The virulence factor OipA in Asian isolates of Helicobacter pylori

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The virulence factor OipA in Asian isolates of *Helicobacter pylori*

A thesis submitted in partial fulfillment of the requirement for the degree of Bachelor of Science in Biology Department from The College of William and Mary

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Abstract

Persistent infection by *Helicobacter pylori*, a gram-negative bacterium, is associated with gastric cancer. The outer membrane protein of *H. pylori* OipA has been identified as a virulence factor that induces inflammation and facilitates adhesion to epithelial cells by some studies. Our lab has determined that two of our collections of eight Asian strains of *H. pylori* possess a second copy of *oipA*, a condition that is not found among European, African or other western strains of *H. pylori*. The potential significance of this observation arises from the fact that the prevalence of gastric cancer is much higher in Asia than that of the west. We hypothesized that the frequent merodiploid nature (two gene copies in an otherwise haploid genome) of *oipA* among Asian *H. pylori* strains may lead to increased induction of the pro-inflammatory cytokine IL-8 and translocation of the effector protein CagA into epithelial cells. Such increase in virulence of Asian *H. pylori* strains may partially explain the high prevalence of gastric cancer in Asia. However, our results indicate that knocking out each *oipA* allele individually or ablating both alleles in a single mutant has no effect on IL-8 secretion by AGS cells, adherence ability to AGS cells and CagA translocation of *H. pylori* strain 98-46. However, our results of the mRNA levels of *oipA* in our mutant collection indicate transcript levels vary among the mutants.

Introduction

*H. pylori’s high prevalence in Asia*

*H. pylori* is a gram negative bacterium that has colonized half of human population since our earliest evolutionary times (Kronsteiner et al., 2016). The infection usually happens
during early childhood and is transmitted between close family members (Sun et al., 2013; Chmiela et al., 2014). Without antibiotic treatments, the infection can last for the host's lifetime (Chmiela et al., 2014). Despite its high prevalence, only 5-10% of those infected develop serious gastric disease, such as gastric cancer or peptic ulcer disease. Due to its association with gastric diseases and chronic inflammation, *H. pylori* was classified as a human carcinogen by the International Agency for Research on Cancer (IARC), responsible for ~60% of gastric adenocarcinoma cases (IARC working group, 1994; Parkin et al., 2005; Wang, F. et al., 2014). Thus, the prevalence of *H. pylori* and the development of gastric cancer are closely associated (Chang, A.H. and Parsonnet, J., 2010).

However, the prevalence of *H. pylori* is not evenly distributed worldwide. In developed countries, the prevalence of *H. pylori* is decreasing due to antibiotic treatment and a more hygienic food and drinking water environment, whereas in many developing countries, including many countries in Asia, the prevalence exceeds 50% (Hooi et al., 2017; Peleteiro et al., 2014). Even among Asian countries, the prevalence varies significantly. Developed countries such as Japan and Korea, have much lower prevalence of *H. pylori* than that of developing countries India and Thailand (Hooi et al., 2017). Interestingly, Asian countries possess significantly higher incidence of gastric cancer. Gastric cancer cases of Asia, especially Eastern Asia, contributed half of the world’s total gastric cancer cases (Rahman et al., 2014). Phylogenetically, *H. pylori*, with its great genetic diversity is divided into seven populations corresponding to geographic origins (Falush, D. et al., 2003). hspEAsia, the major population distributed in East Asia, is associated with much severe clinical outcomes than Western strains. However, increased virulence among the hspEAsia isolates infecting
patients in Asia may contribute to the well-documented health burden of gastric cancer in that region. The mechanisms involved in this geographic disease anomaly remain elusive.

**H. pylori pathogenesis**

The ability of *H. pylori* to establish a persistent infection is associated with its diverse virulence factors, including the *cag* pathogenicity island (*cag*PAI) and its encoded translocated effector protein CagA as well as multiple outer membrane proteins, which allow adhesion to gastric epithelial cells (Kronsteiner et al., 2016). At the onset of infection, the *H. pylori* enzyme urease is utilized at the mucus layer and helps to increase the pH level locally (Eaton et al., 1991). Sheathed flagella allow the pathogen to penetrate through mucus layer and contact the epithelial cell layer and into the gastric pits as well. Once this contact is established, *H. pylori* can use an unusually large number of adhesion proteins including SabA, BabA, AlpA, OipA and HopZ to facilitate its adhesion to epithelial cells (Kronsteiner et al., 2016).

Strains possessing the Cag (cytotoxin-associated gene) pathogenicity island (*cag*PAI), an approximately 40-kb DNA insertion possibly acquired through horizontal transmission (Censini et al., 1996), are associated with higher IL-8 induction. IL-8 is a pro-inflammatory cytokine and the subsequent recruitment of leukocytes such as neutrophils and macrophage results in the characteristic gastric inflammation associated with virtually all infections by *cag*PAI possessing *H. pylori* strains. IL-8, acting on endothelial cells expressing receptors CXCR1 and CXCR2, is associated with the development of cancer (Li, A. et al., 2005; Holmes et al., 1991). It has been shown that expression of IL-8 correlates with angiogenesis (Li, A. et al., 2003; Lee et al., 2013), and such vascular development is an important hallmark
of cancer development (Hanahan, D. and Weinberg, R.A., 2000). Figure one summarizes potential function of IL-8 on endothelial cell, tumor cell, stromal cell and inflammatory cell.

![Figure 1. Role of IL-8 in cancer development. Adapted from Lee et al. (2013). IL-8 affects endothelial cell, tumor cell and stromal cell, inducing growth factor, migration and proteases which are highly associated with angiogenesis and metastasis, two important hallmarks of cancer.

