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# The Contact Dependent Nature of the Expression of Outer Membrane Protein Genes in Helicobacter pylori

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**Abstract**

*Helicobacter pylori* is a gram-negative bacterium that colonizes the gastric mucosa of about 50% of the world's human population. *H. pylori* utilizes a diverse variety of outer membrane proteins for survival, colonization, and adherence to host human gastric epithelial cells in human stomach. Outer membrane proteins involved in adhesion, referred to as adhesins, are critical for bacterial survival and pathogenesis. Previous studies have shown that contact with host human gastric epithelial cells alters the transcription levels of several *H. pylori* genes. However, whether or not contact induces alterations in transcription and the mechanisms through which *H. pylori* senses contact with gastric epithelial cells are unknown. The present study investigates changes in the gene expression of several outer membrane proteins induced by contact with human gastric adenocarcinoma (AGS) cells, and the possible role that two-component systems (TCS) play in this sensing contact. We showed that *H. pylori* cells that adhered to AGS cells possessed increased *oipA* transcript levels compared to non-adherent *H. pylori* cells. In addition, AGS attachment assays revealed that attachment to AGS cells was significantly higher in *H. pylori* strains possessing a phase-on allele of *oipA* compared to an isogenic strain that had *oipA* experimentally turned phase off. ELISA assays revealed that *H. pylori* producing a functional OipA protein and containing the *cag* Pathogenicity Island (*cag*PAI) were positively correlated with both higher levels of attachment and higher levels of IL-8 production by AGS cells, suggesting that OipA interacts with the *cag*PAI to induce a pro-inflammatory reaction. Additional experiments revealed that contact with AGS cells upregulated *babA* mRNA levels in adherent *H. pylori* and repressed *sabA* mRNA levels in nonadherent *H. pylori*. Subsequent experiments using conditioned media possessing AGS cell secretions showed a significant decrease in *sabA* mRNA levels in free-swimming *H. pylori* when compared to *H.*

*pylori* incubated in unconditioned media. We hypothesize that *sabA* repression by AGS cell secretions aids in the separation of the two populations of *H. pylori* that occur in the stomachs infected individuals (adherent and non-adherent, motile *H. pylori* cells). Finally, we showed that mRNA levels of *babA*, encoding a well characterized outer membrane protein adhesin, are significantly reduced when the histidine kinases of the three known two-component systems are knocked out. Furthermore, we found no statistical difference between the mRNA level of *babA* in naïve *H. pylori* cells and the triple histidine kinase knock out mutation strain. and that two-component systems have a role in sensing contact with host gastric epithelial cells. This data suggests that contact dependent changes in *babA* expression is regulated via a two-component system. Our data also suggests that *oipA* and *sabA* are not regulated by two-component systems in a contact dependent manner. This indicates that two-component systems have a role in mediating contact dependent alterations in the gene for at least one *H. pylori* outer membrane protein.

## Introduction

In 1982, Barry J. Marshall and J. Robin Warren identified *Helicobacter pylori* as a major cause of gastric disease. Marshall, a physician, noticed that several of his patients suffering from gastric illnesses shared a common, but uncharacterized and uncultivated bacterial species. Investigating this bacterium along with Warren, a pathologist interested in gastritis, they successfully isolated *H. pylori* from a patient suffering from dyspepsia and Marshall subsequently drank a brew containing two culture plates of *H. pylori* colonies. Within weeks, he suffered from severe active gastritis with polymorphonuclear infiltrate and epithelial damage, and identified *H. pylori* as the culprit (Marshall & Adams 2008). Previous to their findings, peptic ulcers were thought to be induced by stress and lifestyle and the stomach was believed to

be a near-sterile environment due to its highly acidic nature (Nobel Media 2014). For their discovery, Marshall and Warren were awarded the Nobel Prize in Physiology or Medicine. It is now understood that *H. pylori* is the causative agent for over 90% of duodenal ulcers and almost 80% of gastric ulcers (Nobel Media 2014; Marshall & Adams 2008).

Further studies characterized *Helicobacter pylori* as a spiral-shaped, gram negative, microaerophilic bacteria species (Marshall & Warren 1984) that inhabits the human gastric epithelium, associated with chronic gastritis, gastric ulcers, duodenal ulcers, and gastric cancer (Watari et al. 2014). Studies indicate that *H. pylori* acquisition typically occurs in early childhood and hosts remain infected for the for the remainder of their lives, unless treated with antibiotics. Although the exact mechanism(s) for *H. pylori* transmission is/are yet to be fully understood, *H. pylori* transmission frequently occurs from mother to child via oral-oral and fecal-oral routes and is acquired at higher rates in developing countries compared to developed countries (Brown 2000). Although more than 50% of the world's human population is infected with *H. pylori*, about 80% of those infected exhibit no symptoms (Blaser 2006). Interestingly, studies suggest that *H. pylori* may even offer protection against asthma, allergies, and resistance to acid reflux disease (Amedei et al. 2010, Lim et al. 2016). *Helicobacter pylori* is thought to have an important role in the human microbiota as part of the stomach's natural flora due to its tendency to cause disease in a minority of its infected hosts, protective qualities, persistence and roughly 60,000 year old relationship with humans (Atherton & Blaser 2009).

Although most carriers of *H. pylori* show no symptoms and are able to live unaffected, many are adversely impacted by the effects of this bacteria. In 2017, the World Health Organization (WHO) listed clarithromycin resistant *H. pylori* as a "high priority" pathogen (Branswell 2017). Due to its increasing antibiotic resistance, high rates of infection, and potential

to cause life-threatening illnesses, *Helicobacter pylori* is an increasing concern and a worthwhile pathogen to study in order to design novel treatments.

**Survival and Persistence in the Human Stomach**

The human stomach is a considerably hostile environment, with a pH ranging from 1.5 to 3.5 (Teyssen et al. 1995), proteolytic enzymes, and the frequent shedding of the gastric epithelial and mucus layers, the human digestive system is a harsh environment for microorganisms (Marieb & Hoehn 2010, Nardone & Compare 2015, Hansson 2012). *H. pylori* has a distinct set of adaptations that allow it to survive and persist in this harsh environmental niche. One way *H. pylori* survives the acidic environment of the stomach is through its production and utilization of the enzyme urease, a $Ni^{2+}$ containing enzyme. Urease is produced by numerous and diverse bacterial species, and is critical to *H. pylori* metabolism, colonization of the gastric mucosa, survival, and virulence (Konieczna et al. 2012, Mobley 2001). Urease, also known as urea amidohydrolase, catalyzes the hydrolysis of urea into $NH_3$ and carbamate. Carbamate decomposes into carbonic acid and another molecule of ammonia. In aqueous solutions, the carbonic acid is in equilibrium with the two molecules of ammonia in their protonated and deprotonated forms. This results in an overall increase in pH, creating a more alkaline environment (Konieczna et al. 2012). The urease of *H. pylori* has a higher affinity to urea than of other bacterial species ($K_m$ = 0.8mM). Because of its high affinity urease activity, *H. pylori* hydrolyzes the small amounts of urea present in the stomach, creating an alkaline microenvironment around itself as a protective buffer against the harsh gastric acid (Bhattacharyya et al. 2000). These buffering compounds help maintain cytoplasmic pH near neutrality by capturing acidic protons that leak into the cytoplasm (Pflock et al. 2006).

