An Analysis of Outer Inflammatory Protein A in cag Pathogenicity Island Negative and Positive Strains of Helicobacter pylori

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An Analysis of Outer Inflammatory Protein A in cag Pathogenicity Island
Negative and Positive Strains of Helicobacter pylori

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A thesis submitted in partial fulfillment of the requirements of the degree of Bachelor of Science
with Honors in Biology from The College of William & Mary in Virginia.

Williamsburg, Virginia

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A thesis submitted in partial fulfillment of the requirements for the degree of Bachelor of Science with Honors in Biology from The College of William & Mary

by

Danielle N. Horridge

Accepted for Honors

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Abstract

OipA, outer inflammatory protein A, is an outer membrane protein virulence factor of the gastric pathogen *Helicobacter pylori*. oipA expression is regulated by phase variation at a CT dinucleotide repeat located within the 5’ end of the gene, such that the gene is alternatively in-frame (Phase On) or out-of-frame (Phase Off). *H. pylori* isolates lacking the *cag* Pathogenicity Island (*cagPAI* negative), the primary virulence determinant of *H. pylori*, induce less host inflammation and almost uniformly possess an oipA allele that is phase off. Meanwhile, *cagPAI* positive *H. pylori* isolates almost always possess phase on oipA alleles. The *cagPAI* encodes a type IV secretion system (T4SS) that elicits the secretion of the pro-inflammatory cytokine, Interleukin 8 (IL-8), by infected gastric epithelial cells. OipA has been shown to play a role in inflammation and as an adhesin that assists in the attachment of *H. pylori* to host cells. Based on the current study, we hypothesize that the highly conserved, albeit apparently non-expressed, oipA in *cagPAI* negative strains can affect the adherence character of these isolates of reduced virulence when altered to a phase on status; however OipA’s role in inflammation may be dependent upon the presence of the *cagPAI*.

We sought to determine the role of OipA in *cagPAI* positive and negative *H. pylori* infection of human gastric adenocarcinoma (AGS) cells using the *cagPAI* positive strain, 26695, and *cagPAI* negative strains, J68 and J75. The oipA CT repeat length was experimentally altered in *H. pylori* 26695 in order to turn oipA phase off, and turn oipA phase on in J68 and J75. Real time quantitative PCR (RT-qPCR) was used to determine the relative quantity of oipA mRNA. Experimentally turning oipA phase off in strain 26695 resulted in a >5-fold decrease in oipA transcript levels compared to the wild type. The novel phase on oipA J68 mutant resulted in a
>6-fold increase in oipA transcription, and Western blotting using antibodies to a FLAG epitope tagged inserted within the J68 oipA allele revealed protein expression at the whole cell level.

AGS attachment assays revealed that *H. pylori* adherence was significantly increased in both the cagPAI positive and negative strain when oipA was phase on compared to isogenic oipA phase off strains. Quantification of IL-8 by enzyme-linked immunosorbent assay showed that this variation in *H. pylori* attachment was only linked to changes in IL-8 production by AGS cells when infected by the cagPAI positive strain, not cagPAI negative. Taken together, these results indicate that oipA, although phase off in cagPAI negative isolates of *H.pylori*, can still serve as an adhesin independent of a cag T4SS. However, both OipA and the cagPAI are necessary to induce the host pro-inflammatory cytokine response.

Using RT-qPCR, we quantified oipA transcription in response to attachment of *H. pylori* to AGS cells. oipA transcript levels were at least 7-fold higher in the *H. pylori* cells attached to AGS cells compared to non-adherent *H. pylori* cells from the same experiments. This apparent attachment selection favoring bacteria expressing higher levels of oipA was found in both phase on and phase off mutants of the cagPAI positive strain 26695. This finding further adds to the role OipA plays in attachment to host cells.
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Introduction

*Helicobacter pylori*

In 1982 researchers Barry Marshall and Robin Warren challenged the common perception that gastric ulcers were caused by stress. Beginning with Marshall’s self-ingestion experiments with samples isolated from patients, which resulted in transient gastritis, they proved A) that the stomach is not a sterile environment as previously thought, and B) that the isolated bacterial species, *Helicobacter pylori*, is the etiological agent of Peptic Ulcer Disease and other gastric ailments including gastric cancer. For their work, Marshall and Warren were awarded with the 2005 Nobel Prize in Physiology or Medicine (Nobel Media 2014).

Further characterization revealed that *H. pylori* is a gram negative spiral shaped bacterial species (Marshall & Warren 1984). *H. pylori* resides exclusively in the human stomach and has been associated with chronic gastritis, gastric ulcers, duodenal ulcers, and gastric cancer. The exact mode of transmission is largely unknown, but it typically occurs in childhood, either via vertical transmission from mother to child, or via oral-oral or fecal-oral routes (Brown 2000).

While more than half of the world’s population is infected with *H. pylori* in their upper gastrointestinal tract, over 80% of those hosts show no negative symptoms (Blaser 2006). In fact, studies have shown that this infection has the potential to confer desirable effects such as resistance to acid reflux disease and to childhood asthma (Lim et al. 2016), and it is thought that *H. pylori* may play an important role in the natural flora of the stomach. While the prevalence of this bacterium is decreasing with the rise of antibiotic use in Western countries, *H. pylori* colonization is still widespread in the developing world (Gerrits et al. 2017).

Though not always problematic, gastric colonization by *H. pylori* can have many detrimental effects and impacts the lives of many. In fact, the World Health Organization (WHO)
listed clarithromycin resistant \textit{H. pylori} as a “high priority” member of its 2017 list of bacteria for which new antibiotics are urgently needed (Branswell 2017). With pervasive antibiotic use and a concomitant increase in antibiotic resistance in many \textit{H. pylori} strains, it is important to continue studies of its pathogenesis and mechanisms of persistence in the stomach in order to develop novel treatments.

\textbf{\textit{H. pylori} Persistence in the Stomach}

Prior to the studies of Marshall & Warren less than 40 years ago, it was thought that the stomach was a nearly germ-free environment, and that the only microbes inhabiting it were remnants from food and oral microbiota (Nardone & Compare 2015). This belief stemmed from the lack of perceived ability of microbes to withstand the hostile environment of the stomach, including low pH levels, proteolytic enzymes, and shedding of the gastric mucus and underlying epithelial cells. \textit{H. pylori}’s ability to not only reside in the stomach, but to persist, often over the entire lifespan of the host, and to cause chronic illness and even cancer, has only recently begun to be understood.

The acidic nature of the stomach is one of the preliminary features of the innate immune response and inhibits the growth of the vast majority of pathogens that might find their way into the stomach. In fact, it is likely a key factor in protecting more distal portions of the gastrointestinal tract from infection by pathogenic bacteria, viruses, and parasites (Smith 2003). Using chemotaxis, \textit{H. pylori} is able to sense the pH gradient as it burrows through the mucus layer using its flagella, and moves towards the less acidic epithelial cells underneath the mucus (Rolig et al. 2012). \textit{H. pylori} also has the ability to neutralize the acid in its immediate vicinity by producing urease, which breaks down urea to carbon dioxide and ammonia which can react with strong acids to produce a neutral environment immediately surrounding the bacterium.
(Schreiber et al. 2004; Suzuki et al. 2002). The bacterium can then live freely in the mucus or adhere to gastric epithelial cells via a variety of outer membrane proteins. Two particularly well-documented adhesins include SabA and BabA, which play a role in sialic acid-binding and blood group antigen-binding, respectively (Yamaoka 2008; Ishijima et al. 2011).

This adherence function also helps the bacterium persist in the face of a second major barrier to colonization— the shedding of the mucus lining. However, in a given population of *H. pylori* in the stomach, only a small percentage adheres to the epithelial cell surfaces at a given time, while the majority are motile in the mucus layer. The adherent cells are more efficient in the delivery of bacterial proteins into host cells (Oleastro & Ménard 2013). Without these functions, chronic colonization would not be possible.

**H. pylori** pathogenesis

The global prevalence of *H. pylori* infection is about 50% of the population, making it the most widespread infection in the world. However, in many developing countries, more than 80% of the population may be *H. pylori* positive (Khalifa et al. 2010). Prevalence is less than 40% in industrialized countries, and that number continues to decrease due to antibiotic use, access to adequate health care, and emphasis on hygiene (Nagy et al. 2016). While most people never show symptoms of *H. pylori* colonization, in some individuals it is associated with peptic ulcers as well as gastric cancer.

Infection of *H. pylori* in the stomach can result in chronic gastritis, or inflammation of the stomach lining, mainly caused by an induced host inflammatory response at the site of infection (Kusters et al. 2006). Additionally, the ammonia produced as *H. pylori* regulates pH is toxic to epithelial cells, and additional *H. pylori* proteins such as VacA (vacuolating cytotoxin A) can disrupt tight junctions and lead to damage in epithelial cells, and even cause apoptosis.
(Palframan et al. 2012). Chronic gastritis may progress to peptic ulcer disease when the epithelial layer of the stomach is broken down by the inflammatory response and stomach acid and pepsin are able to penetrate and damage tissue (Dumreise et al. 2009).