The association between cagPAI and IL-8 induction is mediated by a needle like type IV secretion system (T4SS) encoded by cagPAI. Deletion of crucial genes such as cagE or cagG results in significantly reduced IL-8 secretion (Hsu et al., 2002). Through this T4SS virulence factors such as CagA and peptidoglycan, are delivered into epithelial cells (Tan et al., 2009; Hutton et al., 2010).

cagA (cytotoxin-associated gene A), encoded at one end of cagPAI, is an important virulence factor associated with IL-8 induction. Once delivered into epithelial cell through T4SS, CagA can be phosphorylated by tyrosine kinase at glutamate –proline –isoleucine -
tyrosine-alanine (EPIYA) motifs and induces the expression of pro-inflammatory cytokine interleukin 8 (IL-8) (Backert et al., 2001). The phosphorylated CagA activates several signal pathways, such as MEK (mitogen-activated protein kinase) and NF-κB (nuclear factor κB) pathway, each of which is involved in proliferation of epithelial cells (Xu, X. et al., 2012). Phosphorylated CagA is also associated with altered activity of SHP-2 (Src homology phosphatase 2), inducing actin cytoskeleton rearrangement and alteration of epithelial cell polarity resulting in an altered morphology called the “hummingbird” phenotype. A study done by Chang et al. (2016) demonstrated that fragmentation of CagA resulted in less severe hummingbird phenotype. The disruption of cell polarity caused by CagA allows H. pylori to replicate directly on the apical cell surface (Tan et al., 2009; Backert et al., 2001). In addition, CagA also targets tumor suppressor RUNX3 and induces the ubiquitination of RUNX3 (Tsang et al., 2010). The induction of growth factor, alteration of epithelial cell morphology and facilitation of cell proliferation all play important roles in H. pylori associated disease pathogenesis.

Peptidoglycan (PG), another important virulence factor, also leaks through T4SS into host epithelial cell cytoplasm. Inside the cell, PG is detected by nucleotide-binding oligomerization domain protein (NOD1), which then activates NF-κB inducing secretion of IL-8 (Hutton et al., 2010).

**Increased virulence among Asian H. pylori strains**

The virulence of H. pylori is greatly affected by the presence or absence of the cagPAI. While cagPAI positive strains induce severe inflammation and are associated with more severe cell damage and gastric diseases, cagPAI negative strains usually display mild
asymptomatic inflammation (Kronsteiner et al., 2016). Nevertheless, the prevalence of cagPAI positive strains showed large geographic variations (Yamaoka et al., 2002). Approximately 60-70% of Western H. pylori strains contain cagPAI, whereas nearly 100% of Asian strains possess this key virulence locus (Tomb et al., 1997; Ando et al., 2002b).

The CagA protein also exhibits interesting geographic variations. The EPYIA motif located at C-terminus of CagA is found in four different patterns (EPIYA-A, EPYIA-B, EPYIA-C and EPYIA-D). EPYIA patterns vary according by geographic regions. The majority of Western H. pylori strains contain combinations of EPIYA-A, -B, and -C CagA motif, whereas Asian H. pylori strains were dominated by combinations of EPIYA-A, -B, and -D motif (Covaccci, A. et al., 1993; Argent et al., 2008). The dominant Asian EPIYA motifs have been shown with higher affinity of SHP-2, which induces significantly more morphological change and mobility of epithelial cells (Higashi, H. et al., 2002). Thus, Asian H. pylori strains with Eastern CagA are considered having higher virulence.

Geographic variation in H. pylori genetic architecture is not limited to the cagPAI and CagA. oipA, encoding an outer membrane protein adhesion implicated in host inflammatory response, also shows genetic polymorphisms associated with geographic variations (Ando et al., 2002b; Zhang et al., 2014). The expression of OipA is regulated by a slipped-strand repair mechanism (SSM) with varied CT (cytosine-thymidine) tract near the 5’ end of oipA gene (Miftahussurur M, Yamaoka Y, 2015). Depending upon the number of CT repeats in this region, the oipA gene may be expressed, i.e., phase on, or non-expressed, i.e., phase off. CT repeat patterns within oipA are associated with geographic origins (Zhang et al., 2014). In European H. pylori isolates, the 6 CT repeat variants were dominant. In contrast, 3 CT repeat
variants dominated among *H. pylori* strains isolated from patients in Asian countries (Zhang et al., 2014). The reduced repetitive nature of the CT tract region among Asian strains likely decreases the frequency of SSM mediated CT tract mutation (Ando et al., 2002b; Zhang et al., 2014). Thus, despite the hypermutable CT repeat, these Asian strains possessing a collapsed polyCT tract showed little phase variation of *oipA* gene *in vitro*, indicating a conserved characteristic of OipA expression.

OipA phase variation is also closely related to the status of the primary virulence determinant of this pathogen, the cagPAI. A study performed by Ando et al. (2002b) showed that among 109 *H. pylori* isolates from nine countries, 96% of cagPAI-positive strains possessed an *oipA* allele in frame (phase on), while none of the cagPAI-negative strains contain in-frame *oipA*. A recent study in our lab (Horridge et al., 2017) demonstrated that disrupting or turning phase off of *oipA* gene of *H. pylori* strain 26695 resulted in reduced IL-8 production and severely reduced or eliminated CagA translocation. Our results suggested that such a close relationship between *oipA* phase variation and cagPAI might be associated with our demonstrated role of OipA in CagA translocation into gastric cells *in vitro*.

**Outer membrane protein A and its duplication in Asian *H. pylori* strains**

The limited phase variation of *oipA* among Asian isolates of *H. pylori* and the association of phase on alleles of *oipA* with the presence of the cagPAI, suggests an important function. OipA may function synergistically with cagPAI, the Vacuolating Cytotoxin (VacA) and other virulence factors such as adhesins (Su et al., 2016; Dossumbekova et al., 2006; Yamaoka et al., 2000). A study by Sun and colleagues demonstrated that gastric cancer patients exhibited
a higher OipA seropositivity, suggesting a potential role of OipA as a gastric cancer biomarker (Sun et al., 2013).