Via chemotaxis, *Helicobacter pylori* uses its two to six flagella to burrow deep into the more neutral gastric mucosa (Suerbaum 1995, Mobley 2001). The bacteria are then able to live in the mucus or attached to gastric epithelial cells via interactions between the host epithelial cell and various *H. pylori* outer membrane proteins. About 4% of the *H. pylori* genome encodes for an extraordinarily large set of diverse outer membrane proteins (OMPs) with unique and important functions for survival, such as pH regulation and adhesion (Oleastro & Ménard 2013). SabA and BabA, two well studied *H. pylori* outer membrane protein adhesins, allow the bacteria to resist the shedding of the gastric mucus lining (Ishijima et al. 2011). The majority of the *H. pylori* cells are motile within the gastric mucus lining, while only a small percentage of *H. pylori* cells adhere to epithelial cell surfaces, predominantly at intercellular junctions (Hazell et al. 1986). This balance between adherent and free-swimming forms in the mucus layer is vital to *H. pylori* colonization and persistence as it prevents the bacteria from being eliminated from the stomach, allows the bacteria to evade the host's immune system, and transport proteins into the host gastric epithelial cells (Oleastro & Ménard 2013).

### *H. pylori* Pathogenesis

Globally, *Helicobacter pylori* infects approximately 50% of the world's human population. *H. pylori* has a high degree of genetic diversity and pathogenesis seems to vary depending on factors such as geographic location, socioeconomic status, race, ethnicity, and age, with most infections occurring in childhood, and morbidity associated with older ages (Shan et al. 2014, Salama et al. 2000). Infection rates appear to be higher in developing countries, with an estimated rate of 80% infection (Khalifa et al. 2010), and lower in industrialized countries, with infection rates estimated to be below 40% (Nagy et al. 2016). Although the majority of infected

individuals never show any symptoms of *H. pylori* infection, some are affected negatively in the form of gastritis, peptic and duodenal ulcers, and gastric cancer (WHO 2017).

The exact mechanisms of *H. pylori* pathogenesis are far from completely understood, but several pathogenic determinants have been the subject of many experimental investigations. Mechanisms of pathogenesis include the use of adhesins, secreted cytotoxins, and a variety of enzymes (Kusters et al. 2006). Infection can result in chronic gastritis or inflammation of the gastric lining, induced by host inflammatory responses (Kusters et al. 2006, Solnick & Schauer 2001, Ansari & Yamaoka 2018). The toxic *H. pylori* protein VacA (vacuolating cytotoxin A) damages cells and disrupts normal cell-cell communications by inducing excessive vacuolation and disrupting tight junctions. Additionally, VacA can cause changes in plasma membrane permeability and mitochondrial membrane permeability, and induce apoptosis (Jones et al. 2010). Furthermore, the ammonia produced by *H. pylori* urease to acclimate to acidic pH values is toxic to gastric epithelial cells (Palframan et al. 2012). Chronic gastritis induced by *H. pylori* can advance to peptic ulcer disease when the gastric epithelium is damaged by the host's inflammatory response, and the combination of stomach acid and the digestive enzyme pepsin leaks through and damages tissue (Dumrese et al. 2009).

Additionally, *H. pylori* can gain different pathogenic and metabolic abilities via the acquisition of foreign DNA segments via horizontal transfer. These genomic segments, also called "islands", can encode for a diverse range of functions including, but not limited to, metal-uptake systems, metabolic enzymes, and adhesins (Hacker et al. 1997). Pathogenicity islands (PAIs) are a type of genetic island that carries genes that encode one or more virulence determinants (Gal-Mor & Finlay 2006). The *cag*PAI is a 40-kb chromosomal region unique to that *H. pylori* and encodes virulence factors including the Cytotoxin-Associated Gene (Cag)

proteins (Hacker & Kaper 2000). Numerous studies have established a strong association between the effector protein CagA encoded within the *cag*PAI and *H. pylori* induced disease (Blaser 1998, Covacci et al. 1997, Jiménez-Soto & Haas 2016). The *cagPAI* also encodes a type IV secretion system (T4SS) that is specific to *Helicobacter* and necessary for the translocation of the bacterial protein CagA into the host gastric epithelial cell (Mobley 2001). The T4SS binds to the cell adhesion protein Integrin Beta 1 (ITGB1) on the surface of the host epithelium cell and tight adhesion is facilitated via the various adhesins produced by *H. pylori*, including BabA, SabA, and OipA (Blaser 1998, Backert et al. 2011, Acio-Pizzarello et al. 2017).

Due to its association with gastric diseases, CagA is one of the most extensively studied *H. pylori* proteins. Following a direct "injection" from the *H. pylori* cell via the T4SS, CagA localizes to the inner leaflet of the cell membrane in the host gastric epithelium cell (Kaplan-Turkoz et al. 2012). It is then tyrosine phosphorylated on an EPIYA motif in its carboxy-terminal variable region (Handa et al. 2007) and binds to the host cell's SHP-2 protein, a cytoplasmic tyrosine phosphatase that regulates signal transduction events, including growth factors and cytokines (Higashi et al. 2001, Hatakeyama 2017). This complex dephosphorylates and inactivates the Src family kinases in the host cell and induces cytoskeletal reorganization of the host cell (Higashi et al. 2001, Safari & Zaer 2017, Hatakeyama 2004). The new phenotype that arises as a result of the CagA-induced cytoskeletal rearrangement (shown both *in vivo* and *in vitro)* (Wessler et al. 2011) is known as the "Hummingbird Effect", in which the cell spreads and displays elongated growth, as well as thin sheets of actin and finger-like protrusions containing actin protrude from the edges of the cell. The "Hummingbird" phenotype in gastric epithelium cells is considered to be precancerous (Segal et al. 1999).

Additionally, CagA has been implicated in a number of hypotheses enhancing *H. pylori* pathogenesis due to its interaction with multiple intracellular target proteins, increased cellular invasiveness and alterations in monolayer polarity and permeability (Tan et al. 2009, Hatakeyama 2017). A study by Tan et al. (2009) indicated that CagA was required for bacterial replication on the apical surface of polarized epithelial cells, while *cagA* mutant strains were unable to replicate on the apical cell surface (although they were able to replicate on the basolateral surface of the host cell). Tan et al. 2009 proposed that CagA contributes to *H. pylori* replication on the apical surface of the cell because of the ability of CagA to disrupt gastric epithelial cell polarity and disrupt cell junction barriers, manipulating the polarity of gastric epithelial cells (Johnson et al. 2012).

Another proposed method for *H. pylori* pathogenesis is via the high rates of host cell mutation as a result of increased production of inflammation regulators, including cyclo-oxygenase-2 and reactive oxygen and nitrogen species (ROS/RNS). These pro-inflammatory factors can activate a number of cellular cascades, such as the NF-kB pathway, an important regulator of inflammation. In addition to being a pro-inflammatory factor, ROS can modify protein function and cause mutations (Sepulveda 2013). Tsuji et al. (2003) proposed a "perigenetic pathway" mechanism by which prolonged host inflammatory response, such as the production of the inflammatory cytokine tumor necrosis factor a (TNF-a), affects epithelial cell-cell adhesion by increasing intracellular radical production and degrading E-cadherin (a tumor suppressor) (Singhai et al. 2011) and β-catenin complexes between epithelial cells. Additionally, they suggested that *H. pylori* promotes the dispersion and migration of infected epithelial cells by activating *src* oncogenes (Tsuji et al. 2003).

