP0394   | Predicted gene        | 1.0201                      |
|                 | HP0428   | Predicted gene        | 2.0485                      |
|                 | HP0430   | Predicted gene        | 1.7632                      |
|                 | HP0431   | ptc1                  | 2.2646                      |
|                 | HP0432   | Predicted gene        | 2.4158                      |
|                 | HP0434   | Predicted gene        | 2.3603                      |
|                 | HP0440   | Predicted gene        | 1.307                       |
|                 | HP0441   | VirB4 homolog         | 1.5219                      |
|                 | HP0453   | Predicted gene        | 1.505                       |
|                 | HP0462   | hsdS                  | 1.5466                      |
|                 | HP0463   | hsdM                  | 1.5851                      |
|                 | HP0472   | omp11                 | 1.916                       |
|                 | HP0483   | Predicted gene        | 1.0174                      |
|                 | HP0522   | cag3                  | 1.0262                      |
|                 | HP0524   | cag5                  | 1.5355                      |
|                 | HP0525   | cagux                 | 1.6885                      |
|                 | HP0526   | CagZ                  | 1.2767                      |
|                 | HP0527   | cag7                  | 2.8708                      |
|                 | HP0538   | cag17                 | 1.4644                      |
|                 | HP0543   | cag22                 | 1.1474                      |
|                 | HP0601   | flaA                  | 3.1409                      |
|                 | HP0602   | Endonuclease III      | 1.881                       |
|                 | HP0603   | Predicted gene        | 2.5156                      |
|                 | HP0611   | Predicted gene        | 1.6473                      |
|                 | HP0613   | ABC transporter ATP-binding protein | 1.1885 |

(Continued)

| Gene expression | Gene no. | Gene name or function | log<sub>2</sub> (fold change) |
|-----------------|----------|-----------------------|-----------------------------|
|                 | HP0621   | mutS2                 | 1.1332                      |
|                 | HP0638   | Membrane protein      | 1.1037                      |
|                 | HP0651   | Fucosyltransferase    | 1.4555                      |
|                 | HP0652   | serB                  | 1.9143                      |
|                 | HP0666   | gtpC                  | 1.321                       |
|                 | HP0673   | Predicted gene        | 1.131                       |
|                 | HP0675   | xerC                  | 1.6417                      |
|                 | HP0690   | facA                  | 1.1721                      |
|                 | HP0711   | Predicted gene        | 1.6394                      |
|                 | HP0713   | Predicted gene        | 2.6885                      |
|                 | HP0728   | Predicted gene        | 1.2074                      |
|                 | HP0751   | flaG                  | 2.6091                      |
|                 | HP0752   | flID                  | 2.6449                      |
|                 | HP0753   | flS                   | 1.2148                      |
|                 | HP0754   | 5-formyltetrahydrofolate cyclo-ligase | 2.0228 |
|                 | HP0755   | Predicted gene        | 1.3991                      |
|                 | HP0757   | Beta-alanine synthetase homolog | 1.2021 |
|                 | HP0758   | Membrane protein      | 1.8156                      |
|                 | HP0759   | Membrane protein      | 1.6024                      |
|                 | HP0821   | uvrC                  | 1.3838                      |
|                 | HP0846   | hsdR                  | 1.8459                      |
|                 | HP0860   | gmdB                  | 1.369                       |
|                 | HP0896   | omp19                 | 1.8069                      |
|                 | HP0897   | Predicted gene        | 1.124                       |
|                 | HP0922   | Membrane protein      | 1.2871                      |
|                 | HP0939   | yckJ                  | 1.4377                      |
|                 | HP0941   | alr                   | 1.331                       |
|                 | HP0942   | dagA                  | 1.1314                      |
|                 | HP0943   | dadA                  | 1.293                       |
|                 | HP0985   | Predicted gene        | 1.0004                      |
|                 | HP1000   | Para                  | 1.4507                      |
|                 | HP1002   | Predicted gene        | 1.3929                      |
|                 | HP1017   | rocE                  | 1.704                       |
|                 | HP1020   | ispDF                 | 1.2427                      |
|                 | HP1021   | cheY                  | 1.6802                      |
|                 | HP1022   | Predicted gene        | 1.0345                      |
|                 | HP1027   | fur                   | 1.1997                      |
|                 | HP1047   | rfbA                  | 1.0606                      |
|                 | HP1051   | Predicted gene        | 1.1147                      |
|                 | HP1080   | Membrane protein      | 1.3598                      |
|                 | HP1081   | Predicted gene        | 1.5337                      |
|                 | HP1095   | trnP                  | 1.4743                      |
|                 | HP1119   | figK                  | 3.4284                      |
|                 | HP1120   | Predicted gene        | 3.1475                      |
|                 | HP1121   | BspBIM                | 1.8038                      |
|                 | HP1148   | trmD                  | 1.1276                      |
|                 | HP1165   | tetA                  | 1.2466                      |
|                 | HP1167   | Predicted gene        | 4.0239                      |
|                 | HP1215   | Predicted gene        | 2.1289                      |
|                 | HP1233   | Predicted gene        | 2.281                       |
|                 | HP1238   | amiF                  | 1.387                       |