OipA was designated as outer inflammatory protein in 2000 by Yamaoka et al. Their study demonstrated that knockout of oipA led to 50% reduction of IL-8 secretion of AGS cells, hence the name outer inflammatory protein. However, the role of OipA in IL-8 induction remains controversial. Several studies, included one from our lab, showed that OipA plays an important role in inducing the pro-inflammatory cytokine IL-8 secretion (Yamaoka et al., 2000; Horridge et al., 2017; Odenbreit et al., 2009). Nevertheless, many other studies demonstrated that disrupting of oipA gene does not affect IL-8 production (Dossumbekova et al., 2006; Ando et al., 2002a). Interestingly, a study showed that a duplication of oipA gene (oipA merodiploidy) was found in some Asian and Amerindian H. pylori isolates (Kawai et al., 2011), but not in isolates from other geographical regions. Thus, we hypothesized that the contradictory results of OipA’s role in IL-8 induction were partially due to the unknown nature of oipA merodiploidy.

The mechanism of duplication of oipA is not clear. Furuta et al. (2011) hypothesized that the diploid oipA genes in Asia and Amerindian H. pylori strains was induced by “Duplication Associated with Inversion (DDAI). DDAI could result in the generation of a copy of DNA at a distant locus, which was followed by inversion of two loci through homologous recombination. They also constructed a phylogenetic tree (Fig. 2A) based on inversion events in H. pylori, which identified duplication of oipA gene as an early event in the evolution of this pathogen. This hypothesis may explain why some Asian and Amerindian H. pylori strains are merodiploid for oipA, while strains from all other geographic regions are haploid for
oipA.

Figure 2. Phylogenetic and inversion events of H. pylori. Adapted from Furuta et al. (2011). (A) Phylogenetic tree constructed based on all inversions in H. pylori. Letter A in panel A indicates the event of oipA gene duplication. (B) DNA inversions in H. pylori over evolutionary time. Circles indicate bacterial genome. Triangles indicate inversions occurred through DDAI.

Together, the high prevalence of the virulence determinant cagPAI, modified oipA CT tract to perhaps an invariant phase on status and duplication of oipA gene suggest a potential increased virulence among Asian H. pylori strains may in part be attributed to OipA expression. Thus, this study aims to explore the relationship between the virulence of Asian strains and the frequent diploid nature of oipA among H. pylori strains from this region. We hypothesized that Asian strains merodiploid for oipA may correlate with higher epithelial cells attachment and facilitate more CagA translocation via the cagPAI encoded T4SS and higher IL-8 induction. Such virulence determinants may be associated with the documented higher incidence of gastric cancer in Asia.

Materials and Method

H. pylori isolates
Eight *H. pylori* strains used in this study were isolated from Japanese gastric disease patients: four from gastritis patients, four from patients with adenocarcinoma of the distal stomach. These isolates were generous gifts from Dr. Takafumi Ando and Dr. Richard M. Peek Jr of Vanderbilt University Medical Center. All eight strains: 98-8, 98-10, 98-11, 98-18, 98-19, 98-20, 98-23, and 98-46 are *cagPAI*-positive. However, the nature of *oipA* merodiploidy was not determined prior to their receipt by our lab.

**H. pylori cultivation**

*H. pylori* strains were cultured on tryptic soy agar II with 5% sheep blood (BBL) for 48 to 72 hours. Incubation was carried in 37°C with 5% CO₂. The genome of *H. pylori* was extracted by using gBAC mini Genomic DNA Kit (IBI), suspended in elution buffer, stored at -20°C for PCR analysis. Sulfite-free Brucella broth (SFBB) at pH7 supplemented with cholesterol (Gibco by Life Technologies) and 20µg vancomycin/mL was used as the liquid culture for *H. pylori* cultivation at 37°C in 5% CO₂ with 150rpm.

**Sequencing of oipA1 and oipA2**

All 8 Japanese strains were tested for duplication of *oipA* gene. Approximately 200 ng genomic DNA was used as template with GoTaq Master Mixes (Bio-Rad) and specific primers (Table 1) were used for polymerase chain reaction (PCR) in a final volume of 25µl in to amplify the DNA region of interest. The PCR products were then analyzed by electrophoresis with 1.0% agarose gel supplemented with ethidium bromide. For strain 98-46 and 98-19, whose PCR amplicon sizes suggested a potential second copy of *oipA*, sequencing reactions were performed for both *oipA1* and potential *oipA2* gene by BigDye Terminator Cycle Sequencing Kit.
Cloning and mutagenesis of oipA1 and oipA2

From both *H. pylori* strains 98-46 and 98-19, oipA1 and oipA2 gene were each cloned into pGEM-T Easy Vector (Bio-Rad). Vector and amplified *oipA1* or *oipA2* DNA were ligated using T4 DNA Ligase (New England Biolabs), and then transformed into JM109 competent cells. Transformed cells were grown in LB Broth supplemented with 100 μg ampicillin/mL for 24 hours, from which plasmids (designated as pOipA1 and pOipA2) were harvested by using IBI Mini Plasmid Kits. For mutation of *oipA* alleles of strain 98-46, inverse PCR primers (Table 1) was designed based upon *oipA1* and *oipA2* sequences determined by sequencing theses alleles from *H. pylori* 98-46. These primers, with unique and novel BamHI restriction sites include in their 5’ ends (Table 1), were used to simultaneously delete ~450 bp internal to each *oipA* allele from pOipA1 and pOipA2 and introduce a unique BamHI sites. These restriction sites were used to clone in either chloramphenicol resistance (Chloramphenicol Acetyl Transferase or CAT) or kanamycin resistance (*aphA3* or kan) were inserted to create antibiotic resistant plasmids designated as pΔOipA1::kan and pΔOipA2::CAT.