In 2018, gastric cancer was the sixth most common form of cancer in the world and the third most common cause of cancer related death (WHO 2018). Individuals infected with *H. pylori* are more than ten times more likely to develop gastric cancer than uninfected individuals (Wroblewski et al. 2010) and an estimated 75% of all gastric cancer cases in the world can be traced to *H. pylori* infection (Suganuma et al. 2012). In fact, the International Agency for Research on Cancer (IARC), a part of the World Health Organization, identified *H. pylori* as the only known bacterial carcinogenic agent in the world (American Cancer Society 2018). Because of its relevance, persistence, and pathogenicity for such a large percentage of the global population, *H. pylori* is an incredibly important bacterial species to study.

**Contact Dependent Gene Expression of *H. pylori***

Recognizing environmental factors at the appropriate physiological sites in hosts is an incredibly important evolved trait that many bacterial pathogens have evolved in order to sense changes and correctly express certain pathways and pathogenicity determinants (Bhattacharya et al. 2016). Studies have demonstrated that contact with eukaryotic cells is a stimulus that alters gene expression in *H. pylori* (Johnson et al. 2012, Kim et al. 2004), although many of the specific dynamics of *H. pylori* behavior in relation to contact with the gastric epithelial cell surfaces are still largely unknown. However, contact has been observed to alter gene transcription in ways that seem to optimize persistent colonization and proliferation (Bhattacharya et al. 2016, Johnson et al. 2012, Amsterdam et al. 2003). One study found that contact with gastric epithelial cells stimulated an increase in the vacuolating cytotoxin gene *vacA* transcription, suggesting that the altered expression of *vacA* is necessary for *H. pylori* adaptation and survival in its harsh gastric environment (Amsterdam et al. 2003). Another study found that the urease subunit gene *ureB* expression is upregulated following attachment to epithelial cells

(Johnson et al. 2012), as well as operons within the *cag*PAI (Sharma et al. 2010). Experiments by Kim et al. (2004) revealed that *H. pylori* was not only upregulating 22 genes upon contact with AGS cells, but also down regulating 21 other genes. The genes analyzed in this study included genes encoding outer membrane proteins, genes related to transcription and translation, genes important for transport and metabolic functions, and genes encoded for by the *cag*PAI. Furthermore, Bhattacharya et al. (2016) found that *HP0102*, a highly conserved gene with roles in chemotaxis, was significantly upregulated in several strains of *H. pylori* after infection. They also demonstrated that mutant strains with a deletion of the *HP0102* gene exhibited low acid-escape response and poor colonization efficacy, indicating the importance of the expression of this gene during colonization. In our previous study (Horridge et al. 2017), we found that *H. pylori* that were attached to AGS cells during infection assays possessed higher levels of *oipA* (Outer Inflammatory Protein A), and that strains producing viable OipA proteins were able to adhere to AGS cells at a significantly higher rate than strains that were *oipA* phase off, and thus could not express OipA.

Comprehensively, these studies provide compelling evidence illustrating that *H. pylori* alters the expression of numerous genes upon contact with gastric epithelial cells in manners assumed to be advantageous to residence upon a gastric epithelial cell (Johnson et al. 2012). There are three hypotheses aimed at explaining the contact dependent nature of *H. pylori* gene expression (Johnson et al. 2012). The first postulates that specific factors, such as ions and peptides secreted by epithelial cells are sensed by the adherent *H. pylori*. Alternatively, if those specific factors are bound or internalized by epithelial cells, the overall concentration of these factors are reduced and this reduced concentration may be sensed by the adherent *H. pylori*. The second hypothesis proposes that alterations in gene expression are triggered by the binding of *H.*

*pylori* to epithelial cells in ways that are advantageous to *H. pylori* survival, such as possible increased adhesion to epithelial cells to avoid being washed away during the regular shedding of the lining of the gastric mucosa. Finally, it has been posited that bacterial metabolism and growth are stimulated by uptake of factors released by epithelial cells. Johnson et al. (2012) found that contact with epithelial cells can promote bacterial replication and the formation of microcolonies though the acquisition of iron (Tan et al. 2011) and other nutrients from host epithelial cells. Additionally, they suggest that *H. pylori* cells undergo changes in physiology and morphology upon contact with host epithelial cells, such as the formation of pilus-like structure at the bacterial-host cell interface (Johnson et al. 2012).

Bacteria are able to acquire nutrients from infected epithelial cells, and this uptake of nutrients facilitates growth of adherent bacteria. Bacterial iron acquisition has been studied extensively due to its importance in understanding pathogenesis and survival (Tan et al. 2011, Johnson et al. 2012). Iron is critical because it is a necessary component of a range of cellular processes, including DNA replication, energy generation, and protection against oxidative stress. (Skaar 2010).

**The *Helicobacter pylori* Two-Component System ArsRS**

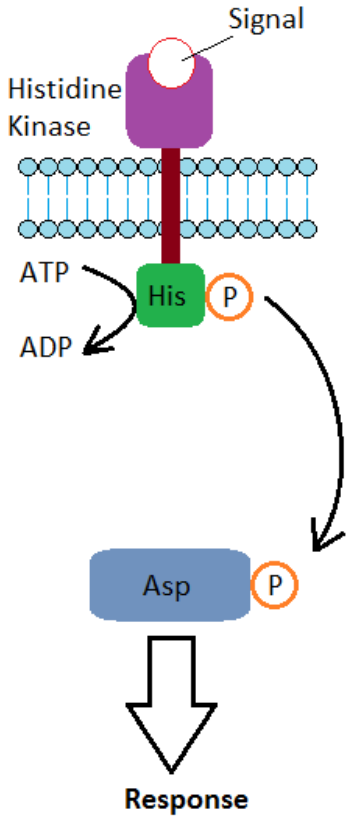


*Figure 1:* ***Two-component system as adapted from Bretl et al. 2011****. In the ArsRS two-component system, the sensory histidine kinase ArsS is activated and autophosphorylated in acidic environments, which in turn, phosphorylates the cognate response regulator ArsS.*