(Continued)
### TABLE 2 | Continued

| Gene expression | Gene no. | Gene name or function | log₂ (fold change) |
|-----------------|----------|-----------------------|--------------------|
| HP1258          | Predicted gene | 1.0603                  |                    |
| HP1321          | ATP-binding protein | 1.45                    |                    |
| HP1390          | Predicted gene | 3.3507                  |                    |
| HP1391          | Predicted gene | 1.915                   |                    |
| HP1440          | Predicted gene | 3.7316                  |                    |
| HP1505          | Predicted gene | 1.123                   |                    |
| HP1519          | Predicted gene | 2.1358                  |                    |
| HP1523          | recG       | 1.2919                  |                    |
| HP1589          | Predicted gene | 1.091                   |                    |
| HP0003          | kdsA       | −1.0474                 |                    |
| HP0004          | icfA       | −1.4707                 |                    |
| HP0010          | groEL      | −1.7937                 |                    |
| HP0011          | groES      | −1.2866                 |                    |
| HP0015          | Predicted gene | −1.0676                 |                    |
| HP0033          | clpA       | −1.0831                 |                    |
| HP0035          | Predicted gene | −1.1711                 |                    |
| HP0036          | Predicted gene | −1.1712                 |                    |
| HP0057          | Predicted gene | −2.2975                 |                    |
| HP0072          | ureB       | −1.48                   |                    |
| HP0073          | ureA       | −1.5839                 |                    |
| HP0091          | hsdR       | −1.0385                 |                    |
| HP0099          | tipA       | −1.1019                 |                    |
| HP0100          | Predicted gene | −1.0583                 |                    |
| HP0102          | Predicted gene | −1.5088                 |                    |
| HP0103          | tipB       | −1.5548                 |                    |
| HP0109          | dnaK       | −1.8288                 |                    |
| HP0110          | GrpE       | −1.142                  |                    |
| HP0111          | Predicted gene | −1.1914                 |                    |
| HP0118          | Predicted gene | −1.3158                 |                    |
| HP0145          | fsxO       | −1.0377                 |                    |
| HP0153          | recA       | −1.5738                 |                    |
| HP0154          | eno        | −1.0973                 |                    |
| HP0157          | aroK       | −1.7674                 |                    |
| HP0213          | gidA       | −2.3523                 |                    |
| HP0229          | omp6       | −1.3943                 |                    |
| HP0243          | napA       | −1.9605                 |                    |
| HP0289          | lmaA       | −1.3117                 |                    |
| HP0290          | lysA       | −1.5474                 |                    |
| HP0291          | Predicted gene | −1.2657                 |                    |
| HP0292          | Predicted gene | −1.0587                 |                    |
| HP0294          | amiE       | −2.2148                 |                    |
| HP0296          | rplU       | −1.4814                 |                    |
| HP0297          | rpmA       | −1.4625                 |                    |
| HP0298          | dppA       | −4.9784                 |                    |
| HP0299          | dppB       | −4.1717                 |                    |