Gastric cancer is the fourth most common cause of cancer related death in the world (WHO 2017). *H. pylori* positive individuals develop gastric cancer more often than uninfected individuals, and *H. pylori* infection increases the risk of gastric cancer by a minimum of ten-fold (Wroblewski et al. 2010). Additionally, *H. pylori* has been designated a class I carcinogen by the WHO, making it a rare “living carcinogen.” It is estimated that in the developed world, up to 75% of gastric cancers are related to chronic *H. pylori* infection (Suganuma et al. 2012). While the exact mechanism by which *H. pylori* causes cancer is not known, one proposed mechanism suggests that the enhanced production of free radicals at the site of infection contributes to an increased rate of host cell mutation (Sepulveda 2013). Another mechanism proposes a transformation of the host cell phenotype via interactions with the bacterium. This “perigenetic pathway” suggests that prolonged inflammatory response can alter gastric epithelial cell-cell adhesion and dispersion without changes in tumor suppressor genes (Tsuji et al. 2003). Additionally, infection with strains of *H. pylori* containing a crucial virulence locus, the cag Pathogenicity Island, and thus the oncogenic protein cytotoxin-associated antigen A (CagA), is strongly associated with gastric cancer (Hatakeyama 2004).

**The cag Pathogenicity Island**

Pathogenicity islands (PAIs) are mobile genetic elements involved in the virulence of bacterial pathogens and are acquired through horizontal gene transfer (Juhas 2015). Horizontal gene transfer is the means by which genetic material is transferred between microorganisms, other than from parent to offspring. This process can occur via plasmids, or small DNA
molecules separate from chromosomal DNA (transformation), via bacteriophage (transduction), or via conjugative transposons (conjugation) (Zhaxybayeva & Doolittle 2011). Often PAIs encode proteins involved in virulence; in fact, this is one means by which bacteria pass on antibiotic resistance genes, a major challenge in modern medicine (Schmidt & Hensel 2004).

The cagPAI of \textit{H. pylori} is a 40-kb chromosomal region, identifiable due to a lower GC content, 29.6\%, compared to the rest of the genome, 39\% (Terry et al. 2005). The cagPAI encodes a type IV secretion system (T4SS), and its presence in the \textit{H. pylori} genome is positively associated with the presence of highly active alleles of the secreted Vacuolating Cytotoxin (VacA). VacA is shown to enhance colonization and pathogenesis by means such as stimulation of vacuole formation in host cells (Foegeding et al. 2016). The name of the cagPAI stems from the gene for its effector protein, CagA, or cytotoxin associated gene A. There are 31 open reading frames in the PAI, and \textit{cagA} is located on the 3’ end of the island. CagA is translocated into host stomach epithelial cells via the T4SS, also encoded within the cagPAI, upon attachment (Noto & Peek 2012). These secretion systems involve conjugative structures to transport proteins through a complex channel structure directly through the membrane into the cytoplasm of the host cell (Backert & Tegtmeyer 2017). Chaperones stabilize effectors like CagA, and energize protein export by ATP hydrolysis. Once inside the cell, CagA is phosphorylated by a host kinase, and proceeds to interact with systems leading to host cell junction damage, cytoskeletal changes, and cell proliferation (Figure 1A) (Hatakeyama 2008).

In a separate sequence of events, non-translocated proteins of the T4SS encoded by the cagPAI are involved in inducing pro-inflammatory cytokine, most importantly Interleukin 8 (IL-8), production by host cells (Figure 1A) (Fischer et al. 2002). This pro-inflammatory response depends on the activation of transcription factor NF\(\kappa\)B. Gastric epithelial cells secrete IL-8 in
order to recruit an innate immune system response, resulting in strong inflammation in the gastric mucosa. The cagPAI is found disproportionately in \textit{H. pylori} isolates from patients with chronic active gastritis, peptic ulcer disease, and gastric cancer, indicating that it is an important virulence factor in the bacterium (Cover 2016). Thus, cagPAI positive strains containing the PAI are naturally more virulent, while cagPAI negative strains lacking the island are naturally much less virulent.

**Outer Membrane Inflammatory Protein A**

Outer membrane inflammatory protein A, or OipA, is an outer membrane protein unique to \textit{H. pylori}. Approximately 4% of the \textit{H. pylori} genome encodes for a large set of outer membrane proteins (OMPs), many with unique functions including adhesion and pH regulation (Oleastro & Ménard 2013). OipA, encoded by the gene \textit{oipA} (previously \textit{hopH – HP0638}), is originally named for its role in inducing inflammation in the host, as evidenced by high mucosal IL-8 levels. The particular means by which OipA induces inflammation are unclear as the evidence often overlaps with cagPAI-mediated pathways (Figure 1B). It is hypothesized that IL-8 gene transcription related to OipA involves the transcription factor NFκB, activator protein 1, and an interferon-stimulated responsive element within the IL-8 promoter (Yamaoka 2010).

OipA is now known to play roles in adherence to host cells and host colonization, in addition to promoting host inflammation (Figure 1B) (Dossumbekova et al. 2006). OipA is a member of the Hop (\textit{Helicobacter Outer Proteins}) outer membrane family of proteins, all of which promote binding to the gastric epithelium in some way (Oleastro & Ménard 2013). Regarding the role of OipA in adhesion, the host gastric epithelial cell receptor is unknown, but has been hypothesized to be in the integrin family, which normally mediate cell-cell interactions (Posselt et al. 2013).
OipA is a protein ~ 34 kDa in size encoded by the gene *oipA*, which exhibits phase variation that is regulated by slipped strand mispairing within a hypermutable CT dinucleotide repeat motif located in the 5’ region of the gene (Miftahussurur & Yamaoka 2015). Slipped strand mispairing is a mutagenic process that occurs during DNA replication when DNA strands are displaced, resulting in the mispairing of complementary bases (Torres-Cruz & van der Woude 2003). This mechanism is most common in repetitive DNA sequences, such as the CT tract in *oipA*, and typically involves the deletion or insertion of one repeat unit. Such changes in *oipA* result in a frame shift that drives phase variation in protein expression. While there is great variation in the number of CT’s in the dinucleotide repeat of *oipA* between strains, slipped strand mispairing within strains of *H. pylori* grown *in vitro* is very rare compared to other outer membrane protein genes, such as *sabA* (Yamaoka et al. 2006).

The number of CT repeats determines whether the open reading frame is in or out of frame to be translated into a full-length functional OipA protein, thus phase on or phase off, respectively. In a study of 410 *H. pylori* patient isolates, it was found that this CT sequence can range from 3 to 12 repeats, with short repeats predominating in Eastern Asian countries, where gastric cancer caused by *H. pylori* is much more prevalent compared to strains from Western countries (Yamaoka et al. 2002). Studies show that a functional “phase on” status of *oipA* is associated with increased risk for peptic ulcer disease and gastric cancer. The strong correlation of functional OipA and the virulence of the bacterium has made OipA a candidate for potential vaccines against *H. pylori* (Chen et al. 2002). Additionally, functional OipA is significantly associated with high *H. pylori* density in infected stomachs and severe neutrophil infiltration (Liu et al. 2013).
Figure 1. The pathogenesis of CagA (A) and OipA (B) signaling in host gastric epithelial cells. CagA is translocated into host cells via a T4SS encoded by the cagPAI. CagA causes proliferation and inflammation, as does OipA, in addition to its role as an adhesin. If and how OipA works in conjunction with the T4SS or CagA to induce inflammation is widely unknown. (Adapted from Yamaoka 2010)
The relationship between OipA and the cagPAI

OipA and the cagPAI are two important *H. pylori* virulence factors and there is great interest in the relationship they have to each other. Interestingly, Ando et al. (2002) confirmed that while the vast majority (>96%) of cagPAI positive isolates contained *oipA* “phase on,” 0% of cagPAI negative isolates contained the gene in its phase on status, yet the gene was always present and highly conserved. With such relatively small genomes, it is rare for a bacterial genome to contain extraneous genetic information that is not expressed or somehow utilized. While OipA and the cagPAI are both known to induce the secretion of cytokines such as IL-8 by host epithelial cells, most likely via a mechanism involving transcription factor NF-κB, how they work together (or if they work together) is widely unknown (Matsuo et al. 2017). The present study aims in part to determine the relationship between *oipA* and the cagPAI, and to shed light on why *oipA* is so highly conserved in cagPAI negative strains in which the protein is apparently not expressed.