*H. pylori* uses various two-component systems to sense its environment and respond to a variety of stressors. Two-component systems are generally comprised of two proteins: an integral membrane protein, a sensory histidine kinase, and a cytoplasmic response regulator protein (Parkinson 1993). Communication occurs through a sequence of phosphorylation events in response to environmental conditions (Perraud et al. 1999). Although the ammonia-producing enzyme urease is a major contributor to the survival of *Helicobacter pylori* in its acidic environment, the two-component system ArsRS is thought to have a considerable role in survival, and acid acclimation beyond its role in controlling the expression of the urease pathway. Acid acclimation is the capability of *H. pylori* to maintain periplasmic pH near

neutrality under acidic conditions in order to maintain the cytoplasmic pH at physiological levels (Scott et al. 2007). ArsRS (previously designated HP0166-HP0165), short for *a*cid *r*esponsive *s*ignaling, is proposed to play an essential role in controlling acid responsive gene regulation during the colonization of and subsequent persistence in its host. ArsRS consists of an OmpR-family response regulator ArsR and the cognate histidine kinase ArsS, which is autophosphorylated in response to acidic environments (Pflock et al. 2006, Wen et al. 2007). The operon encoding this TCS is autoregulatory, thus the expression of ArsRS is increased under acidic conditions. The phosphoryl group of the phosphorylated histidine of ArsS is subsequently transferred to a highly conserved aspartic acid in the cognate response regulator, ArsR which then binds to regions overlapping promoters in the *ureAB* and *ureIEFGH* operons encoding for the enzyme urease (UreAB) and the urea transport pathway (UreIEFGH), two components essential for the incorporation of the metal cofactor $Ni^{2+}$ into the urease enzyme and thereby the synthesis of an active urease enzyme (Pflock 2005). By interacting with the promoters of these two operons, ArsRS controls acid-induced transcription of the machinery that plays a major role in maintaining homeostatic pH values in the face of gastric acidity. In addition, ArsRS is involved in acid responsive transcriptional regulation for other ammonia producing genes, such as *amiE*, *amiF* (Pflock 2005, Jones et al. 2018). *amiE* and *amiF*, which both encode amidases, are significantly upregulated in response to *H. pylori* exposure to acidic environments and are suggested to play a role in acid resistance (Bury-Mone et al. 2003, Jones et al. 2018).

Previous studies have shown that both components in the two-component system ArsRS are critical to *H. pylori* colonization. *H. pylori* with an inactivated histidine kinase ArsS is incapable of colonizing a mouse model of infection (Panthel et al. 2003). Additionally, ArsRS has been recently identified as a key part of *H. pylori* biofilm formation (Servetas et al. 2016),

although biofilm formation and roles in pathogenesis are currently unknown (Hathroubi et al. 2018). ArsRS is thought to be essential for growth and may have distinct functions in *H. pylori* survival and colonization (Beier & Frank 2000, Jones et al. 2018).

**Two-Component System CrdRS**

The two-component system CrdRS, also known as HP1364/HP1365, consists of the sensor histidine kinase CrdS and the response regulator CrdR. CrdRS is necessary for *H. pylori* $Cu^{++}$ resistance and has been hypothesized to detect and respond to $Cu^{++}$ concentration alterations in its environment (Waidner et al. 2005). $Cu^{++}$ functions as cofactors for electron transport, oxidases, and hydroxylases in bacterial metabolism (Argüello et al. 2013). Because of its various functions in maintaining homeostasis, proper $Cu^{++}$ metabolism is crucial for bacteria survival and required for colonization (Mobley 2001, Waidner et al. 2005).

Although copper is essential for survival, mechanisms to maintain $Cu^{++}$ ions below toxic concentration levels are critical. $Cu^{++}$ ions catalyze Fenton-like reactions that generate toxic hydroxyl radicals (Waidner et al. 2005). *H. pylori* maintains cytoplasmic copper concentration via the P-type ATPase, CopA. which facilitates the transport of copper ions out of the cytoplasm and into the periplasm (Ge & Taylor 1996). Additionally, copper resistance determinants CrdA, CrdB, CzcB, and CzcA contribute to copper resistance by forming a Czc-like metal export system to detoxify accumulating copper ions (Waidner et al. 2002). Interestingly, the response regulator CrdR is required for the transcriptional regulation of *crdA*, a copper resistance determinant (Waidner et al. 2005). RNA profiling has revealed that transcription of CrdA is significantly upregulated by changes in environmental copper concentrations (Waidner et al. 2002). The Crd system of *H. pylori*, which is orthologous to the Cus system in *E. coli*, is

proposed to transport copper ions from the periplasm to the outer membrane of the cell (Franke et al. 2003).

The CrdRS two-component system has also been shown to respond to nitric oxide (NO) stress and facilitate transcriptional changes in several genes. Under conditions of NO stress, *crdA* is upregulated significantly, along with *fecA*, an iron(III) dicitrate transport protein, proteins involved in electron transfer, DNA repair enzymes, and proteins involved in the flagellar system (Hung et al. 2015). Given its multiple roles in metal ion homeostasis and chelation, it is hypothesized that the CrdRS two-component system is involved in responding to and counteracting host innate immunity mediated by NO (Flint et al. 2016).

**Two-Component System FlgRS**

The two-component system FlgRS (previously designated HP0703-HP0244) is comprised of the sensory histidine kinase FlgS and its cognate response regulator FlgR. Unlike the two previously discussed two-component systems ArsRS and CrdRS, both of which possess their sensory kinases as integral membrane proteins, the histidine kinase FlgS is cytoplasmically localized (Kao et al. 2016). FlgS is the only known cytoplasmic histidine kinase of *H. pylori* (Wen et al. 2008) and is known to mediate flagellar gene expression and motility via its response regulator FlgR, an essential virulence factor (Niehus et al. 2004).

In order to survive the acidic conditions of its biological habitat, *H. pylori* must be able to regulate cytoplasmic pH levels. Previously, FlgS was thought to only regulate flagellar motility through its cognate response regulator FlgR. A 2008 study by Wen et al. indicated that FlgS is also responsible for sensing and regulating gene expression in response to a decrease in cytoplasmic pH by mediating the expression of acid acclimation genes including *ureA*, *ureB*, *ureI*, *ureF*, *amiE*, *rocF*, and *ansB* (Wen et al. 2003, Wen et al. 2008). Their results suggest that

FlgS is a cytoplasmic pH-responsive histidine kinase that has multiple functions in *H. pylori* survival and virulence (Dunne et al. 2014).

**Outer Membrane Inflammatory Protein A**

*O*uter *I*nflammatory *P*rotein *A*, or OipA, is an important virulence factor of *H. pylori* and is associated with increased mucosal interleukin-8 (IL-8) secretion and subsequent inflammation (Yamaoka et al. 2000). OipA is a part of the Hop (Helicobacter Outer Proteins) family of outer membrane proteins. Several Hop proteins are involved with adherence and binding to the gastric epithelium and some undergo phase variation as a means to regulate protein production (Oleastro & Ménard 2013, Harvey et al. 2015, Yamaoka et al. 2018). OipA is encoded for by the *oipA* gene (previously known as *hopH* or *HP0638*) and gene expression is subject to phase variation regulated by the slipped-strand mispairing mechanism based on a hypermutable CT dinucleotide repeat motif section in the 5' region of the *oipA* gene (phase on results in functional OipA while phase off results in nonfunctional OipA) (Yamaoka et al. 2000, Horridge et al. 2017). The exact mechanisms by which OipA induces inflammation are currently unknown due to a dependency upon *cag*PAI pathways. However, it is hypothesized that binding sites for the transcription factor NF-κB, activator protein 1 (AP1), and interferon-stimulated responsive element (ISRE)-like element in the IL-8 promoter region engage in regulation of IL-8 transcription in gastric epithelial cells infected with *H. pylori* and this process is mediated by the *cag*PAI oncoprotein effector, CagA (Yamaoka 2010). In addition, Ando et al. (2002) found that, despite *oipA* being highly conserved across all *H. pylori* strains, the vast majority (>96%) of cagPAI positive strains contained *oipA* in its "phase on" status, although no *cag*PAI negative strains contained the gene in its phase on status.