(Continued)
were delivered through the T4SS to gastric epithelial cells, and in wild-type strain. During infection, CagA or LPS metabolites genes from each operon were expressed more in wild-type strain compared with the ΔdppA strain, which suggested that the Dpp transporter might play an important role in bacterial adhesion. To test this hypothesis, we first confirmed the expression of the OMPs involved in bacterial adhesion. Our results showed that, compared with H. pylori 26,695, the expression of adhesion genes (babA, hopZ, oipA, and sabA) was higher in a ΔdppA strain, while alpAB, hpA, hopQ, and sabB showed similar expression levels between the wild-type and ΔdppA strains (Figure 5A). This suggested that DppA caused a lower expression of OMPs. We also investigated the expression of OMPs in ΔdppB, ΔdppC, ΔdppD, and ΔdppF strains, and showed higher expression of BabA, HopZ, OipA, and SabA than in wild-type (data not shown), which confirmed that the Dpp transporter causes a lower expression of OMPs. Next, to verify whether the Dpp transporter altered the adhesion of H. pylori to AGS cells, AGS cells were infected with H. pylori 26,695 wild-type and ΔdppA cells; subsequently, we investigated the number of bacteria bound to AGS cells. Our results showed that ΔdppA cells had a higher binding capacity compared with wild-type 26,695 cells (Figure 5B). H. pylori NCTC11637 and its isogenic mutant ΔdppA were also analyzed, and we found that in the H. pylori NCTC11637 strain, deletion of dppA also resulted in a higher bacterial adhesion level (Figure 5C). This suggested that in H. pylori, the Dpp transporter caused a reduced expression level of OMPs, including babA, hopZ, oipA, and sabA, thereby reducing the adhesion of H. pylori to AGS cells.

The Dpp Transporter Inhibits H. pylori Activation of Gastric Epithelial NF-κB

Upon adhesion to AGS cells, H. pylori directly activates NF-κB through the T4SS, which delivers the effector protein CagA, peptidoglycan, or ADP-ribose cell wall. We investigated the effect of the Dpp transporter on H. pylori-induced NF-κB activation in AGS cells. We performed a dual-luciferase reporter assay using an NF-κB-luc reporter plasmid. After 4 h of infection with H. pylori, NF-κB was activated in wild-type infected cells. We also found that ΔdppA, ΔdppB, ΔdppC, and ΔdppD infection activated NF-κB to a level 50% higher than infection with a wild-type strain (Figure 6A). However, infection with the ΔdppF strain failed to activate NF-κB, likely due to low activity of ΔdppF for its low

We also tested the ΔdppC background, and found that all 10 genes from each operon were expressed more in ΔdppC than in wild-type strain. During infection, CagA or LPS metabolites were delivered through the T4SS to gastric epithelial cells, and this was dependent on the direct interaction between T4SS proteins and α5β1 integrins (Kwok et al., 2007). To investigate if the Dpp transporter also influenced the binding of T4SS to α5β1 integrin, a bacterial pulldown assay using purified α5β1 integrin was performed, and we measured the amount of α5β1 integrin bound by H. pylori. The results showed that the ΔdppA strain bound significantly more α5β1 integrin compared with H. pylori 26,695 wild-type strain (Figures 4D,E). These results suggested that deficiency in Dpp transporter resulted in a higher expression of T4SS genes and an increase in T4SS binding to α5β1 integrin.