Objectives

The first step in resolving the question of why *oipA* is present in cagPAI negative strains of *H. pylori*, yet seemingly never expressed, is to determine a) if it is possible to artificially alter the expression of the gene *oipA* and b) document and quantify any impacts of OipA expression on *H. pylori* phenotypes. *In silico* correction of the CT repeat tract in *oipA* indicates that cagPAI negative isolates of *H. pylori* are capable of producing a protein with >95% amino acid identity to orthologs from cagPAI positive isolates (Figure 2). Based on this, a primary objective of this study was to alter the poly CT tract of *oipA* in the cagPAI negative strains J68 and J75 in order to turn *oipA* “phase on,” and conversely, to alter the poly CT tract of *oipA* in the cagPAI positive strain 26695 in order to turn it “phase off.” A second objective was to quantify changes in *oipA*
expression subsequent to experimental phase shifts by using real time quantitative PCR and Western Blots. The third objective of this project was an analysis of the effects of these changes on virulence phenotypes when mutant strains were used to infect gastric adenocarcinoma cells (AGS cells). The virulence phenotypes analyzed included the bacterium’s ability to adhere to host cells, quantified using attachment assays to a cultured gastric cell line in vitro, as well as the ability of these infected cells to produce pro-inflammatory cytokines, such as IL-8, in response to infection with these oipA mutants.

Our hypothesis was that by altering the poly CT tract to turn oipA phase on in the cagPAI negative strains J68 and J75, transcript levels of the gene would increase, and a functional protein would be produced. Additionally, when used to subsequently infect AGS cells, these mutants would display increased attachment ability, and induce greater IL-8 production by host cells. We hypothesized the opposite results when using a mutant where we experimentally turned oipA “phase off” in the cagPAI positive strain 26695.

Figure 2. OipA is highly conserved in four different strains of H. pylori. The amino acid sequences of OipA after in silico correction of the gene to phase on status in 3 cagPAI negative strains were compared to OipA in the cagPAI positive strain, 26695 (A), via ClusalW alignment. The cagPAI negative strains are: B) J68, C) J75, and D) J195.
**Materials and Methods**

**H. pylori culture**

*H. pylori* was cultured on tryptic soy agar II with 5% sheep’s blood, otherwise known as blood agar plates (BAPs) from BBL for 24-72 hours at 37°C in an ambient air/5% CO₂ atmosphere. Liquid cultures of *H. pylori* were grown in sulfite-free Brucella broth (SFBB) supplemented with 1X cholesterol (Gibco by Life Technologies), and 20 µg vancomycin/mL shaking at 150 rpm.

**AGS cell culture**

AGS cells were a gift from Timothy Cover of Vanderbilt University Medical Center. Cells were grown in Roswell Park Memorial Institute (RPMI) medium 1640 supplemented with HEPES (Gibco by Life Technologies), 10% fetal bovine calf serum (FBS) or newborn calf serum (NCS), and penicillin/streptomycin (P/S). Cultures were grown at 37°C in an ambient air/5% CO₂ atmosphere on either 6-well tissue culture treated plates (CytoOne) or T-75 tissue culture flasks (PRIMARIA by Fischer Scientific).

**Cloning of metronidazole resistant plasmids**

In creating the *oipA* mutant strains of *H. pylori*, an antibiotic counter-selection method designed by Mark McClain at Vanderbilt University Medical Center (Loh et al. 2011) was employed with the goal of introducing a specific mutation into the gene without leaving any antibiotic resistance genes behind. While a traditional method of inserting mutations involves attaching the desired alteration to an antibiotic resistance gene, this also introduces the possibility that any phenotypic changes observed may be due to the presence of the resistance gene.

The first step of this method was to create a metronidazole resistant (Mtz⁺) strain of both the cagPAI positive strain of *H. pylori* 26695, and the cagPAI negative strains J68 and J75.
Unless otherwise specified, all plasmids and *H. pylori* mutants regarding the strain J68 were created by another student in the Forsyth lab, Allison Begley (Unpublished data 2016).

Metronidizole is a pro-drug that must be reduced by a bacterial enzyme in order to become bacteriocidal. In *H. pylori* RdxA is the enzyme that accomplishes this reduction. Thus, deleting an internal portion of *rdxA*, and thereby rendering it non-functional, generates an *H. pylori* Mtz\(^R\) mutant.

This was accomplished by first amplifying a 1556 bp amplicon containing the full length *rdxA* gene (HP0954) using the HP0955 Fwd and HP0953 Rev primers (Table 1) in a standard PCR. All primers were synthesized by Integrated DNA Technologies unless otherwise specified. The amplicon was cloned into a TOPO TA cloning vector pCR4 (Invitrogen) according to the manufacturer’s protocol. The resultant plasmids isolated from *E. coli* were named prdxA (Table 2).

Inverse PCR was performed on these plasmids to delete a 390bp internal portion of *rdxA* using the iPCR *rdxA* Fwd and Rev primers (Table 1) with 5’ phosphorylation to aid in ligation. The resultant linear product was checked for size on an agarose gel, the PCR product was purified using a spin column kit for PCR Purification (IBI) and digested with *DpnI* restriction endonuclease to destroy the prdxA template, and then ligated together using T4 DNA ligase (Quick Ligation Kit - New England Biolabs) and transformed into *E. coli* DH5α competent cells and selection for ampicillin resistance was used to isolate clones containing the mutant plasmid. Clones were screened by purifying and isolating plasmids, which were screened via PCR using the HP0955 Fwd and HP0953 Rev primers and compared to the amplicons generated from the parental plasmid prdxA. Successful deletions were identified by a decrease in amplicon size of
390 bp and confirmed via sequencing reactions performed using the Big Dye Sequencing Kit (Applied Biosystems), and the successful plasmid construct was named pΔrdxA (Table 2).

The confirmed ΔrdxA plasmid was used in natural transformation of the H. pylori strains 26695, J68, and J75. Mtz\(^a\) colonies capable of growing on 5µg metronidazole/mL SFBB 10% NCS plates were selected, and the mutation was confirmed by performing PCR on extracted gDNA using the HP0955 Fwd and HP0953 Rev primers and performing agarose gel electrophoresis to confirm the presence of a deletion. The deletion was further confirmed with sequencing reactions performed using Big Dye (Invitrogen) with the primer HP0955 Fwd, and strains were named 26695, J68, and J75 ΔrdxA, respectively (Table 3).

**Cloning of rdxA complement / chloramphenicol resistant plasmids**

Separately from the Mtz\(^a\) clones, a chloramphenicol (Cm) resistance gene (CAT or chloramphenicol acetyl transferase) together with an intact version of rdxA as a cassette, was inserted into the oipA gene in 26695, J68, and J75. The purpose of this cassette insertion is twofold. One, it will serve as a traditional “knock out” or null mutation of the oipA gene, as the large cassette will prevent functionality of the gene product. Two, placing an intact, functional copy of rdxA within oipA will return Mtz sensitivity to H. pylori, while simultaneously adding the CAT gene will confer Cm resistance. Thus, in a subsequent natural transformation/ allelic exchange reaction, when a mutant ΔoipA plasmid is naturally transformed into strains of H. pylori with both the rdxA deletion, and the CAT-rdxA cassette, the mutant ΔoipA allele can recombine to replace the oipA::CAT-rdxA cassette, and the ΔrdxA mutant can be selected for as those clones will return to a Mtz\(^a\) phenotype.

The first step in this process was to amplify a region containing the entire oipA gene, as well as the untranslated regions both upstream and downstream, in a PCR using the oipA
universal Fwd and Rev primers designed based on a consensus sequence built from N=4 *H. pylori* sequenced genomes (Table 1). The resultant amplicon of ~2300bp was cloned into the TOPO TA cloning vector, pCR4 (Invitrogen), and the resultant plasmid was named pOipA (Table 2). Next, the GeneArt Site-Directed Mutagenesis System (Invitrogen) was utilized according to the manufacturer’s protocol to insert a *Bam*HI site into the cloned allele of *oipA* using the *oipA* *Bam*HI Fwd and Rev mutagenic oligos (Table 1). Mutagenesis reaction products were used to transform XL10-Gold Competent *E. coli* cells and clones were selected for using ampicillin resistance and screened with Big Dye (Invitrogen) sequencing. This plasmid was named pOipA.*Bam*HI (Table 2). Neither *oipA* nor the pCR4 vector contain a naturally occurring *Bam*HI site. Once the *Bam*HI site was inserted, plasmids were cut at the *Bam*HI site to facilitate subsequent cloning of selectable markers; chloramphenicol acetyl transferase (CAT) and *rdxA*.

The CAT-*rdxA* cassette was isolated as a *Bam*HI fragment from pMM672 (Loh et al. 2011) (Table 2), a gift from Drs. Mark McClain and Timothy Cover of Vanderbilt University Medical Center. Digestion reaction of the plasmid was run on an agarose gel, and the CAT-*rdxA* cassette was isolated by gel purification (IBI). Once isolated, the cassette was cloned into pOipA.*Bam*HI using T4 ligase, facilitated by the presence of *Bam*HI sites on both the linear pOipA.*Bam*HI plasmid and the CAT-*rdxA* cassette. This new plasmid (pOipA::CAT-*rdxA*, Table 2) was transformed into *E. coli*, selected for based on Cm resistance, isolated and purified. The presence of the cassette was confirmed with a PCR using the *oipA* universal primers and comparing the mutants to controls. This result was corroborated with sequencing as well.