Bacterial genomes are relatively small, and it is uncommon for them to carry extraneous genes that are not expressed or utilized. OipA and the *cag*PAI both have roles in inducing inflammatory cytokines such as IL-8 by host gastric epithelial cells, likely by a mechanism involving the transcription factor NF-κB. The explicit mechanisms and interactions are yet to be fully understood (Matsuo et al. 2017). This study aims to understand the relationship between *oipA* and the *cag*PAI, why *oipA* is highly conserved even in *cag*PAI negative strains in which the protein is always in its phase off status.

OipA assumes a role in adhering to host gastric epithelium cells and colonization, as well as stimulating inflammation (Horridge et al., 2017). The host cell receptor for OipA binding is currently unknown but has been hypothesized to be in the integrin family. Integrins are typically expressed at the basolateral side of polarized epithelial cells and may be accessed by *H. pylori* upon disruption of intercellular adhesions by Cag proteins (Posselt et al. 2013). Studies have shown that a functional, phase on OipA is strongly correlated with increased risk for ulceration and gastric cancer and positively correlated with the presence of the *cag*PAI, making OipA a protein of interest for medical research (Chen et al. 2002, Ando et al. 2002). Furthermore, functional OipA is significantly linked high *H. pylori* density in the stomachs of infected individuals (Liu et al. 2013).

**Sialic Acid Binding Adhesin**

*S*ialic *A*cid *B*inding *A*dhesin, or SabA, is another outer membrane protein (part of the Hop family of proteins) and adhesin of *H. pylori*. SabA plays significant roles in facilitating *H. pylori* pathogenesis through its adhesive properties. The sialyl-Lewis X/A antigens (sLeX and sLeA) on the surface of gastric epithelial cells function as receptors for SabA (Odenbreit et al. 2009). In a healthy gastric mucosa, sialylated glycoconjugates are typically sparse, but are replaced by

naturally produced Lewis antigens (sialylated glycans sLeX and sLeA) upon *H. pylori* infection induced inflammation (Yamaoka 2008, *SabA*). Increased expression of sialylated antigens are reported in gastric cancer (Sakamoto et al. 1989). Recently, it was discovered that SabA also binds to gangliosides in the human stomach, adding to its credentials as a major candidate for further studies (Benktander et al. 2018).

Several studies have shown that the regulation of SabA is complex. Like the other *hop* genes, *sabA* is thought to be regulated by phase variation through alterations in the poly(T) tract in the promoter region of the gene (Harvey et al. 2014) and dinucleotide repeats near the 5' end of the coding region (Goodwin et al. 2008). Moreover, the two-component system ArsRS has been shown to be involved in the repression of *sabA* transcription *in vitro* under acidic conditions (Goodwin et al. 2008). It is speculated that this adhesin is downregulated to reduce bacterial adherence to gastric mucosa to prevent bacterial loss along with the shedding epithelial cells, or to avoid attachment to receptors on mucins in acidic pH conditions in order to promote motility and swim to and more neutral pH environment (Arnqvist 2016).

**Blood Group Antigen Binding Adhesin A**

*B*lood *g*roup *A*ntigen *B*inding *A*dhesin, or BabA, is an adhesin that has been well characterized in its ability to bind to blood antigens. Fucosylated ABO blood group antigens ($Le^b$, $ALe^b$, and $BLe^b$) are recognized and bound by BabA (Ilver et al. 1998). Like *sabA* and *oipA*, *babA* is typically regulated through phase variation via slipped-strand mispairing, although this is not always the case as some alleles lack the hyper-mutable polynucleotide repeats. The formation of *babA* and *babB* chimeras (*babA/B* and *babB/A)* is thought to play a role in translational regulation by inducing differential binding affinities for different blood group antigens (Yamaoka 2008, *BabA*).

**Objectives**

In every human stomach colonized with *Helicobacter pylori*, two populations of *H. pylori* exist; a major population motile in the gastric mucus overlying the epithelium and a secondary minor population adhered to epithelial cell surfaces (Kusters et al. 2006). The outer membrane proteins expressed by *H. pylori* facilitate colonization of the host gastric epithelial cells by serving as adhesins; binding proteins that interact with host cell receptors to facilitate bacterial adherence. Examination of the behavior of these genes upon contact with host cells potentially provide a detailed insight into the contact-dependent behavior of *H. pylori*. One objective of this project was to document and quantify changes in *oipA* expression in *H. pylori* strain 26695 to determine whether contact with AGS (human gastric adenocarcinoma) cells influenced bacterial gene expression. Another aim of this project was to examine the role of OipA in *H. pylori* adherence to human gastric cells *in vitro* using AGS cells and *H. pylori* strains 26695 and J68 as well as documenting the role of OipA in IL-8 secretion by gastric epithelial cells. While strain 26695 possesses the *cag*PAI and an *oipA* allele that is phase on and thus functional, *H. pylori* strain J68 is a clinical isolate that naturally lacks the *cag*PAI and possesses a phase off allele of *oipA*. We experimentally altered the *oipA* phase status of each strain, to examine the effects of the presence and absence of a functional OipA protein on *H. pylori*'s ability to adhere to AGS cells. Another objective of the current study was to ascertain if bacterial contact with human AGS cells changes the expression of other *H. pylori* outer membrane protein adhesins (SabA and BabA). The final objective was to determine if changes in expression are facilitated by any of the three complete two-component systems, ArsRS, CrdRS, and FlgRS, possessed by *H. pylori* by using a triple deletion strain 109' $\Delta^3$ with all three systems genetically ablated.

We hypothesized that the expression of the outer membrane proteins of interest (OipA, SabA, and BabA) would be expressed at different levels in *H. pylori* cells attached to AGS cells

vs those cells that remained unattached. Furthermore, we hypothesized that differences in gene expression are due to the signal transduction mechanisms flowing through one the two-component systems used by *H. pylori* as a means to sense and respond to environmental changes and stressors. We hypothesized that ArsRS was likely to be the two-component system that had the greatest effect on sensing cell-cell contact with the host AGS cell due to its ability to sense changes in pH and its location on the cytoplasmic membrane of the cell, thereby inducing change in gene expression of the various outer membrane proteins.

## Materials and Methods

### H. pylori *culture*

*H. pylori* strains were cultured on tryptic soy agar II with 5% sheep's blood, also known as blood agar plates or BAPs, and incubated in humidified ambient air/5% $CO_2$ at 37°C for 24-72 hours.