**oipA1 null and oipA2 null 98-46 mutant**

Natural transformation was used as the mechanism to accomplish allelic exchange using plasmid pΔOipA1 and *H. pylori* strain 98-46 wild type. Via allelic exchange, recombination selected for the exchange of the native *oipA* first copy with ΔOipA1::kan allele on the plasmid. Mutant *H. pylori* strains were selected on 10μg kanamycin/mL SFBB plates after 7 days of incubation. Resultant mutants were confirmed via both PCR of the *oipA1* locus and sequencing reaction. *oipA2* null mutant for 98-46 was constructed based on same protocol as
that of \textit{oipA1} null mutant. \textit{p\Delta}OipA2::cat was naturally transformed into wild type 98-46, creating a chloramphenicol resistant \textit{H. pylori} strains. Mutants are designated as \textit{oipA1}⁻ and \textit{oipA2}⁻ respectively (Table 3).

\textbf{\textit{oipA1}}:\textit{oipA2} \textbf{null mutant}

\textit{p\Delta}OipA1::kan was naturally transformed into the \textit{H. pylori} 98-46 \textit{oipA2}⁻ mutant strain. After 7 days of incubation on 10μg kanamycin/mL SFBB plates, isolated colonies were selected and screened. The chloramphenicol/kanamycin resistant mutant strain was confirmed by PCR amplification at \textit{oipA1} gene locus and sequencing. The mutant is designated as \textit{oipA2}⁻/\textit{oipA1}⁻ (Table 3).

\textbf{\textit{cagE}} \textbf{null mutant}

\textit{cagE} null mutants were created to impair the function of \textit{cagPAI} T4SS, and thus interfere with CagA translocation and IL-8 secretion by cultured AGS gastric adenocarcinoma cells. This creates an isogenic control strain for use in experiments on IL-8 induction and CagA translocation. The plasmid pICB::CAT (Tummuru et al., 1995), possessing a chloramphenicol resistance gene within the gene \textit{cagE} was naturally transferred into 98-46 wild type \textit{H. pylori} strains. Mutants designated as \textit{cagE}⁻ were confirmed via PCR and sequencing reaction (Table 3).

\textbf{AGS cell culture}

The human gastric epithelial adenocarcinoma cell AGS (a gift from Timothy Cover of Vanderbilt University Medical center) was grown in Roswell Park Memorial Institute 1640 medium—RPMI medium (Life Technologies) with 10 mM HEPES 10% newborn calf serum (NCS) and 1X penicillin/streptomycin (Life Technologies). AGS cells were incubated in T-25
tissue culture flasks (Thermo Fisher) in 37°C incubator with 5% CO₂.

**AGS cell infection**

2.5x10^5 AGS cells were inoculated into six-well tissue culture plates (CytoOne) in 3ml of RPMI supplemented with 10 mM HEPES 10% NCS and penicillin/streptomycin. Incubated at 37°C incubator with 5% CO₂, AGS cells typically achieved ~90% confluency at 5 days. Each well was washed twice by 1 ml of RPMI supplemented with 10 mM HEPES 10% NCS without addition of antibiotics. *H. pylori* cells were harvested from TSAII/5% sheep blood agar plates and suspended in RPMI medium without antibiotics. The density of bacterial culture was determined by spectrophotometer. The AGS cells were then incubated with *H. pylori* at a multiplicity of infection (MOI) of 100:1 for 5 hours at 37°C with 5% CO₂. Supernatant was collected and centrifuged to remove non-adherent *H. pylori* and detached AGS cells.

**IL-8 ELISA assay**

ELISA assay was performed to assess IL-8 level. Supernatant of AGS infection was collected, centrifuged at 4360 x g to remove cells, and the supernatant stored at -80°C. IL-8 capture antibody (BioLegend) was used to coat the 96-well plate overnight at 4°C. The next day, 96-well plate was washed with PBS with 0.05% Tween 20 and blocked by 1X assay diluent at room temperature for one hour. Supernatants from AGS infection were added and incubated at room temperature for 2 hours. Then microplate was washed and was incubated with detection antibody at 1:500 dilution for one hour. HRP-avidin conjugated, secondary antibody at 1:1000 dilution, was added to microplate and then incubated for 15 minutes. The 96-well plate was read at 490nm by microplate reader (Bio-Rad).
CagA translocation assay

Western blot assays of phosphorylated tyrosine and CagA were used to assess CagA translocation. After infection of AGS cells in 6-well tissue culture plates with *H. pylori* at an MOI of 100 for 14 hours, non-adherent *H. pylori* cells were removed, and each well washed three times by 3 ml 4°C PBS. The AGS cell monolayer-*H. pylori* complex was lysed using 400μl lysis buffer (50mM Tris-HCL, pH7.4, 150mM NaCl, 0.1mM EDTA, and 1% of NP-40) supplemented with 2 mM sodium orthovanadate and a protease inhibitor cocktail (Thermo Fisher). After the addition of lysis buffer and protease inhibitors, the monolayer was dislodged using a cell scraper and the mixture was incubated at 4°C with agitation at 150 rpm for 1.5 hours. Protein concentration of the resulting lysate was determined by Pierce BCA Protein Assay Kit (Thermo Fisher) after removing insoluble materials at 12,000 x g centrifugation. 20μg of lysate protein was loaded identically into two 7% TGX polyacrylamide gels (Bio-Rad) and separated by electrophoresis. Blotting proteins from polyacrylamide gels to nitrocellulose membranes was done overnight with 30V and blots were then blocked using 5% Bovine Serum albumin (BSA) in Tris-buffered saline with 0.05% Tween 20 (TBST) supplemented with 2mM of sodium orthovanadate. One blot was treated with a 1:500 dilution of rabbit monoclonal antibody to phosphotyrosine (AbCam), the other blot was treated with a 1:1000 diluted mouse monoclonal anti-CagA antibody (AbCam). Both antibody incubations were overnight at 4°C with gentle agitation. Horseradish peroxidase conjugated anti-rabbit and anti-mouse IgG were used as secondary antibodies. SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher) was added after antibody blotting to accomplish chemiluminescence detection of phosphorylated or total
CagA.