Lastly, this *oipA::CAT-rdxA* plasmid was naturally transformed into the appropriate Δ*rdxA* strains of *H. pylori* (26695, J68, and 175 Δ*rdxA*) and selected for using SFBB plates with 10µg Cm/mL. Resultant strains were confirmed via PCR of the *oipA* locus and sequencing, and
named according to transformed \textit{H. pylori} strain and $\Delta rdxA/oipA::$CAT-$rdxA$ (Table 3). These strains, (26695, J68, and J75 $\Delta rdxA/oipA::$CAT-$rdxA$), serve as both intermediates in the isolation of markerless mutants and as $oipA$ knockouts, and are each Mtz$^\text{R}$ and Cm$^\text{S}$.

\textbf{Cloning of $oipA$ mutant plasmids}

\textit{oipA} mutagenic oligos were designed specific to each strain to alter the number of CT dinucleotide repeats in the 5’ region of \textit{oipA} in order to turn the gene phase off in the \textit{cagPAI} positive \textit{H. pylori} strain 26695, and phase on in the \textit{cagPAI} negative \textit{H. pylori} strains J68 and J75. This was accomplished by deleting one CT in strain 26695 to go from 6 CT repeats to 5, resulting in a frame shift in \textit{oipA} from phase on to phase off, adding one CT in the J68 \textit{oipA} allele from 10 to 11 dinucleotide repeats (\textit{oipA} phase off to phase on), and deleting one CT in the J75 \textit{oipA} allele from 7 to 6 to turn \textit{oipA} from phase off to phase on. Sequences of wild type and mutant \textit{oipA} in all three strains, as well as the corresponding amino acid sequence, are depicted in Figure 3. Mutagenesis was performed using the 26695.$oipA$OFF, J68.$oipA$ON, and J75.$oipA$ON mutagenic primers (Table 1), respectively, and several clones were screened via sequencing. Plasmids were named p26695.$oipA$OFF, pJ68.$oipA$ON, and pJ75.$oipA$ON (Table 2).

Confirmed clones were naturally transformed into the appropriate \textit{H. pylori} strain $\Delta rdxA/oipA::$CAT-$rdxA$. Mutated \textit{oipA} plasmids were able to recombine with the $oipA::$CAT-$rdxA$ locus on the recipient chromosome to replace the CAT-$rdxA$ cassette. Such allelic replacement mutants were selected for with SFBB plates containing 5$\mu$g metronidazole/mL. The loss of the CAT-$rdxA$ cassette during allelic replacement leaves the strain with only a defective $rdxA$ locus due to the earlier deletion in a location distant from the $oipA$ gene on the genome. These strains, 26695 $\Delta rdxA/oipA$OFF, J68 and J75 $\Delta rdxA/oipA$ON (Table 3), are Mtz$^\text{R}$/Cm$^\text{S}$, and have a mutated \textit{oipA} gene with no antibiotic resistance genes left behind in the same region.
Cloning of FLAG epitope mutant plasmids

In order to examine oipA expression beyond relative transcript levels, we aimed to observe protein expression at the whole cell level in the mutant strains using Western blotting. While creating an antibody specific to oipA was not feasible in the time frame of this honors project, we were able to design mutagenic primers containing a FLAG tag (Sigma-Aldrich) and insert this seven amino acid epitope encoding sequence into the gene oipA (Table 1). These primers were used in site-directed mutagenesis with 26695, J68, and J75 plasmids containing wild type oipA as well as mutant oipA. Successful plasmids were confirmed via sequencing and named with the appropriate strain and phase of oipA, i.e. p26695oipAOFF.FLAG (Table 2).

Plasmids were naturally transformed into the appropriate H. pylori strain ΔrdxA/oipA::CAT-
rdxA and again confirmed with sequencing. These strains (Table 3) were later used in Western blotting using ANTI-FLAG antibodies (Sigma-Aldrich).

**Cloning of cagE KO mutant plasmids**

In addition to altering the phase of oipA based on the presence or absence of the cagPAI, we also wanted to create an artificial cagPAI T4SS knockout in the naturally cagPAI positive strain 26695. Previous studies have shown that the most efficient way to accomplish this is to knockout one essential component of the cagPAI, cagE, which will render the type IV secretion system non-functional. A plasmid containing this knockout, pICB:CAT (Table 2), was created by Tummuru et al. (1995) by inserting a chloramphenicol resistance gene, CAT, into the gene cagE, previously named picB. This plasmid was naturally transformed into both 26695 ΔrdxA with oipA unaltered and thus phase on, and 26695 ΔrdxA, oipA phase off. Clones with the mutation were selected for using SFBB plates with 10µg Cm/mL, and the CAT insertion within the chromosomal copy cagE was confirmed via PCR and Big Dye sequencing using the cagE Universal Fwd and Rev primers (Table 1). Strains were named 26695 oipAON.cagEKO and 26695 oipAOFF.cagEKO (Table 3).

**Cloning of J75 5’ UTR indel deletion plasmids**

In order to delete the 11bp indel in the 5’ UTR of J75, complementary primers were designed to match the sequence surrounding, but excluding, the indel. These J75 5’ UTR deletion mutagenic oligos (Table 1) were used in mutagenesis reactions using a QuikChange Lightning Site-Directed Mutagenesis System (Agilent Technologies) with pJ75.oipAON and pOipA.J75 as templates. Several clones were analyzed via DNA sequencing using the oipA.Fwd.J75 primer (Table 1). The successful plasmids were named pJ75.oipAOFF.5’Δ and pJ75.oipAON.5’Δ (Table 2). These plasmids were naturally transformed into J75 ΔrdxA/
oipA::CAT-rdxA, and selected for using SFBB plates with 5µg Mtz/mL. Resultant strains were confirmed via PCR and sequencing, and named J75.oipAOFF.5’Δ and J75.oipAON.5’Δ (Table 3).

**Plasmid Purification and Isolation**

5mL of liquid LB/100µg ampicillin ml⁻¹ broth were inoculated with a single isolated E. coli colony from antibiotic plates or grown from freezer stock (LB + 30% glycerol). Liquid culture was incubated at 37°C shaking at 225rpm for 16-24hours. Cells were spun down at 5000 xg. Cells were lysed and plasmids were isolated using Mini Hi-Speed Plasmid Kit (IBI).

Concentrations of plasmid dsDNA were quantified using a NanoPhotometer (Implen).

**H. pylori Natural Transformation**

*H. pylori* strains desired to be transformed were grown up from freezer stock and passed to 4-6 BAPs to grow for 24-36 hours. All growth was harvested into 10mL of SFBB/10µg vancomycin ml⁻¹ and centrifuged at 4360rpm for 5 minutes. The appropriate plasmid was heat sanitized at 80°C for 20 minutes to reduce potential contamination. Between 7-13µg of plasmid were added to resuspended *H. pylori* cells, and 30µL of the suspension was spotted onto the center of 5 BAPs, and incubated face up for 2-4 hours at standard conditions. Spots were streaked across the plates and incubated face down overnight. Each BAP was harvested and passed to its own SFBB plate with the desired antibiotic for selection. Plates were incubated for 3-5 days. Individual colonies (4-7) were isolated and harvested from their own BAP for genomic DNA extraction.

**Genomic DNA Extraction**

*H. pylori* was grown on BAPs for 24-48 hours under standard conditions. Cells were harvested in 1mL of Phosphate Buffered Saline (PBS) and centrifuged at 6,000 rpm for 5
minutes. gDNA extraction was performed via the Mini gBAC genomic DNA kit (IBI) by the manufacturer’s protocol.

**Amplified Fragment Length Polymorphism (AFLP) Analysis**

AFLP was employed to determine natural variation in the *oipA* CT dinucleotide repeat tract of *H. pylori* grown *in vitro*. AFLP primers were designed to amplify a 270 bp PCR product beginning upstream of *oipA* and including the CT dinucleotide repeat tract (Table 1). Separate primers were designed to amplify a 294bp control region of the gene from a non-repeat bearing region to serve as a control. In each case, reverse primers contained fluorescent VIC tags on the 5’ end (Applied Biosystems). Genomic DNA was obtained from desired strains of *H. pylori*. Each sample was assayed in triplicate in a PCR with the VIC tagged primers to amplify the desired regions of *oipA*, and amplicons were generated according to the manufacturer’s protocol. One µL of 1:50 diluted PCR samples were added to 12µL Hi-Di Formamide plus 0.25µL GeneScan Rox500 size standard in 96 well plates. Samples were denatured at 95°C for 3 minutes and analyzed with ABI 3130 genetic analyzer to quantify amplicon frequency. For each run, the individual area under the curve for each amplicon was summed to determine the total area.