The Dpp Transporter Causes Lower Expression of Outer Membrane Proteins and Reduces the Adhesion of H. pylori to AGS Cells

Our transcriptomic study revealed that several outer membrane proteins related to adhesion were differentially expressed in ΔdppA strain. This suggested that the Dpp transporter might play an important role in bacterial adhesion. To test this hypothesis, we first confirmed the expression of the OMPs involved in bacterial adhesion. Our results showed that, compared with H. pylori 26,695, the expression of adhesion genes (babA, hopZ, oipA, and sabA) was higher in a ΔdppA strain, while alpAB, hpA, hopQ, and sabB showed similar expression levels between the wild-type and ΔdppA strains (Figure 5A). This suggested that DppA caused a lower expression of OMPs. We also investigated the expression of OMPs in ΔdppB, ΔdppC, ΔdppD, and ΔdppF strains, and showed higher expression of BabA, HopZ, OipA, and SabA than in wild-type (data not shown), which confirmed that the Dpp transporter causes a lower expression of OMPs. Next, to verify whether the Dpp transporter altered the adhesion of H. pylori to AGS cells, AGS cells were infected with H. pylori 26,695 wild-type and ΔdppA cells; subsequently, we investigated the number of bacteria bound to AGS cells. Our results showed that ΔdppA cells had a higher binding capacity compared with wild-type 26,695 cells (Figure 5B). H. pylori NCTC11637 and its isogenic mutant ΔdppA were also analyzed, and we found that in the H. pylori NCTC11637 strain, deletion of dppA also resulted in a higher bacterial adhesion level (Figure 5C). This suggested that in H. pylori, the Dpp transporter caused a reduced expression level of OMPs, including babA, hopZ, oipA, and sabA, thereby reducing the adhesion of H. pylori to AGS cells.
FIGURE 3 | Effects of DppA on the expression of CagA. (A) mRNA level of CagA expressed in H. pylori 26,695 and Dpp transporter mutant strains. Values represent the relative mRNA level of CagA normalized to H. pylori 26,695. (B–D) CagA expression level determined by Western blot. Total protein represents the cell lysate resolved by SDS-PAGE. Protein bands representing CagA are indicated, and the position of a 130 kDa size marker is indicated by an arrow. (C, E) Quantification analysis of CagA bands. Densitometry was normalized to total protein. Values are shown as averages ± SD (n = 3). ***P < 0.001, **P < 0.01, *P < 0.05.

FIGURE 4 | Effects of DppA on the expression of CagT4SS. (A) Hierarchical cluster analysis of T4SS gene expression in 26,695 and ΔdppA strains. (B) Determination of mRNA levels of T4SS components in H. pylori 26,695, ΔdppA, and ΔdppC (C). Values represent the relative mRNA level of each gene normalized to H. pylori 26,695. (D) αβ1 integrin bound by H. pylori 26,695 and ΔdppA. Bands representing β1 integrin are indicated, and total bacterial protein load is shown. (E) Quantification analysis of β1 integrin bands. Densitometry was normalized to total protein. ***P < 0.001, **P < 0.01, *P < 0.05.
growth ability as shown in Figure 1E. We also checked *H. pylori* NCTC11637 and its isogenic mutant ΔdppA, and found that NF-κB-luc was expressed at a level 70% higher than in wild type (Figure 6B). This suggested that the Dpp transporter in *H. pylori* reduced the ability to activate NF-κB in AGS cells. Activation of NF-κB directly expresses the expression of the inflammatory factors, including IL-8 (Brandt et al., 2005). Thus, we next utilized by ELISA and qPCR to examine IL-8 expression in AGS cells infected by *H. pylori*. Our results showed that IL-8 expression in AGS cells induced by *H. pylori ΔdppA, ΔdppB, ΔdppC, and ΔdppD* was significantly higher than that induced by wild-type *H. pylori* 26,695 (Figures 6C,D). As shown in Figure 6A, ΔdppF failed to activate the expression of IL-8 in AGS cells. In *H. pylori* NCTC11637, ΔdppA also induced a higher level of IL-8 expression compared with wild-type *H. pylori* NCTC11637 (Figures 6E,F). This indicated that the Dpp transporter repressed NF-κB and IL-8, thereby reducing the inflammatory response of AGS cells induced by *H. pylori*.