**Attachment assay**

*H. pylori* attachment assays were performed to assess the adhesion ability of *H. pylori* to AGS cells. AGS cell monolayers were infected with wild type 98-46 and isogenic mutant *H. pylori* strains 98-46 oipA1, 98-46 oipA2, and 98-46 oipA2/oipA1 in 6-well tissue culture plate (Cyto One) at a multiplicity of infection of 100:1 (*H. pylori*: AGS cells). After 5 hours incubation at 37°C with 5% CO₂, non-adherent *H. pylori* cells were removed by aspiration. Each well was washed with 1ml of PBS three times. AGS cells were then lysed with 1ml of 0.1% Saponin in PBS for 15 minutes at 37°C with 5% CO₂ with 150 rpm agitation. Lysates of each well were serially diluted in sterile PBS to 10⁻⁴. From each dilution, 20 μL was spotted on trypticase soy sheep blood agar plates in triplicate and incubated for 7 days under the standard conditions. Bacterial titer was calculated from colony forming units (CFU).

**RNA extraction**

*H. pylori* 98-46 wild type and the three isogenic mutants (98-46 oipA1, 98-46 oipA2, and 98-46 oipA2/oipA1) were cultured in SFBB supplemented with 20 μg vancomycin per ml and 1X cholesterol (Gibco/BRL) at 0.2 OD₆₀₀ in 6-well plates. After 12 hours incubation with 150rpm agitation, OD₆₀₀ was measured, adjusted to 0.4 for each sample, and incubated for 5 hours. 1.0 x10⁹ cells were collected in microcentrifuge tubes, centrifuged at 6000rpm for 5 minutes, and supernatants were discarded. To each sample, cell pellets were resuspended in 1ml of RNAzol RT (Molecular Research Center, Inc.) and stored at -80°C.

For RNA extraction, 1ml of sample was transferred into screw caped microcentrifuge lysing tubes and centrifuged for 20 seconds. 400 ml molecular grade water was added and incubated
at room temperature for 15 minutes. Then samples were centrifuged for 15 minutes at 12,000 x g and transferred to new microcentrifuge tubes with 400µL 75% ethanol added. Samples were next incubated at room temperature for 10 minutes and centrifuged at 12,000 x g for 8 minutes. Supernatants were discarded and 600µL 75% ethanol was added. Samples were centrifuged at 8,000 x g for 2 minutes. Samples were washed again by ethanol and air dried for 10 minutes. 50 µL molecular grade water was added to the RNA pellet to resuspend. Each sample was quantified using a NanoDrop Spectrophotometer. 1µg of RNA was used with 4µl of iScript (Bio-Rad) to synthesize cDNA.

**Real-Time Quantitative PCR (RT-qPCR)**

The expression level of *oipA* gene of wild type 98-46 and mutants (98-46 *oipA*\( ^+ \), 98-46 *oip2*\( ^+ \) and 98-46 *oipA2* \(/oipA1^-\) ) was measured by RT-qPCR. Reactions were performed in technical triplicate in Applied Biosystems StepOne apparatus. *ftsZ* was used as the housekeeping gene to normalize variability in cDNA levels. Relative expression level was compared to that of wild type 98-46 *oipA* gene and calculated by the DataAssist software (Applied Biosystems).

**Results**

**Screening of 8 Japanese *H. pylori* isolates**

Eight Japanese *H. pylori* isolates were obtained and screened by PCR for a second copy of *oipA* in the region of the genome found in several other Asian *H. pylori* genomes available in the public databases. A second allele of *oipA* in two *H. pylori* strains, 98-19 and 98-46 was amplified by specific primers (Table 1) designed based upon highly conserved sequences
rpoN and hydrolase-encoding gene flanking the oipA2 allele among oipA merodiploid database entries. These two strains were isolated from patients with diagnoses of gastric cancer and gastritis, respectively (98-19 from gastritis patient and 98-46 from gastric cancer patient). In contrast, Japanese H. pylori isolate strains 98-8, 98-10, 98-11, 98-18, 98-20, and 98-23 did not yield and oipA2 gene using these same primers, indicating these strains did not possess a second oipA gene within the rpoN-hydrolase locus. However, the position of oipA2 allele is not necessarily constrained to this one locus (Fig 3). As exhibited in figure 3, due to variation of oipA2 position in gene, it is possible that oipA2 gene may be present somewhere else in the genome of other six Japanese strains. So it remains undetermined if these strains are truly oipA haploid.

Figure 3. Two alternate architectures for oipA1 and oipA2 gene. A) Represents one possible architecture for the oipA1 and oipA2 loci derived from the current study of H. pylori strain 98-46. rpoN and hydrolase are flanking the oipA2 allele. Primers for PCR amplification was designed based upon to this architecture as this scenario is found in

Both the oipA1 and oipA2 loci of 98-46 and 98-19 were cloned into plasmids (Table 2), and sequenced. Sequencing data revealed that for both strains, oipA1 and oipA2 are in frame, and thus capable of expressing functional OipA protein. For strain 98-46, oipA1 and oipA2 alleles contain 3 CT repeats. This is typical of oipA among H. pylori isolates of Asian origin. For strain 98-19, while oipA2 possesses typical 3 CT repeats, oipA1 possesses 5 CT repeats. However, both oipA1 and oipA2 in this strain are phase on and thus capable of expression of functional OipA. Despite the addition of two additional CT repeat units, thus 4 nucleotides, the reading frame of oipA1 is maintained due to compensatory nucleotide change in the region to maintain a phase on status to this allele. Sequencing data also demonstrate that oipA1 and oipA2 alleles are highly conserved within each strain (99.7% identity in strain 98-46 and 98.2% identity in strain 98-19). For strain 98-46, oipA1 and oipA2 alleles only differ by 2 nucleotides of the total nucleotides. For strain 98-19, oipA1 and oipA2 alleles differ at 16 nucleotides.