**Attachment Assay**

These assays were performed to quantify the ability of *H. pylori* to adhere to AGS cells, a cell line from human gastric adenocarcinoma. AGS cells were routinely cultured in T75 flasks containing sterile RPMI medium supplemented with 10% fetal bovine (FBS) or newborn calf serum (NCS) as well as HEPES (10mM) (Gibco) with penicillin and streptomycin. For adherence assays, each well of a 6-well tissue culture plate (Cyto One) was seeded with 2.5x10^5 AGS cells and grown for 24 hours to reach 5x10^5 AGS cells per well. Wells were washed three times with RPMI supplemented with 10% FBS with no antibiotics. AGS cells were infected with
H. pylori at a multiplicity of infection of 100:1 (H. pylori: AGS) and incubated at standard conditions and 50rpm shaking for 5 hours. At 5 hours, culture medium was harvested for subsequent ELISA for IL-8, and wells were washed three times with PBS and then lysed with 0.1% Saponin in PBS for 15 minutes at standard conditions and shaking. Lysates were collected and serially diluted in PBS to 10⁻⁷. Dilutions were plated on BAPs in 20 μL spots in triplicate and incubated at standard conditions for 5 days. After 5 days, colony forming units (CFU) were counted and bacterial titer calculated.

**Post-Attachment oipA expression**

In order to determine oipA expression in H. pylori that had adhered to AGS cells as compared to those unattached in the supernatant, attachment assays were performed as above. After the 5 hour incubation, all 3mL of supernatant were collected from each well and centrifuged at 4360rpm, 4°C for 10 minutes. Supernatants were manually removed by pipetting, and the pellets were resuspended in 1mL of RNAzol RT (Molecular Research Center, Inc.). This preparation contains the non-adherent H. pylori cells. The AGS monolayer containing attached H. pylori was then washed twice with PBS to remove non-attached bacteria, and lysed with 1mL of 0.1% saponin for 15 minutes at standard conditions. Cells were scraped and lysates were collected. Wells were washed twice with PBS. Lysates were centrifuged at 4360rpm, 4°C for 10 minutes, supernatants were removed manually, and pellets were resuspended in 1mL of RNAzol RT. RNA was extracted, cDNA created, and samples used in RT-qPCR to quantify relative quantity of oipA transcript (see methods below).

**RNA Extraction**

Preparation: H. pylori was grown from freezer stock. Samples were collected in 5mL of SFBB/ 10μg Vancomycin ml⁻¹ at 0.2 OD₆₀₀ and incubated at standard conditions, shaking at
150rpm for 12-24 hours. OD \(_{600}\) values were determined, and 8 \times 10^8 cells were collected and centrifuged at 4360 rpm for 10 minutes at 4\(^\circ\)C. Supernatants were discarded and cell pellets were suspended in 1mL of RNAzol RT (Molecular Research Center, Inc.). Total RNA was extracted from each pellet according to the manufacturer’s protocol. Suspensions were beadrupted for 45 seconds. Molecular grade water was added to each sample and allowed to incubate for 15 minutes. Samples were spun down for 15 minutes at 12,000 x g. 1mL of supernatant was added to 75% ethanol and incubated for 10 minutes. Samples were centrifuged at 10,000 xg for 8 minutes and supernatant was decanted. Two additional ethanol washes were carried out and RNA pellets were allowed to dry for 20 minutes. Pellets were resuspended in 20-70 μL of PCR grade H\(_2\)O. Samples were then transferred to ice and RNA concentrations quantified using a NanoDrop.

RNA samples were then used in cDNA synthesis. 1 μg of purified RNA was combined with iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad) and cDNA was synthesized using the manufacturer’s cDNA synthesis protocol. cDNA was used for RT-qPCR.

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

The expression of the *H. pylori* gene *oipA* was compared to the housekeeping gene *ftsZ* and/or *gyrB*, encoding the cell division protein FtsZ and DNA gyrase subunit B, respectively, using a TaqMan Gene Expression assay (Life Technologies) performed on the Applied Biosystems StepOne apparatus. The assays were performed in technical triplicate for each gene and each strain according to the manufacturer’s protocol using custom TaqMan Custom Gene Expression assays (Thermo-Fisher), including the *oipA*.Taq, *ftsZ*.Taq, and *gyrB*.Taq probes (Table 1). Relative expression of genes among the various mutants was calculated using the \(2^{\Delta\Delta CT}\)
method as described by Livak and Schmittgen (2001) and processed using DataAssist software (Applied Biosystems).

**Enzyme-Linked Immunosorbent Assay (ELISA)**

Human IL-8 ELISA MAX Deluxe assays (BioLegend) were used via the manufacturer’s protocol in order to quantify the concentration of IL-8 produced by AGS cells in the presence and absence of *H. pylori*. Adhesion assay culture media were centrifuged at 6000 rpm and supernatants were used in ELISA. A 96 well plate was coated in diluted human IL-8 capture antibody overnight at 4°C. After washing four times with wash buffer, assay diluent A was added and incubated for 1 hour. The plate was washed and diluted standards and sample supernatants were added, followed by incubation for 2 hours at room temperature (RT), shaking. The plate was washed and diluted detection antibody was added, and the plate was allowed to incubate for one hour at RT, shaking. After washing, Avidin-HRP was added and incubated for 30 minutes at RT, shaking, followed by 5 washes with wash buffer, and then Substrate Solution C was added and the plate was allowed to incubate in the dark at RT for 15 minutes. Stop solution was added and the plate was read at an absorbance of 450nm on a Bio-Rad iMark Microplate Reader.

**Western Blot**

Mutant and control strains of *H. pylori* were grown on blood agar plates, 0.4 OD600 units of cells were denatured and reduced, and total proteins were separated by SDS-PAGE. Proteins were transferred from the gel to nitrocellulose blotting membranes (Bio-Rad). Proteins were separated via western blot and OipA expression detected using Monoclonal ANTI-FLAG M2 antibody (Sigma-Aldrich), followed by anti-mouse IgG-Peroxidase. Chemiluminescence was
accomplished using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) and visualized with X-Ray film.

**Results**

**Phase Variation in the CT Repeat Tract of oipA is Rare in Strains Grown in vitro**

Outer membrane inflammatory protein (OipA) has been shown to be phase “on” in cagPAI positive strains of *H. pylori*, and phase “off” in cagPAI negative strains, nearly without exception (Ando et al. 2002). As previously described, OipA expression is regulated via slipped-strand mispairing in a CT dinucleotide repeat tract in the 5’ end of the gene. This hypermutable sequence can be used to generate phase variation in the gene. Typically, the longer the hypermutable sequence, the more likely that variation will occur (Harvey et al. 2014). In order to determine if there was natural variation in this CT repeat length when strains of *H. pylori* are grown in vitro, we employed amplified fragment length polymorphism (AFLP) analysis.

Results shown in Figure 4 suggest that despite the ability of slipped strand mispairing in the CT tract to generate variability, there was little variation in the number of CT repeats present within populations of the tested *H. pylori* strains. In the cagPAI positive strain, 26695, >97% of the population contained 6 CT’s, creating a phase on allele of *oipA*. In the cagPAI negative *H. pylori* strain, J68, while the majority of the population is phase off with 8 CT’s, there is a more significant minority population, compared with strains 26695 and J75, that is phase on with 6 CT’s, ~9% (Figure 4). In the cagPAI negative strain, J75, >95% of the population contained 7 CT’s, making the oipA allele in this less virulent strain phase off. Overall, this apparent paucity of *in vitro* variation in the poly CT tract suggests that the relationship between the presence/absence of the cagPAI and the expression phase of *oipA* tends to be well maintained.
Experimental Alterations to the CT Tract Result in Changes in oipA Expression

In order to quantify the effect of changes to the CT tract on oipA expression, RNA was isolated from the \textit{H. pylori} oipA mutants and controls, converted to cDNA, and used in RT-qPCR to measure oipA transcript levels. Transcription and translation are coupled in bacteria, and transcription often undergoes rho dependent termination when translation halts. In prokaryotes, ribosomes begin translating mRNA emerging from RNA polymerase, but dissociate when they encounter a stop codon. Rho factor binds to sites on naked RNA, pulling the nascent RNA away.
from RNA polymerase and the DNA template and thus terminating transcription (Boudvillain et al. 2013). Transcription termination in *H. pylori* is most likely rho dependent (Washio et al. 1998). For example, if *oipA* is phase off due to the number of CT dinucleotide repeats, the mRNA will thus contain in-frame, cryptic stop codons which will cause ribosomes to dissociate from the emerging transcript, which will allow rho transcription terminators to catch up to RNA polymerase and stop transcription. Therefore, although *oipA* can be transcribed whether the gene is phase on or phase off, a cell that is phase off will not only have no protein, but will also have less mRNA transcripts. In this way, we have used RT-qPCR to indicate a relative rate of protein translation via the relative quantity of transcript (Acio-Pizzarello et al. 2017, in press).

Deleting the internal portion of *rdxA* in order to create a metronidazole resistant control within wild type, phase on *oipA* in strain 26695 did not produce a significant difference in *oipA* transcript levels compared to the wild type *H. pylori* strain by a Welch’s unpaired t-test of unequal variance, indicating that it is a reliable control (Figure 5). Altering the number of CT’s in *oipA* in 26695 such that the gene is phase off decreases the transcript levels of the gene by more than 80% compared to the phase on control. Additionally, the relative quantity of *oipA* transcript when the gene is phase off is not significantly different from the relative quantity when the gene is “knocked out” by insertion of a CAT-*rdxA* cassette, thus creating a null mutant (Figure 5).