Taked together, this work was the first study to show the role of the Dpp transporter in the regulation of virulence of *H. pylori*. Our study demonstrated that the Dpp transporter is important for the growth of *H. pylori*, suggesting that dipeptides might serve as an important nutrient source for this bacterium. Although Dpp transporter-deficient strains proliferated slower, they were associated with higher bacterial adhesion and T4SS expression and induced a stronger inflammatory response in AGS cells. The Dpp transporter also activated the expression of CagA, illustrating the complex role of Dpp transporters in subtle control of bacterial virulence.

**DISCUSSION**

The host tissue is a rich source of nutrients for bacteria, providing nutrients such as sugars and amino acids. To acquire the nutrients from host, pathogens produce specific virulence factors and causes host damage. It is important to understand the interaction between metabolism and bacterium pathogenesis since bacterial growth is the main goal for the pathogen to colonize in the host (Rohmer et al., 2011). Peptide transporters are important to acquire carbon from host sources for pathogen’s growth. Moreover, these transporters are also responsible for importing environmental cues to coordinate bacterial behavior (Garai et al., 2017). Human pathogens always face various environmental stresses, such as temperature variation, pH, nutrient changes, and oxidative stress (Shao et al., 2005). Understanding these transporters and their cognate substrates may help in unraveling the mechanisms of bacterial adaptation through changes in bacterial behavior, including virulence.

In this study, we found that the Dpp transporter was important for the bacterial growth (Figure 1). However, *H. pylori ΔdppD and ΔdppF* grew significantly slower compared to wild type, ΔdppA, ΔdppB, or ΔdppC cells. DppD and DppF are both dipeptide ABC transporter ATP binding subunits, which suggests that ΔdppD and ΔdppF might completely abolish the function of Dpp transporters, resulting in a shortage of nutrients. Specifically, we noticed that the ΔdppF strain grew much slower compared with other strains. DppF acts as an ABC transporter ATP binding subunit, which might be critical for the growth of *H. pylori*, causing ΔdppF with its decreased capacity to induce an inflammatory response in AGS cells (Figures 6A,C,D).

In this study, we found that the CagA expression was largely dependent on the expression of the Dpp transporter (Figure 3). During the infection of gastric epithelial cells, *H. pylori* translocates CagA using the T4SS. Through interactions with SH2 domains, CagA activates their function to promote the Ras-Erk signaling pathway to activate oncogenesis of gastric epithelial cells (Tohidpour, 2016; Hatakeyama, 2017; Naumann et al., 2017). The CagA protein is strongly associated with development of gastric cancer, and regulation of CagA expression is closely related to gastric cancer development (Hatakeyama, 2017). Studies have shown that CagA expression varies depending on the growth stage and conditions (Karita et al., 1996). It has also been shown that high-salt concentrations induce the expression of CagA, which is related to gastric cancer development. Iron and pH also regulate CagA expression (Odenbreit et al., 1999).
Our study provided new evidence on the regulation of CagA expression in H. pylori, suggesting that the nutrient status of the environment affects CagA expression and H. pylori-related gastric cancer. Specifically, when grown in environments with abundant nutrients, H. pylori might express high levels of CagA.

In the early stages of infection, H. pylori activates NF-κB in a CagT4SS-dependent manner. The regulation of T4SS expression might contribute to H. pylori-induced inflammatory response in AGS cells. In this study, we found upregulated expression of T4SS components in the ΔdppA strain (Figures 4A,B), which was also replicated in a ΔdppC strain (Figure 4C). Among these genes, RNA sequencing data and qPCR results showed that genes were upregulated in ΔdppA strain to a different degree, which suggested that although these genes are all responsible for the T4SS apparatus, they might be regulated by different mechanisms. A previous study investigated the expression of T4SS and found that these genes responded differently to growth phase, temperature, pH, iron, and cell contact. Some environmental signals even exert pleiotropic effects on these genes. This suggests that T4SS expression and assembly are controlled by sophisticated mechanisms, but more studies are necessary. The T4SS machinery translocates CagA, ADP-heptose, peptidoglycan, and other substrates to host cells. Some studies have shown that ADP-heptose is a novel pathogen-associated molecular marker in H. pylori, and it is the main factor activating NF-κB in a T4SS-dependent manner. We speculate that T4SS expression is repressed under rich nutrient conditions, and H. pylori reduces the translocation of ADP-heptose or other effector molecules. Under poor nutrient condition, T4SS expression is activated and results in a high level of activation of the NF-κB response.