Based on sequencing data, predicted amino acid sequences of OipA1 and OipA2 were generated and ClusalW alignment was performed (Fig 4 and 5).
The OipA1 and OipA2 in this isolate differ at only two amino acid positions, indicating virtually no divergence since the oipA duplication event that occurred prior to human migration into North America over 12,000 years ago.

To compare more oipA merodiploid \textit{H. pylori} strains, genome sequences of the seven other Asian \textit{H. pylori} oipA merodiploid strains in the U.S. Department of Energy's Joint
Genome Institute; Integrated Microbial Genomes and Microbiomes database were collected and compared. All seven strains showed 100% DNA sequence identity of oipA1 and oipA2 sequences within any strain. In addition to comparison for oipA gene within one strain, the similarity of oipA gene across different strains was also assessed (Fig. 6). The similarity of oipA gene sequence among different strains is also very high. As indicated by Furuta et al. (2011), the duplication of oipA gene occurred early in evolution of H. pylori. Thus, numerous mutations resulting in divergence within and between genomes could easily be predicted, whereas the striking similarity of oipA gene within one strain and among different strains may suggest strong negative selection affecting oipA.

Figure 6. Sequence comparison of predicted OipA1 sequences across seven H. pylori strains merodiploid for oipA. All seven strains from the database have completely
identical \textit{oipA1} and \textit{oipA2} alleles within the genomes. \textit{OipA1} among different strains are still highly conserved.

Thus, we hypothesized that deletion of one or both \textit{oipA} alleles might result in reduced attachment, less CagA translocation between \textit{H. pylori} and AGS cells and reduced IL-8 induction.

\textbf{\textit{oipA} mRNA levels change due to \textit{oipA} gene knockout}

To assess the role of diploid \textit{oipA} alleles in Asian \textit{H. pylori} virulence potential, \textit{oipA1}, \textit{oip2} and \textit{oipA2} /\textit{oipA1} mutants of strain 98-46 were created for this study. \textit{oipA} expression levels of mutants and wild type were determined by Real-Time Quantitative PCR (RT-qPCR) using cDNA made from total RNA extracted from these mutants and wild type. When \textit{oipA1} is ablated by a deletion in the coding region and insertion of an antibiotic resistance gene, the mRNA transcript level is significantly lower compared to wild type 98-46 (Fig. 7). In contrast, when \textit{oipA2} is knocked out in a similar fashion, the mRNA level of \textit{oipA} is relatively similar to that of wild type. Knockout both \textit{oipA1} and \textit{oipA2} result in loss of detectable \textit{oipA} mRNA.

![Relative quantity of oipA expression](image)

Figure 7. Relative quantity of \textit{oipA} mRNA transcript levels of \textit{H. pylori} strain 98-46 wild
type and oipA1', oipA2' and oipA2'/oipA1' mutants. Results shown reflect RT-qPCR results from three independent experiments, each in technical triplicate. The expression level of oipA gene of oipA1 null, oipA2 null, and oipA2/oipA1 null mutants are compared to that of wild type. Error bars indicate standard deviation. Unpaired t-test was used to calculate p values. (*=p≤0.05, **=p≤0.01, n.s.=p>0.05)

IL-8 assay of H. pylori oipA mutants and wild type 98-46

The levels of IL-8 induction by H. pylori 98-46 and isogenic mutants cagE', oipA1', oip2' and oipA2'/oipA1' were determined by ELISA using supernatants from AGS cell infection. AGS cells infected with wild type 98-46 and mutants did not show significant differences (Fig. 8). Although disruption of oipA gene resulted in difference in oipA mRNA levels, it had no significant effect on IL-8 production by infected AGS cells. In contrast, our control cagE null mutant whose cagPAI T4SS function is impaired, failed to induce IL-8 secretion in AGS infections, confirming the important role of cagPAI T4SS in IL-8 induction (Backert et al., 2001; Censini et al., 1996; Hsu et al., 2002).
Figure 8. IL-8 induction of *H. pylori* 98-46 wild type and oipA mutants. IL-8 induction is not affected by disruption of either oipA allele or loss of both alleles. Supernatants collected from AGS cells infection by wild type *H. pylori* 98-46 and mutants were used to analyze levels of IL-8 production. Data is representative of all 5 biological replicate experiments, each done in triplicate. Error bars indicate standard deviation. Unpaired t-test was used to calculate p values. (n.s.=p >0.05, *=p ≤0.05, **=p ≤0.01)

IL-8 induction levels were also compared using each of our 8 Japanese *H. pylori* strains and two cagPAI positive Western strains (Table 3). Japanese *H. pylori* strain 98-19, an oipA merodiploid, induced significantly less IL-8 than 98-18, an oipA haploid strain (Fig 9). *H. pylori* Japanese strains 98-46, also an oipA merodiploid, induced significantly less IL-8 than strain 98-18. Thus, no correlation between oipA duplication and IL-8 induction was found.

**IL-8 of 8 Japanese strains and 2 Western strains**

Figure 9. IL-8 induction is not correlated with oipA diploidy. IL-8 induction by eight Japanese and two Western *H. pylori* strains was measured after AGS cell infections. Strains 98-08, 98-10, 98-11, 98-18, 98-20, and 98-23 are Japanese isolates haploid for oipA gene, whereas 98-19 and 98-46 contain two copies of oipA alleles. Strains B128 and B129 were isolated from western patients (United States). All strains possess cagPAI.
Error bars indicate standard deviation. Data reflect a single independent experiment done in technical triplicate. Unpaired t-test was used to calculate p values. (*=p≤0.05, **=p≤0.01)

In addition, an unpaired t-test was done for analyzing difference between Japanese strains and Western strains IL-8 inducing ability, and no significant difference was found (data not shown). However, as we included only two western strains in this comparison, it is not possible to accurately assess differences in IL-8 induction potential between *H. pylori* isolates from these disparate geographic regions. However, our results are consistent with those of Ando et al. where 24 United States and 39 Japanese *H. pylori cagPAI* positive strains were compared (Ando et al., 2002a). Thus, IL-8 induction level is independent from *oipA* gene diploidy and geographic variations.