Similarly, there is no significant difference between the cagPAI negative J68 *ΔrdxA* control with *oipA* phase off, and the J68 *oipA* knock out created in the same manner (Figure 6). When the CT dinucleotide repeat tract was altered such that *oipA* was in frame and thus phase on in J68, there was a significant increase in *oipA* transcript levels (Figure 6), further indicating that changes to the CT tract lead to altered gene expression compared to the control.
Figure 5. Phase off status of oipA in *H. pylori* strain 26695 causes a significant decrease in the relative quantity of transcripts expressed.

Real time quantitative PCR was used to determine the relative expression of oipA in the *H. pylori* 26695 ΔArxA control strain with oipA phase on, compared to wild type, as well as to the oipA phase off and oipA knock out mutants. The data shown here is representative of the results obtained in three independent experiments, each conducted in technical triplicate. Error bars show standard deviation. Statistics were calculated using a Welch’s unpaired *t*-test of unequal variance with 26695 oipA phase on as the control (** = *p* ≤ .01, n.s. = *p* > .05).
Figure 6. Phase on status of oipA in J68 leads to a significant increase in the relative quantity of oipA transcripts expressed.

Real time quantitative PCR was used to determine the relative expression of oipA in the H. pylori J68 ΔArdxA control strain with oipA phase off, compared to wild type, as well as to the oipA phase on and oipA knock out mutants. The data shown here is representative of the results obtained in three independent experiments, each conducted in technical triplicate. Error bars show standard deviation. Statistics were calculated using a Welch’s unpaired t-test of unequal variance with J68 oipA phase off as the control (* = p ≤ .05, n.s. = p > .05).

Phase On Status of oipA in the cagPAI Negative H. pylori Strain J68 Results in Expression of Full Length OipA Protein

Though RT-qPCR results indicated that oipA mRNA transcripts were at higher levels and thus potentially translated, we employed anti-FLAG western blots to see if the OipA protein was being expressed at the whole cell level. A FLAG protein epitope tag was inserted into the oipA allele of H. pylori strain J68 via site directed mutagenesis and allelic exchange, and a monoclonal antibody to this epitope was used to detect OipA protein in western blots. In the
cagPAI negative and naturally oipA phase off *H. pylori* strain J68, when oipA was experimentally turned to phase on, there was an ~34 kDa protein detected (Figure 7). This indicates a successful switch from phase off to on, and expression of the protein of the predicted size of OipA (34,184 Daltons) (performed by Allison Begley, our high school protégée, 2016).

**Phase On oipA Confers an Increased Host Adherence Phenotype**

Once it was demonstrated that altering the number of CT dinucleotide repeats resulted in successful phase change of oipA with a concomitant expression of the OipA protein, we next wanted to investigate whether the demonstrated decrease in oipA transcript level in the cagPAI positive strain 26695 (Figure 5) would lead to a decrease in *H. pylori* adherence to gastric epithelial cells, and vice versa in the cagPAI negative strain J68. When oipA was turned phase off in 26695, attachment levels decreased from ~1.0x10⁵ CFU/mL to less than 2.0x10⁴ CFU/mL (Figure 8). oipA phase was also strongly correlated to *H. pylori* attachment capability in J68, as attachment levels more than doubled when oipA was turned phase on (Figure 9). These results indicate that OipA has the ability to mediate attachment to host cells independent of the cag pathogenicity island and demonstrate that the cagPAI negative strain J68 allele of oipA is fully functional when turned phase on.
OipA Effects on IL-8 Production

Due to the differences in attachment ability observed between *H. pylori* strains with *oipA* phase on and phase off, and given OipA’s theorized involvement in the host inflammatory response, we hypothesized that the attachment results might be associated with similar changes in epithelial cell IL-8 production. To quantify this in terms of IL-8 concentration, attachment assay culture medium was collected and the undiluted cell-free conditioned media were used to
conduct IL-8 ELISAs in technical triplicate. AGS cells infected with the cagPAI positive strain 26695 with oipA experimentally switched to phase off produced nearly 90% less IL-8 than those infected by H. pylori with oipA phase on (Figure 10). There was no significant difference in the amount of IL-8 produced by AGS cells infected with strain 26695 with oipA knocked out via the CAT-rdxA cassette as compared to oipA phase off, and both of these concentrations of IL-8 were comparable to the amount of IL-8 produced by uninfected AGS cells (Figure 10).

![Bar graph showing IL-8 production](image)

**Figure 10. Phase off status of oipA in H. pylori strain 26695 correlates with decreased adhesion and decreased gastric epithelial cell IL-8 production.** Cell medium from AGS cells infected with 26695 oipA phase on, or oipA knock out, or oipA phase off H. pylori, as well as from uninfected AGS cells was collected after a 5 hour infection and used in ELISA to detect AGS cell IL-8 production. IL-8 concentrations were compared. The data shown here is representative of the results obtained in three independent experiments, each conducted in technical triplicate. Error bars show standard deviation. Statistics were calculated using a Welch’s unpaired t-test of unequal variance (\(* * = p \leq 0.01\), n.s. = \(p > 0.05\)).
Surprisingly, in *H. pylori* strain J68 there was no significant difference in IL-8 production by AGS cells, whether infected with *oipA* phase on, *oipA* phase off, *oipA* knock out strains, or uninfected (Figure 11). This stark contrast between *cagPAI* positive and *cagPAI* negative strains indicates that, while OipA has demonstrated the ability to mediate IL-8 production in the presence of the T4SS encoded by the *cag* pathogenicity island, OipA cannot itself alone mediate the host inflammatory response in the absence of the *cagPAI*.

**Figure 11. Increased adhesion via *oipA* phase on status in J68 does not affect gastric epithelial cell IL-8 production.** Cell medium from AGS cells infected with J68 *oipA* phase off, or *oipA* knock out, or *oipA* phase on *H. pylori*, as well as from uninfected AGS cells was collected after a 5 hour infection and used in ELISA to detect AGS cell IL-8 production. IL-8 concentrations were compared. The data shown here is representative of the results obtained in three independent experiments, each conducted in technical triplicate. Error bars show standard deviation. Statistics were calculated using a Welch’s unpaired *t*-test of unequal variance (n.s. = *p* > 0.05).
**oipA mRNA Transcript Levels in Adherent vs. Non-adherent H. pylori**

While the traditional attachment assays can provide information on attachment capability and IL-8 production, we also wanted to know if there was a difference in the amount of oipA mRNA transcripts in *H. pylori* that had attached to AGS cells, versus those that were also incubated with host cells, but remained unattached. In order to determine this, attachment assays were performed as before, however after the 5-hour incubation, whole infected AGS cell culture supernatants were collected separately, as were adherent *H. pylori* after lysing infected AGS cells. RNA was extracted from both the non-adherent *H. pylori* (those in the cell culture supernatants) samples as well as from cells bound to AGS cells. cDNA was then synthesized, and RT-qPCR was used to quantify relative oipA expression.

Due to the variation in *H. pylori* cell numbers in these two populations and subsequently mRNA and cDNA amounts compared to traditional mRNA extraction preparations, two housekeeping genes were used in RT-qPCR experiments. *ftsZ* was used as a normalizing gene as per usual, and *gyrB* was employed as a comparison to oipA. Relative quantities of the housekeeping gene *gyrB* are not predicted to vary due to *H. pylori* attachment or non-attachment to AGS cells.

Results from this experiment revealed that 26695 oipA phase on *H. pylori* cells that were attached to AGS cells after the 5 hour infection had 7 times the amount of oipA transcript compared to those cells that had not attached, while housekeeping gene *gyrB* transcript levels did not vary (Figure 12). This may indicate that there is selection occurring within the *H. pylori* population that confers an adherence advantage to *H. pylori* cells that produce more OipA; one hypothesis being that they could better adhere to host cells and avoid adverse effects such as mucosal layer shedding. Interestingly, this trend also holds for 26695 oipA phase off (Figure 12).
We hypothesize that this may be due to selection for phase on variants of *oipA* in the presence of AGS cells via the slipped strand mispairing mechanism. An alternative hypothesis is that contact with AGS cells may trigger increased transcription of *oipA* as a response to the presence of the gastric epithelium.