### H. pylori *mutant strains*

To analyze the expression of *oipA* in *H. pylori* cells dependent on attachment to AGS cells and the effect of OipA on adherence to AGS cells, a mutant strain where OipA was experimentally turned off was used. A phase "on" 26695 strain producing a functional OipA was used as a comparison. Additionally, *H. pylori* J68, a strain that naturally has *oipA* turned phase off, was experimentally turned phase "on" to produce a functional OipA protein (Horridge et al. 2017). As each of the mutant strains created possessed an ablation of the gene *rdxA*, the control strain used to analyze the gene expression of outer membrane proteins SabA, BabA, and OipA, *H. pylori* 26695/*ΔrdxA*, has an *rdxA* deletion, rendering it metronidazole (Mtz) resistant as well (Loh et al. 2011). The experimental strain of *H. pylori*, designated 109' $\Delta^3$, was created from the

26695/*ΔrdxA* strain and has in-frame deletions in the genome of the genes encoding each of the three histidine kinases: ArsS, CrdS, and FlgS. This strain was created by Dr. Forsyth (Forsyth, Unpublished).

***Cloning of metronidazole resistant plasmids***

To create *oipA* mutant *H. pylori* strains, we used an antibiotic counter-selection method designed by Mark McClain at Vanderbilt University Medical Center (Loh et al. 2011) to introduce a specific mutation into a gene without leaving residual antibiotic resistance genes. The traditional method of inserting mutations includes attaching the desired modification to an antibiotic resistance gene, risking the likelihood of undesired phenotypic changes that may occur as a result of the presence of the antibiotic resistance gene. First, we created a metronidazole resistant (MtzR) strain of the *oipA* "on" strain of *H. pylori* 26695 and the *oipA* "off" strain J68. All plasmids and *H. pylori* mutants involving the strain J68 were created by my colleague in the Forsyth lab, Allison Begley (Horridge et al. 2017).

Metronidazole is a pro-drug that becomes bactericidal after being reduced by a bacterial enzyme in the cytoplasm (Mura et al. 2011). In *H. pylori*, the RdxA enzyme does this reduction. Therefore, deletion of the internal portion of *rdxA* renders it non-functional and generates an *H. pylori* metronidazole resistant ($Mtz^R$) mutant. We accomplished this by amplifying a 1556 bp amplicon containing the full length *rdxA* gene (HP0954) using HP0955 Fwd and HP0953 Rev primers (Table 1) in a standard PCR. All primers were synthesized by Integrated DNA Technologies unless otherwise stated. The amplicon was cloned into a TOPO TA cloning vector pCR4 (Invitrogen) according to manufacturer's protocol. The resulting plasmids isolated from *E. coli* were named p*rdxa* (Table 2). Inverse PCR (iPCR) was performed on the p*rdxa* plasmids to delete a 390bp internal section of *rdxA* using iPCR *rdxA* Fwd and Rev primers (Table 1) with a

5’ phosphorylation to aid in ligation. The product was examined for size on an agarose gel, and the PCR product was purified using a spin column kit for PCR Purification (IBI) and digested with *Dpn*I restriction endonuclease to destroy the p*rdxA* template, and then ligated together using the T4 DNA ligase (Quick Ligation Kit - New England Biolabs) and then transformed together into *E. coli* DH5α competent cells. Selection of ampicillin resistance was used to isolate clones containing the mutant plasmid.

Clones were screened by purifying and isolating plasmids. Plasmids were screened with PCR using the HP0955 Fwd and HP0953 Rev primers and compared to the amplicons generated from p*rdxA*. Successful deletions were identified by a decreased size of 390bp and confirmed with sequencing reactions using the Big Dye Sequencing Kit (Applied Biosystems), and the successful plasmid was named pΔ*rdxA* (Table 2). The Δ*rdxA* plasmid was used in the natural transformation of the *H. pylori* strains 26695 and J68. Colonies growing on 5µg metronidazole/mL SFBB 10% NCS plates were selected, and the mutation was confirmed via PCR on extracted gDNA using HP0955 Fwd and HP0953 Rev primers (Table 1) and agarose gel electrophoresis in order to confirm the deletion. Additionally, the deletion was further confirmed by Big Dye (Invitrogen) experiments using the primer HP0955 Fwd, and strains were named 26695/Δ*rdxA* and J68/Δ*rdxA* (Table 3).

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

A chloramphenicol (Cm) resistance gene (CAT or chloramphenicol acetyl transferase) with an intact version of *rdxA* as a cassette was inserted into the *oipA* gene of strains 26695 and J68. This cassette insertion served as a “knock-out” null mutation of the *oipA* gene, preventing a functional gene product. Additionally, the functional copy of *rdxA* in the *oipA* gene returns Mtz sensitivity, while the CAT gene adds Cm resistance. Therefore, when the mutant Δ*oipA* plasmid

is naturally transformed into strains of *H. pylori* with both the *rdxA* deletion and the Cat-rdxA cassette, the Δ*oipA* allele can recombine to replace the *oipA::CAT-rdxA* cassette, and the Δ*rdxA* mutant can be selected for using Mtz (5 μg/ml) and resistant colonies screened via PCR using universal Fwd and Rev primers based on *H. pylori* sequenced genomes (Table 1). The resulting amplicon of ~2300bp was cloned into the TOPO TA cloning vector, pCR4 (Invitrogen), and the resulting plasmid was named pOipA (Table 2). The GeneArt Site-Directed Mutagenesis System (Invitrogen) was used in accordance to manufacturer's protocol to insert a *Bam*HI site into the cloned allele of *oipA* using *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 designated pOipA.*Bam*HI (Table 2). *oipA* and the pCR4 vector do not contain a naturally occurring *Bam*HI site. Once the *Bam*HI site was inserted, plasmids were cut at the *Bam*HI site to allow for the cloning of selecTable markers chloramphenicol acetyl transferase (CAT) and *rdxA*. The CAT-rdxA cassette was isolated as a BamHI fragment from pMM672 (Loh et al. 2011) (Table 2), a gift from Drs. Mark McClain and Timothy Cover of Vanderbilt University Medical Center. A digestion reaction of the plasmid was performed on an agarose gel and the CAT-*rdxA* cassette was isolated via gel purification (IBI). Once isolated, the cassette was cloned into pOipA.*Bam*HI using T4 ligase, enabled by the presence of *Bam*HI sites on the pOipA.*Bam*HI plasmid and the CAT-*rdxA* cassette. The new plasmid pOipA::CAT-*rdxA* (Table 2) was transformed into E. coli and selected for using chloramphenicol resistance. It was then isolated and purified. The presence of the cassette was confirmed using a PCR with the *oipA* universal primers and comparing mutants to controls (Table 1). We further confirmed this with sequencing.