**Disrupting oipA gene has no effect on CagA translocation**

Since CagA translocation is closely related with IL-8 induction, CagA translocation was also examined for wild type 98-46 and four mutants by Western Blot. As CagA entered AGS cells, it get phosphorylated by tyrosine (Backert et al., 2001), thus the CagA translocation level was determined by the intensity of tyrosine phosphorylation. Our result revealed that *oipA1, oipA2*, and *oipA2/oipA1* knockout had no effects on phosphorylation of CagA (Fig 10), which suggests that CagA translocation is not impaired in all *oipA* null mutants.
Figure 10. Western blot of CagA translocation into AGS cells infected by *H. pylori* 98-46 wild type and four isogenic mutants. AGS cells were infected with wild type Japanese *oipA* merodiploid strain 98-46, or *oipA1* null, *oipA2* null, *oipA2/oipA1* null and *cagE* null mutants. Uninfected AGS cells are included as a negative control and were cultivated concurrently with the infected monolayers in the same 6-well plates. Two identical polyacrylamide gels were loaded identically and run simultaneously and then western blotted to nitrocellulose. Blots were treated with either anti-CagA or anti-phospho-tyrosine monoclonal antibodies. CagA was translocated from *H. pylori* to AGS cells during infections with wild type, *oipA1* null, *oipA2* null, and *oipA2/oipA1* null mutants, suggesting that disrupting *oipA* gene does not have effects on CagA translocation. Disruption of the T4SS encoded by *cag* PAI by means of a *cagE* knock out, halted the translocation of CagA consistent with the role of the T4SS in this key aspect of *H. pylori* pathogenesis.

However, *cagE* mutant with impaired T4SS was not able to translocate CagA, confirming that CagA translocation is mediated by T4SS (Fischer et al., 2001; Odenbreit et al., 2000).

**Disrupting oipA has no effect on adherence ability**

Due to the change of *oipA* mRNA levels in *H. pylori* strain 98-46 mutants demonstrated in Figure 7, we hypothesized that such difference in *oipA* mRNA transcript level may lead to variations in corresponding *H. pylori*’s adherence ability. Thus, attachment assays were performed using *H. pylori* strain 98-46 and mutants *oipA1*, *oip2* and *oipA2/oipA1*. Our
results showed no significant difference among wild type *H. pylori* 98-46 and mutants in terms of adherence ability to AGS cells (Fig. 11).

**Attachment assay for 98-46 and mutants**

![Attachment assay for 98-46 and mutants](attachment-assay.png)

**Figure 11.** Attachment assay for 98-46 and *oipA* mutants. AGS monolayer infected for 5 hours with wild type 98-46, *oipA1* null, *oipA2* null and *oipA2/1* null mutants were lysed with Saponin, diluted with PBS, and plated for CTU counts. Data represents three independent experiments with technical triplicate. Error bars indicate standard deviation. Unpaired *t*-test was used to calculate *p* values. (n.s.=*p*>0.05)

Our results do not agree with that of Dossumbekova et al. (2006), in which researchers showed that turning *oipA* phase off in 58 western strains (German) *H. pylori* resulted in significantly lower adherence ability to AGS cells. A study done in our lab using *H. pylori* strains 26695 and J68 also demonstrated that turning *oipA* phase off reduced adherence ability (Horridge et al., 2017). However, our results are comparable to that of Yamaoka et al. (2000), in which Asian *H. pylori* strains were used.
Discussion

*H. pylori* has colonized human stomachs for tens or hundreds of thousands of years (Falush et al., 2003). While some *H. pylori* strains behave like commensals and even provide protection for hosts from inflammatory bowel disease, allergies or obesity (Higgins et al., 2011; Codolo et al., 2008), some *H. pylori* strains, especially strains with virulence factor *cag* PAI, are associated with development of severe, even lethal gastric diseases (Chmiela et al., 2014). *H. pylori* has been identified as a carcinogen, the sole human bacterial pathogen to receive such a distinction, and is responsible for 5.5% of all human cancer cases (Parkin et al., 2002). As for East Asia, where prevalence of both *H. pylori* infection and stomach cancer are high, several studies have investigated potential increased virulence of Asian *H. pylori* strains (Ando et al., 2002a; Zhang et al., 2014; Yamaoka et al., 2000; Yamaoko et al., 2006).

OipA, as an important adhesion of *H. pylori*, has been demonstrated to work synergistically with virulence factors such as *cag* PAI, *vacA*, and CagA (Markovska et al., 2011; Yamaoko et al., 2006). *oipA*’s geographic associated genetic polymorphisms include phase variation mediated by SSM and duplication of *oipA2* allele in Asia *H. pylori* strains (Kawai et al., 2011; Zhang et al., 2014), which may suggest a higher virulence among *H. pylori* Asian strains. It is easy to hypothesize that increased the expression of OipA, either by duplication of the locus or the inability to phase vary to a non-expressed form, could result in increased inflammation and increased cancer rates due to increased IL-8 secretion and/or increased translocation of the *H. pylori* virulence protein CagA into gastric cells. It was precisely this hypothesis, based upon observations from ours and other labs of DNA dynamics involving the *oipA* locus among Asian *H. pylori* isolates that prompted the current
In this study, we demonstrated that although duplication of \textit{oipA} gene happened early in the evolution of \textit{H. pylori} (Furuta et al., 2011), \textit{oipA} remains highly conserved among different Asian \textit{H. pylori} strains, and within individual strains. However, \textit{H. pylori} has been well characterized for its intraspecies diversity, with synonymous mutation rate of 2 to $7 \times 10^{-5}$ (Akopyanz, N. et al., 1992; Falush, D. et al., 2001). In a study of Pérez-Losada, M. et al. (2006), they showed that \textit{H. pylori} has the highest population recombination rate (99.2) among 15 bacterial pathogens. Thus, in light of the abundance of studies indicating that genetic diversity is maximized in \textit{H. pylori}, we hypothesized that the striking degree of gene sequence conservation indicates a strong selective force for maintaining OipA sequence and perhaps driving the duplication of this virulence locus.