**Figure 12.** *H. pylori* that attach to AGS cells possess higher levels of *oipA* transcript. Attachment assays were performed using 26695 *oipA* phase on (panel A) and 26695 *oipA* phase off (panel B). For each infection, *H. pylori* in the supernatant was collected separately from the bacteria attached to the AGS monolayer. RNA was extracted, and cDNA synthesized was used in qPCR to determine relative quantities of *oipA* transcripts in attached cells compared to non-attached. The data shown here is representative of the results obtained in independent experiments, each conducted in technical triplicate. Error bars show standard deviation. Statistics were calculated using a Welch’s unpaired *t*-test of unequal variance (* = p ≤ 0.05, n.s. = p > 0.05).
**oipA Status in the cagPAI Negative *H. pylori* Strain J75 does not Correlate to Increases in Transcript Levels**

Despite the strong correlation between *oipA* phase on status and increased *oipA* mRNA transcript levels as well as AGS cell attachment ability in the cagPAI negative strain J68, turning *oipA* phase on in the cagPAI negative strain J75 did not lead to increased transcription of *oipA* (Figure 13) nor attachment (Figure 14). In fact, there was actually a slight decrease in attachment when *oipA* was phase on. Similarly to strain J68, *oipA* phase on status in J75 did not induce greater cytokine production by AGS cells (Figure 15). This is likely due to the documented lack of *oipA* mRNA response to experimental phase on conditions.

![Figure 13. Phase on status of *oipA* in J75 does not lead to increased *oipA* transcription, whether the 5' UTR indel is deleted or not.](image)

Real time quantitative PCR was used to determine the relative expression of *oipA* in the *H. pylori* J75 control strain with *oipA* phase off, compared to *oipA* phase on and 5’ UTR deletion mutants. The data shown here is representative of the results obtained in two independent experiments, each conducted in technical triplicate. Error bars show standard deviation. Statistics were calculated using a Welch’s unpaired *t*-test of unequal variance with J75 *oipA* phase off as the control (n.s. = *p* > .05).
Figure 14. Phase on status of oipA in J75 leads to decreased attachment to AGS cells.
Attachment assays were performed to determine whether turning oipA phase on in the cagPAI negative strain J75 would alter the bacterium’s ability to adhere to AGS cells. Adhesion phenotypes depending on oipA status were compared. The data shown here is representative of the results obtained in three independent experiments, each conducted in technical triplicate. Error bars show standard deviation. Statistics were calculated using a Welch’s unpaired t-test of unequal variance (n.s. = p > 0.05).

Figure 15. oipA phase on status in J75 does not affect gastric epithelial cell IL-8 production.
Cell medium from AGS cells infected with J75 oipA phase off, or oipA knock out, or oipA phase on H. pylori, as well as from uninfected AGS cells was collected after a 5 hour infection and used in ELISA to detect AGS cell IL-8 production. IL-8 concentrations were compared. The data shown here is representative of the results obtained in independent experiments, each conducted in technical triplicate. Error bars show standard deviation. Statistics were calculated using a Welch’s unpaired t-test of unequal variance (n.s. = p > 0.05).
Based on *oipA* expression results, we decided to further investigate the potentially unique nature of *oipA* in the *cagPAI* negative *H. pylori* strain J75 as compared to its *cagPAI* negative counterpart in the study, J68, and the *cagPAI* positive strain 26695. Aligned sequences of the promoters, 5′ untranslated regions (UTRs), and 5′ ends of *oipA* (Figure 16) revealed an 11bp indel in J75 amidst this highly conserved region (disregarding the known variation in the CT tract). Upon further investigation, we discovered that this indel was not unique to J75, but can be found in several sequenced strains of *H. pylori*, although still a minority (data not shown).

We hypothesized that this indel within the 5′ UTR of *oipA* may be the reason that *oipA* transcripts are not produced at higher levels, even when the CT tract is altered such that the gene is phase on. To pursue the role of this 11bp indel, we deleted it in both *H. pylori* strain J75 possessing a wild type phase off *oipA*, and in the mutant strain J75 possessing a phase on *oipA*. These strains were then used in RT-qPCR to determine whether or not *H. pylori* J75 would transcribe *oipA* at higher levels when the CT tract is altered to make the gene phase on, and the indel is no longer present. Interestingly, we found that despite the deletion of the indel, there was still no increase in the relative quantity of *oipA* expressed in J75 (Figure 13). This result further demonstrates the tight control J75 employs to avoid *oipA* expression, despite the fact that the gene is highly conserved.
Discussion

OipA and the cag pathogenicity island are two well documented H. pylori virulence factors. The cagPAI encodes a type IV secretion system (T4SS) that induces the production of pro-inflammatory cytokines by host gastric epithelial cells (Khatoon et al. 2017). OipA acts as an adhesin, and also plays a role in the pro-inflammatory response (Oleastro & Ménard 2013). Studies have shown that cagPAI positive strains of H. pylori contain an oipA allele that is phase on while cagPAI negative strains contain a phase off, yet highly conserved, allele of oipA (Matsuo et al. 2017; Ando et al. 2002). Despite this finding and despite the importance of both virulence factors, the relationship between OipA and the cagPAI remains somewhat mysterious. The goal of my honors research project is to shed light on this relationship by observing the effects of mutating oipA to phase off status in cagPAI positive strain 26695, and mutating oipA to phase on in cagPAI negative strains J68 and J75.

Phase variation in OipA is regulated by a slipped strand mispairing mechanism affecting a CT dinucleotide repeat tract within the 5’ end of the gene (Miftahussurur & Yamaoka 2015). Despite this ability, our amplified fragment length polymorphism (AFLP) results suggest that there is little variation in the CT tract length in populations of H. pylori grown in vitro. This indicates that, while possible, phase variation at this locus may be rare. This is in contrast to phase variation in several other outer membrane proteins, including sialic acid binding adhesin (SabA) (Harvey et al. 2014), in which there are distinct subpopulations with varying CT tract lengths within a population. Over 80% of bacterial genomes are comprised of sequences encoding proteins. Additionally, when bacterial species transition to permanent host interactions, they undergo a major loss of genes, usually along the lines of the “use it or lose it” paradigm (Moran 2002). This occurs as species obtain metabolic compounds from the host and gradually
discard corresponding pathways and genes, resulting in small genomes comprised of the bare essentials. These factors add intrigue to the conundrum that phase off alleles of *oipA* are nearly always found in cagPAI negative strains of *H. pylori* (Ando et al. 2002), yet these strains have not lost the gene for this protein that is apparently never expressed.

*In silico* corrections revealed that the protein produced when the CT tract is altered to generate a phase on allele of *oipA* in the cagPAI negative strains used in this study, J68 and J75, share >95% amino acid identity with the protein produced by 26695. Based on this finding, we hypothesized that turning *oipA* phase on in cagPAI negative strains of *H. pylori* would lead to an increase in *oipA* expression, would result in the production of a full length protein, and would lead to an improved ability of these cells to adhere to gastric adenocarcinoma (AGS) cells and to elicit pro-inflammatory cytokine (IL-8) production in these cells.

Real time quantitative PCR (RT-qPCR) experiments using *oipA* TaqMan probes showed that *oipA* transcript levels were significantly increased when *oipA* was phase on in both a cagPAI positive and a cagPAI negative strain of *H. pylori*, and decreased *oipA* transcript levels were significantly associated with phase off status. The increase in *oipA* mRNA transcript levels in *H. pylori* strain J68 correlated with anti-FLAG western blot detection of OipA::FLAG when the gene was turned phase on, indicating that this cagPAI negative strain is capable of expressing a full length OipA protein. Phase on *oipA* confers an advantage in host adherence ability in both cagPAI positive and negative strains as well, with a significant decrease in the number of *H. pylori* cells that were able to adhere to AGS cells when *oipA* was phase off. These findings show that OipA has the ability to mediate host attachment independent of the cag pathogenicity island.

IL-8 assay results from AGS infection experiments show that phase off status of *oipA* in the cagPAI positive strain 26695 correlates not only with a decrease in host attachment, but also
with decreased host production of this pro-inflammatory cytokine. It is striking that in the absence of OipA expression, there is a significant decrease in IL-8 production even in the presence of a functional cagPAI encoded T4SS. This indicates that the cagPAI cannot mediate IL-8 induction independent of OipA. This finding is also supported by the fact that OipA expression does not support any significant IL-8 secretion by AGS cells when infected by the cagPAI negative strain J68 with oipA experimentally turned phase on. We hypothesize that OipA works in conjunction with the cagPAI to induce the host inflammatory response, and that both H. pylori surface structures must be present to stimulate host pro-inflammatory cytokine production.

Post-infection RT-qPCR experiments where AGS cells were infected by H. pylori strain 26695 revealed that there was a significant increase in the quantity of oipA mRNA transcripts in the H. pylori cells adhered to AGS cells, compared with those incubated with AGS cells, but that were non-adherent. We propose two alternative hypotheses regarding these results. First, we propose that there may be selection within the population of H. pylori used in the infection that allows cells expressing OipA to better adhere to host cells. This might also indicate that there is greater phase variation in systems including host cells. Further experiments could analyze this by performing AFLP on the adherent and non-adherent cell populations and quantifying the number of CT repeats in the gene oipA, and thus the expression phase of this adhesin. Selection experiments could also be employed in which adherent and non-adherent bacteria are isolated, cultured, and used in subsequent infections. Changes in attachment rates may indicate that it is possible to select for specific expression phases of oipA.