Finally, the *oipA*::CAT-*rdxA* plasmid was naturally transformed into the desired Δ*rdxA* strains (*H. pylori* strains 26695 and J68 Δ*rdxA*), selected for using SFBB plates with 10µg Cm/mL, confirmed using PCR of the *oipA* locus, and sequenced (Table 3). The strains (26695, J68, and J75 Δ*rdxA*/*oipA*::CAT-*rdxA*) are $Mtz^S$ and $Cm^R$, and served as intermediates for the isolation of markerless mutants and *oipA* knockouts. Cloning of *oipA* mutant plasmids *oipA* mutagenic oligos were designed specific to each strain in order to alter the number o CT dinucleotide repeats in the 5' region of *oipA* to turn the gene phase off in the naturally phase on *H. pylori* strain 26695, and phase on in the naturally phase off strain J68. We did this by deleting one CT dinucleotide repeat in 26695, reducing the CT repeats from 6 to 5. This resulted in a frame shift in *oipA* from phase on to phase off. Adding one CT in the J68 *oipA* allele increased the number of repeats from 10 to 11, turning *oipA* phase on. Sequences of the wild type and *oipA* in both strains and the corresponding amino acid sequence are depicted in Figure 3. Mutagenesis was performed using the 26695.*oipA*OFF and J68.*oipA*ON mutagenic primers (Table 1), and clones were screened via sequencing. Plasmids were designated p26695.*oipA*OFF and PJ68.*oipA*ON (Table 2). Clones were naturally transformed into the appropriate *H. pylori* strain Δ*rdxA*/ *oipA*::CAT-*rdxA*. Mutated *oipA* plasmids were able to recombine with the *oipA*::CAT-*rdxA* locus on the recipient chromosome, replacing the CAT-*rdxA* cassette. These replacement mutations were selected for using SFBB plates with 5µg metronidazole/mL. The replacement of the CAT-*rdxA* cassette leaves the strain with a defective *rdxA* locus due to the earlier deletion in a section distant from the *oipA* gene. The strains 26695 Δ*rdxA*/*oipA*OFF and J68 Δ*rdxA*/*oipA*ON (Table 3) are $Mtz^R/Cm^S$ and have a mutated *oipA* gene with no antibiotic resistance genes left behind in that region.

### *AGS cell culture*

AGS cells were a gift from Timothy Cover of Vanderbilt University Medical Center. AGS cells, a cell line from a human gastric adenocarcinoma, was grown in RPMI Medium 1640 and supplemented with HEPES (10 mM) (Gibco by Life Technologies) and 10% fetal bovine serum (FBS) or newborn calf serum (NCS). Penicillin and streptomycin were added as well. Cells were cultured in an ambient air/5% $CO_2$ incubator at 37°C for 24-96 hours in T-25 or T-75 flasks (PRIMARIA).

### *Post-Attachment* **oipA** *Expression*

Infection assays were performed to determine the mRNA levels of *oipA* in *H. pylori* that had adhered to AGS cells compared to unattached *H. pylori* in the supernatant and naive *H. pylori* cells that had no exposure to AGS cells, but were maintained in identical tissue culture medium. AGS cells were regularly cultured as stated above. For infection assays, each well of a 6-well tissue culture plate (CytoOne) was seeded with 2.5 x $10^5$ AGS cells per well. After incubating in ambient air/5% $CO_2$ at 37°C for 24 hours to reach 5x$10^5$ AGS cells per well, each well was washed three times with sterile RPMI supplemented with 10% FBS and HEPES (10mM) with no antibiotics. AGS cells were infected with *H. pylori* at a multiplicity of infection of 100:1 (*H. pylori*: AGS), under standard conditions and 50 rpm shaking for 5 hours. At 5 hours, supernatant was harvested for the non-adherent *H. pylori*. Wells were then washed three times with PBS to remove non-adherent *H. pylori* and the AGS monolayer with attached *H. pylori* cells was lysed with 0.1% Saponin in PBS for 15 minutes at standard conditions and shaking at 100 RPM in room temperature. Saponin lyses AGS cells, but *H. pylori* cells are refractory to saponin. Lysates were collected to analyze *H. pylori* that had adhered to AGS cells. Samples were centrifuged at 4360 rpm for 10 minutes at 4°C. Supernatants were discarded and

*H. pylori* cell pellets were suspended in 1 mL RNAzol RT (Molecular Research Center, Inc.) in preparation for RNA extraction.

***Attachment Assay***

Attachment assays were performed in order to measure *H. pylori* adherence ability to AGS cells. AGS cells were cultured in T-75 flasks with sterile RMPI medium supplemented with 10% fetal bovine serum (FBS) or newborn calf serum (NCS), as well as HEPES (10mM) (Gibco) with penicillin and streptomycin. $2.5x10^5$ AGS cells were seeded in each well of a 6-well tissue culture plates (Cyto One) and grown for 24 hours to reach $5x10^5$ AGS cells per well. Wells were washed three time with RMPI supplemented with 10% FBS, without penicillin and streptomycin. Infections were performed at a multiplicity of infection of 100:1 (*H. pylori* to AGS) and incubated at standard conditions, shaking at 50 rpm for five hours. Supernatant was collected for Enzyme-Linked Immunosorbent Assay (ELISA) experiments to measure IL-8 production. Wells were washed three times with PBS and AGS cells were lysed with 0.1% Saponin in PBS at standard conditions and shaking for 15 minutes. Lysates were serially diluted in PBS to $10^{-7}$ and placed in triplicate on BAPs in 20µL spots, then placed in standard conditions for 5 days. Colony forming units (CFU) were then counted and bacterial titer was calculated.

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

To quantify IL-8 production by AGS cells, Human IL-8 ELISA MAXDeluxe assays (BioLegend) were used according to manufacturer's protocol. Adhesion assay culture media was 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. Wells were washed four times with wash buffer and assay diluent A was added and incubated for 1 hour. The plate was washed and diluted sample supernatants were added, and then incubated for 2 hours at room temperature

(RT), shaking. After washing, Avidin-HRP was added and incubated for 30 minutes at RT shaking. This was followed by 5 washes with wash buffer and then followed by Substrate Solution C. The plate was then allowed to incubate in the dark at RT for 15 minutes. Stop solution was added to the plate, and the plate was analyzed at an absorbance of 450nm on a Bio-Rad iMark Microplate Reader.

### ***Post-Attachment* sabA *and* babA *Expression***

Infection assays were performed to determine the expression of *sabA* and *babA* in *H. pylori* that had adhered to AGS cells compared to unattached *H. pylori* in the supernatant and to naive *H. pylori* cells that had no exposure to AGS cells. AGS cells were regularly cultured as stated above. For infection assays, T-75 flasks were each seeded with $6.7 \times 10^6$ AGS cells. Infection assays were performed in in three independent experiments. After incubating in ambient air/5% $CO_2$ at 37°C for 24 hours to reach $1.34 \times 10^7$ AGS cells per flask, each flask was washed three times with sterile RPMI supplemented with 10% FBS and HEPES (10mM) with no antibiotics. AGS cells were infected with *H. pylori* at a multiplicity of infection of 100:1 (*H. pylori*: AGS), at standard conditions and shaking for 5 hours at 50 rpm. Separate flasks without AGS cells were included in each experiment and treated equally for the naive *H. pylori* cells. At 5 hours, supernatant from flasks containing AGS cells and flasks devoid of AGS cells were harvested. AGS monolayers and attached *H. pylori* cells were then washed three times with PBS to remove non-adherent *H. pylori* and AGS cells were lysed with 0.1% Saponin in PBS for 15 minutes at standard conditions and shaking at 100 RPM in room temperature. Lysates were collected to analyze *H. pylori* that had adhered to AGS cells. Samples were centrifuged at 4360 rpm for 10 minutes at 4°C. Supernatants were discarded and cell pellets were suspended in 1 mL RNAzol RT (Molecular Research Center, Inc.) in preparation for RNA extraction.