The results from our RT-qPCR experiments using aour collection of \textit{oipA} mutants in a naturally merodiploid Japanese isolate of \textit{H. pylori}, strain 98-46, indicate that knockout of the \textit{oipA1} allele affects mRNA levels of \textit{oipA}; decreasing it by ~50%. This is to be expected if both \textit{oipA1} and \textit{oipA2} alleles are expressed and contributing equally to total \textit{oipA} mRNA levels. By contrast, knockout \textit{oipA2} gene does not have significant effect on its mRNA levels in this mutant. When both \textit{oipA} alleles are disrupted simultaneously in the same mutant, mRNA transcript level reduced nearly 100%. Such results suggest that when disrupting \textit{oipA2} allele, \textit{oipA1} allele may compensate to maintain \textit{oipA} gene expression. However the \textit{oipA2} allele cannot compensate the disruption of \textit{oipA1}. We hypothesize that the disparity lies in the promoter regions of two alleles. However, our sequencing data revealed nearly identical promoter regions of two \textit{oipA} alleles in strain 98-46. Thus, an alternative hypothesis would be
difference between enhancers which can locate at an atypical distance from oipA gene promoter to affect transcript levels.

Some studies demonstrated that changes of oipA expression might be linked to the adherence abilities of H. pylori to gastric epithelial cells, which might facilitate colonization of mucosal surfaces (Falk et al., 2000, Horridge et al. 2017). Nevertheless, the results of this study indicate that disrupting oipA gene does not affect adherence ability to AGS cells at least for Japanese strain 98-46 to AGS cells in vitro. Even though our results do not agree with studies investigating the role of OipA in adherence using Western H. pylori strains (Dossumbekova et al., 2006), they are consistent with studies using Asian H. pylori strains (Yamaoka et al., 2000). Since, H. pylori, encoding for 33 paralogous putative outer-membrane genes, was known for having diverse adhesins, including SabA, BabA, AlpA, OipA and HopZ (Tomb et al., 1997), we hypothesize that oipA null mutants are not sufficient to reduce adherence due to normal function of multiple other membrane proteins, perhaps expressed in Asian H. pylori strains. This point needs empirical testing to characterize alternate adhesin expression in strain 98-46 and other Japanese H. pylori isolates.

A role for OipA in IL-8 induction, and thus the inflammatory potential of OipA expressing isolates of H. pylori, is supported by several studies (Yamaoka et al., 2000; Horridge et al., 2017). This suggests an association between oipA gene and clinical outcomes. However, in the current study, our results demonstrated that knockouts of oipA genes had no effects on the induction of IL-8 secreted by AGS cells in vitro in H. pylori strain 98-46. Our results are in accordance to a study of Ando et al. (2002a), in which they showed that knockout oipA did not affect IL-8 induction for three Japanese H.pylori strains 98-11, 98-40
and 98-30. Nevertheless, in the Ando et al. (2002a) study, the nature of oipA merodiploidy of three Japanese \textit{H. pylori} strains used in oipA null mutant construction was unknown to the authors as large numbers of genomes of Asian \textit{H. pylori} genomes were not available for analyses.

\textit{oipA} mutants also did not influence CagA translocation, which is comparable to studies of Akanuma et al. (2002) and Ando et al. (2002a). Our results agree with those in studies by Ando et al. (2002a) and Odenbreit et al. (2002), which also demonstrated that \textit{oipA} gene was not correlated with CagA translocation. However, these results are contradictory to those of our recent study using \textit{H. pylori} strain 26695 (Horridge et al., 2017), where we demonstrated that the presence of OipA is necessary for CagA translocation.

However, the conclusion that no association between OipA and IL-8 production and CagA translocation cannot be drawn due to the vast genetic diversity of \textit{H. pylori}. It had been shown that other adhesins BabA and HopQ also mediate adherence to AGS cells and thus facilitate the translocation of CagA (Ishijima et al., 2011; Javaheri et al., 2016). Thus, the inability of OipA to induce IL-8 production and CagA translocation in strain 98-46 may suggest that OipA is not the only outer membrane protein that facilitates adherence and the establishment of T4SS in \textit{H. pylori} strain 98-46. Different adhesins such as BabA and HopQ may represent redundancies in Asian strain \textit{H. pylori}, such that the adherence to AGS cells and the process of CagA translocation depends less on OipA expression. However, further study using additional oipA merodiploid strains, including strain 98-19 determined by our lab, is necessary for the association between oipA merodiploidy and the virulence of Asian \textit{H. pylori} strains to be established.
To conclude, our data to date demonstrate that although the experimental disruption of \textit{oipA} gene or genes results in significant alterations in \textit{oipA} mRNA expression levels, it fails to support a hypothesis the duplication of this virulence factor results in a measurable increase potential virulence using IL-8 induction or CagA translocation \textit{in vitro} as proxies for virulence.

**Acknowledgement**

I would like to use this opportunity to express my gratitude to my advisor, Dr. Mark Forsyth, for his enormous support and guidance along my research. Then I would like to thank my committee members Dr. Beverly Sher, Dr. Helen Murphy, and Kitamura Aiko sensi for their contribution and encouragement. Lastly I thank my labmates Danielle Horridge, JiaJia Chen, June Kim, Neeraja Aravindan, Edward Choi and Justin Reed for their participation and insightful discussions of my honor project.

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**Tables.**

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<table>
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<th>Primer name</th>
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**Table2 plasmids used in this study**

**Table3 H. pylori strains**
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**Reference**