After H. pylori passes through the mucous layer and reaches the gastric mucosa via flagellar movements, OMPs promote close contact between H. pylori and gastric epithelial cells. OMPs play important roles in the establishment of colonization. The OMPs in H. pylori have been gradually unveiled, and their cognate interaction partners have been identified. In this study, we found that expression of BabA, HopZ, OipA, and SabA, and bacterial adhesion were upregulated in the ΔdppA strain (Figure 6). BabA was the first OMP identified to be involved in the adhesion of H. pylori and important for inducing severe inflammation in the stomach. Moreover, studies have shown that T4SS function and CagA translocation are enhanced by BabA. The HopZ gene is involved in the adhesion of H. pylori to gastric epithelial AGS cell line in vitro, but
it did not show any influence on the ability of colonization in the stomachs of guinea pigs (Peck et al., 1999). However, the cognate receptor of HopZ remains unknown. Our study suggested that the Dpp transporter in H. pylori plays an important role in the colonization of the stomach.

Besides the virulence factors investigated in this study, our transcriptomic study by RNA sequencing also indicated that the expression of other virulence genes is also altered in the ΔdppA background. Flagellar coding genes, including flaA, flaB, flaD, flaG, and flaK, were significantly upregulated in ΔdppA cells (Table 2). Flagellar movement is critical for the initial colonization of H. pylori by penetrating gastric mucus layer (Gu, 2017). Indeed, flagellar movement of H. pylori is an important factor in mediating high density colonization and severe infection. Studies have shown that FlaA and FlaB are necessary for H. pylori colonization of animals (Josenhans et al., 1995). ADP-heptose is a lipopolysaccharide synthesis intermediate, which is responsible for H. pylori-induced NF-κB activation (Pfannkuch et al., 2019). RNaseq data showed that upregulated expression of major of LPS-related metabolic genes and the ADP-heptose synthesis gene gmhB in the ΔdppA strain (Table 2). GmhB (Hp0860) is an important synthase gene for the synthesis of ADP-heptose by dephosphorylation of D-glycero-D-manno-heptose-1,7-bisphosphate (HBP) (Stein et al., 2017). This suggests that LPS synthesis and ADP-heptose production might be upregulated in ΔdppA, thereby enhancing H. pylori-induced IL-8 production and NF-κB activation.

In conclusion, we have demonstrated that the Dpp transporter affects the expression of virulence factors such as CagA, T4SS, and OMPs. The Dpp transporter might enable the bacteria to recognize environmental nutrient conditions and change virulence factors such as adhesion and stimulate the release of other virulence factors. Since H. pylori causes a chronic infection and is closely related to gastric cancer, our study suggests that when nutrients are limited, and the Dpp transporter fails to transport dipeptides, H. pylori enhances its ability to colonize and stimulates an inflammatory response to acquire nutrients from the host. Thus, H. pylori tends to repress its ability to stimulate an inflammatory response in gastric epithelial cells while delivering the oncoprotein CagA, which induces gastric cancer.

DATA AVAILABILITY STATEMENT

The original contributions generated for this study are publicly available. This data can be found here: NCBI Gene Expression Omnibus database (GEO; http://www.ncbi.nlm.nih.gov/geo) under accession number GSE164216 (www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE164216).

AUTHOR CONTRIBUTIONS

YW and FS designed the study. XX, JC, and SF performed the experiments. XH analyzed the data. YW, XX, and FS wrote the manuscript. All authors have read and approved the submitted version.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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