An alternative hypothesis is that contact with AGS cells may trigger increased transcription of oipA as a response to the presence of the gastric epithelium. This hypothesis is supported by data showing the same increase in oipA transcript levels in attached cells compared
to non-attached, even when \textit{oipA} is phase off in 26695. Both hypotheses are supported by the difficulties \textit{H. pylori} faces in the stomach regarding mucosal shedding, which ultimately favors mechanisms allowing cells to “stick” to the gastric epithelium (Oleastro & Ménard 2013).

Correlations between \textit{oipA} phase, mRNA transcript levels, and AGS cell attachment levels observed in \textit{H. pylori cagPAI} negative strain J68 were not observed in a second \textit{cagPAI} negative strain, J75. Phase on status of \textit{oipA} in J75 had no effect on \textit{oipA} mRNA transcript levels, nor did it result in increased attachment to AGS cells \textit{in vitro} nor alter IL-8 levels. We hypothesize that this failure to increase transcript levels or attachment ability is due to the fact that the OipA protein is not expressed, even in the \textit{oipA} phase on \textit{H. pylori J75} mutant. In fact, there was a decrease in the amount of AGS cell attachment when \textit{oipA} was altered to phase on status in J75, compared to the phase off control. We hypothesized that the lack of \textit{oipA} expression was due to an 11bp indel within the 5’ untranslated region (UTR) of the J75 allele of \textit{oipA}, absent in either of the other strains used in this study, which may block or otherwise impede transcription or translation. However, when this indel was deleted, there was no apparent change in \textit{oipA} expression.

This result is puzzling as, with the exception of this 5’ UTR indel deletion, the J75 allele of \textit{oipA} is remarkably similar to \textit{oipA} alleles in the other two strains observed in the study, in which \textit{oipA} phase changes had demonstrable phenotypic effects. Taken with the unexpected AGS cell attachment data in which a phase on allele of \textit{oipA} may actually cause a decrease in AGS cell adherence ability, these results suggest that expression of \textit{oipA} may be under negative selection in J75. It is possible that \textit{oipA}, when phase on, causes a decrease in fitness in this strain lacking the \textit{cagPAI} when exposed to host cells. We hypothesize that another genetic defect in strain J75 may drive this inability to express \textit{oipA}. Further study comparing the genomes of
strains J68 and J75 may reveal candidate loci that exert control over OipA expression. This may be an occurrence that may allow us to identify a control mechanism for the expression of this important virulence factor. It may also whether or not regulatory sequences for oipA affect other genes in H. pylori; thus explaining its conservation in an alternate way.

The discovery that a phase on allele of oipA is able to produce the protein in a cagPAI negative strain of H. pylori, which always has the gene present but in a phase off status, and can mediate attachment, only begins to answer the question of why the gene is highly conserved if it is not expressed. This question is even more puzzling in J75, where a phase on status of oipA does not result in higher transcription levels or increased gastric cell attachment. Results from this study indicate that if cagPAI negative strains of H. pylori were to express OipA, the protein would be able to mediate host cell attachment, but not the host inflammatory response. Selection against expression of OipA should lead to the accumulation of mutations in the gene, and even its deletion. We hypothesize that the expression of OipA must be important under a certain set of circumstances during host infection, otherwise we would expect to see evidence of decay and even removal of the gene, rather than persistent conservation in the absence of apparent expression. Future experiments could observe oipA mRNA transcript levels in adherent versus non-adherent cells from AGS cell attachment assays using the cagPAI negative strain J68 with varying phases of oipA, rather than the cagPAI positive strain 26695. This data may shed light on OipA expression at varying points in infection in this cagPAI negative strain of H. pylori.

These experiments demonstrated changes due to oipA phase in strains of H. pylori that naturally possess or do not possess the cag pathogenicity island. A continuing study in our lab examines the effect of changes to the phase of oipA in 26695 possessing a defective allele of cagE (cagE::CAT). CagE, formerly PicB, is one protein encoded by the cagPAI, and is required
for induction of IL-8 in gastric epithelial cells (Tummuru et al. 1995). We generated a null mutant of CagE, which others have shown is incapable of assembling the T4SS and is thus unable to induce IL-8. We are currently working on creating these knock out strains in 26695 with both expression phases of oipA, and will proceed to AGS cell attachment and IL-8 assays. Our hypothesis is that, without a functional cagPAI T4SS, H. pylori strain 26695 will be unable to induce IL-8 secretion no matter the expression phase of oipA.

Lastly, an interesting phenomenon discovered by our lab is that some strains of H. pylori, particularly those from East Asian patients, possess two copies of the oipA gene and are thus merodiploids. We have begun to analyze the role of the second copy of oipA, which is especially fascinating to us due to the fact that H. pylori infected patients from East Asian countries are more likely to develop gastric cancer. We have created a strain of 26695 that contains a second copy of oipA in the same location as the second copy in the Japanese strains 98-40 and 98-19. Another student in our lab, Kexin Fan, is currently working on creating mutants in strain 98-40 where both copies of oipA are knocked out together and separately. She will continue this study to determine what purpose this second copy of oipA may have in more virulent strains of H. pylori.
References


<table>
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<th>Primer name</th>
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<th>Anneal</th>
<th>Ext. Time</th>
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<td>HP0953 Rev</td>
<td>GCTCGGACTCATGGAAATTCGCTCCAT</td>
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<td>PO₂⁻CCTAAAATGGATGCTGGGATGAGC</td>
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<td><strong>gyrB Consensus.Taq</strong></td>
<td>FAM -- TTGCCTGGAAAAATTAG– MGB-NFQ</td>
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Table 2: Plasmids used or created for this study

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<tr>
<th>Name</th>
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<td><strong>prdxA</strong></td>
<td>TOPO TA cloning vector pCR4 containing a1556 bp amplicon of the <em>H. pylori</em> strain 26695 <em>rdxA</em> gene</td>
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<td><strong>pΔrdxA</strong></td>
<td><em>prdxA</em> with a 390bp deletion in the <em>rdxA</em> gene</td>
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<tr>
<td><strong>pOipA (26695, J68, and J75)</strong></td>
<td>TOPO TA cloning vector pCR4 containing a 2300bp amplicon including the entire <em>oipA</em> gene, as well as the untranslated regions both upstream and downstream</td>
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<td><strong>pOipA.BamHI (26695, J68, and J75)</strong></td>
<td>pOipA containing a BamHI site in the cloned allele of <em>oipA</em></td>
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<tr>
<td><strong>pMM672</strong></td>
<td><em>H. pylori</em> 26695 plasmid in which the coding region of <em>rdxA</em> is deleted (Loh et al. 2011)</td>
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<td><strong>pOipA::CAT-rdxA (26695, J68, and J75)</strong></td>
<td>pOipA.BamHI with CAT- <em>rdxA</em> cassette cloned into the BamHI restriction site</td>
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<td><strong>p26695.oipAOFF</strong></td>
<td>pOipA 26695 with CT repeat tract consisting of 5 CT repeats</td>
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<td><strong>pJ75.oipAON.5'Δ</strong></td>
<td>pJ75.oipAON with an 11bp deletion in the 5’ UTR</td>
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Table 3: *H. pylori* strains used or created for this study

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<td>Mtz&lt;sup&gt;S&lt;/sup&gt; and Cm&lt;sup&gt;S&lt;/sup&gt;, contains <em>oipA</em> with a CT repeat tract consisting of 6 CT repeats (phase on)</td>
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<td>J68 Wild Type</td>
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<tr>
<td>J75oipAON.FLAG</td>
<td>J75 ΔrdxA/oipAON containing a FLAG tag in <em>oipA</em>. Mtz&lt;sup&gt;R&lt;/sup&gt; and Cm&lt;sup&gt;S&lt;/sup&gt;</td>
</tr>
<tr>
<td>J75oipAOFF.FLAG</td>
<td>J75 ΔrdxA containing a FLAG tag in <em>oipA</em>. Mtz&lt;sup&gt;R&lt;/sup&gt; and Cm&lt;sup&gt;S&lt;/sup&gt;</td>
</tr>
<tr>
<td>26695 oipAON.cagEKO</td>
<td>26695 ΔrdxA containing <em>cagE</em> with CAT inserted. Mtz&lt;sup&gt;R&lt;/sup&gt; and Cm&lt;sup&gt;R&lt;/sup&gt;</td>
</tr>
<tr>
<td>26695 oipAOFF.cagEKO</td>
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</tr>
<tr>
<td>J75.oipAOFF.Δ</td>
<td>J75 ΔrdxA containing <em>oipA</em> phase off with an 11bp deletion in the 5′ UTR. Mtz&lt;sup&gt;R&lt;/sup&gt; and Cm&lt;sup&gt;S&lt;/sup&gt;</td>
</tr>
<tr>
<td>J75.oipAON.Δ</td>
<td>J75 ΔrdxA containing <em>oipA</em> phase on with an 11bp deletion in the 5′ UTR. Mtz&lt;sup&gt;R&lt;/sup&gt; and Cm&lt;sup&gt;S&lt;/sup&gt;</td>
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