### *AGS Cell Conditioned Media Assay*

To determine if *sabA* was being repressed by soluble molecules secreted by AGS cells into the RPMI media, AGS cells were cultured as stated above in T-75 flasks up to 30-40% confluency. AGS cells were then washed three times using RPMI media without penicillin and streptomycin. Cell cultures were then incubated in 20 mL of RPMI media (no penicillin and streptomycin) for 24 hours in standard conditions. As a control group, a flask with no AGS cells was treated equally. After 24 hours, media was removed from flasks and filtered into new T-75 flasks. $1.34 \times 10^7$ *H. pylori* was added per flask, and both flasks were incubated in standard conditions, shaking for 5 hours at 50 rpm. *H. pylori* cells were collected and centrifuged, then stored in 1 mL of RNAzol RT.

### *RNA Extraction*

RNA was extracted from samples suspended in 1 mL RNAzol RT (Molecular Research Center, Inc.) according to the manufacturer's protocol. Samples were beadrupted for 45 seconds to disrupt cell membranes and to release RNA. After beadruption, each sample was incubated with sterile RNAse free water for 15 minutes at room temperature. Samples were centrifuged for 15 minutes at 12,000 x g. 1 mL of supernatant was added to 400mL of 75% EtOH and allowed to incubate for 10 minutes. Samples were then centrifuged at 10,000 x g for 8 minutes and supernatant was discarded. The samples were washed twice with 75% EtOH, and RNA pellets were air dried for 10-20 minutes. Pellets were resuspended in 30 μL of PCR grade $H_2O$. Samples were then placed on ice and RNA concentrations were quantified using a Nanodrop spectrophotometer.

### *cDNA synthesis*

RNA samples isolated during RNA extraction were used in cDNA synthesis. iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad) and molecular grade water were added to 1 μg of RNA in a final volume of 20 μL, and cDNA was synthesized using a thermocycler at the following temperature conditions: 25°C for 5 minutes, 42°C for 30 minutes, and 85°C for 5 minutes. Samples were stored in -20°C.

### *Real-Time Quantitative PCR (RT-qPCR) to Quantify Relative Gene Expression of Outer Membrane Proteins*

The mRNA level of *oipA* in adherent *H. pylori* was compared to the mRNA level of *oipA* in non-adherent *H. pylori* using RT-qPCR. *oipA* mRNA expression in both cases was compared to the housekeeping gene *ftsZ* and/or *gyrB* (negative control), encoding the cell division protein FtsZ and DNA gyrase subunit B, respectively. Taqman Gene Expression assays (Life Technologies) were performed on the Applied Biosystems StepOne apparatus. Assays were performed in triplicates for each gene for both adherent and nonadherent samples in accordance with the manufacturer's protocol using custom TaqManCustom Gene Expression assays (Thermo-Fisher), including the *oipA*_Taqman, *ftsZ*_Taqman, and *gyrB*_Taqman probes. Subsequent experiments were performed as stated above to compare the relative mRNA levels of *sabA* and *babA* in adherent, non-adherent, and naïve *H. pylori* cells using *sabA*.TaqMan, *babA*.TaqMan probes. Relative expression of genes was calculated and compared using the $2^{\Delta\Delta Ct}$ method as described by Livak and Schmittgen (2001) and processed using DataAssist software (Applied Biosystems).

## Results

### OipA Confers an Increased Host Adherence Phenotype

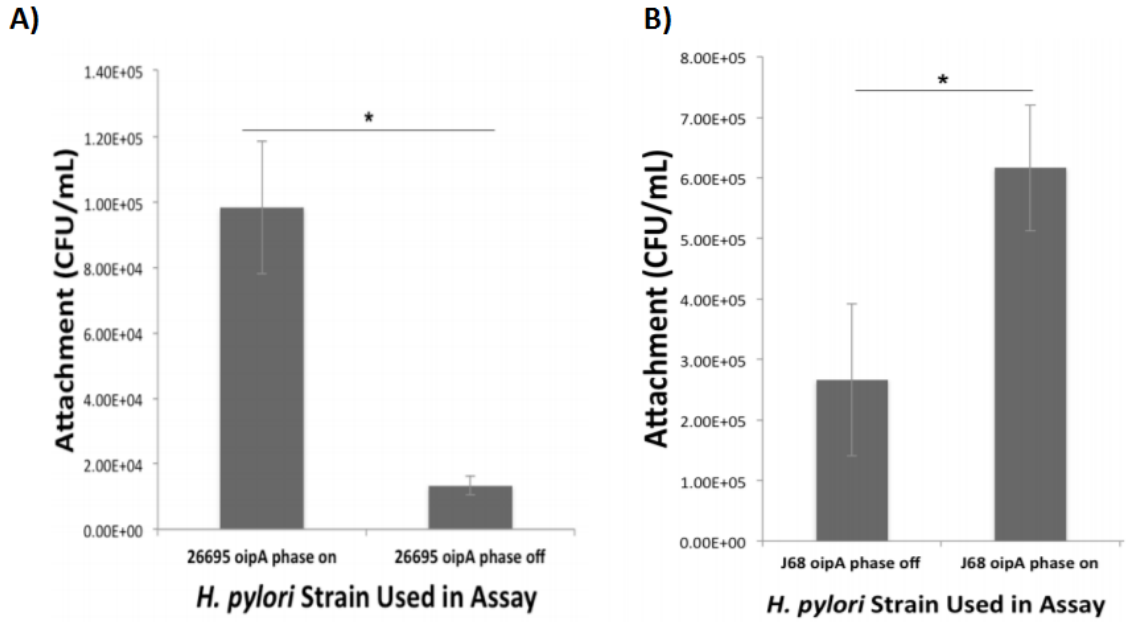


*Figure 2:* ***H. pylori possessing phase on alleles of oipA are more adherent to AGS cells.*** *(A) H. pylori attachment levels were significantly decreased in the 26695 oipA phase off mutant from ~1.0 x105 CFU/mL to less than 2.0 x 104 CFU/mL. (B) Attachment increased more than twofold when oipA was experimentally turned phase on in H. pylori strain J68 (* = p ≤ .05, n.s. = p > .05).*

We investigated the role of OipA in the ability *H. pylori* 26695 to adhere to gastric epithelial cells *in vitro* (Figure 2). *H. pylori* attachment levels were significantly decreased in the *oipA* phase off mutant from ~1.0 x$10^5$ CFU/mL to less than 2.0 x $10^4$ CFU/mL (Figure 2). Additionally, attachment increased more than twofold when *oipA* was experimentally turned phase on in *H. pylori* strain J68. These results suggest that OipA mediates attachment and that a functional OipA increases *H. pylori*'s ability to adhere to host epithelial cells.

### OipA and IL-8 Production

The differences in attachment capabilities between the *oipA* mutant strains and OipA's hypothesized role in inducing host inflammatory response led us to speculate that the results of the attachment assays may correlate with comparable changes in AGS cell IL-8 production. In

order to quantify IL-8 concentration, we collected supernatant from attachment assays and the undiluted cell-free conditioned media were used to conduct IL-8 ELISAs in technical triplicate. AGS cells infected with the *cag*PAI positive strain with *oipA* switched phase off secreted nearly 90% less IL-8 than AGS cells infected with *H. pylori* strain 26695 with *oipA* phase on (Figure 3A).

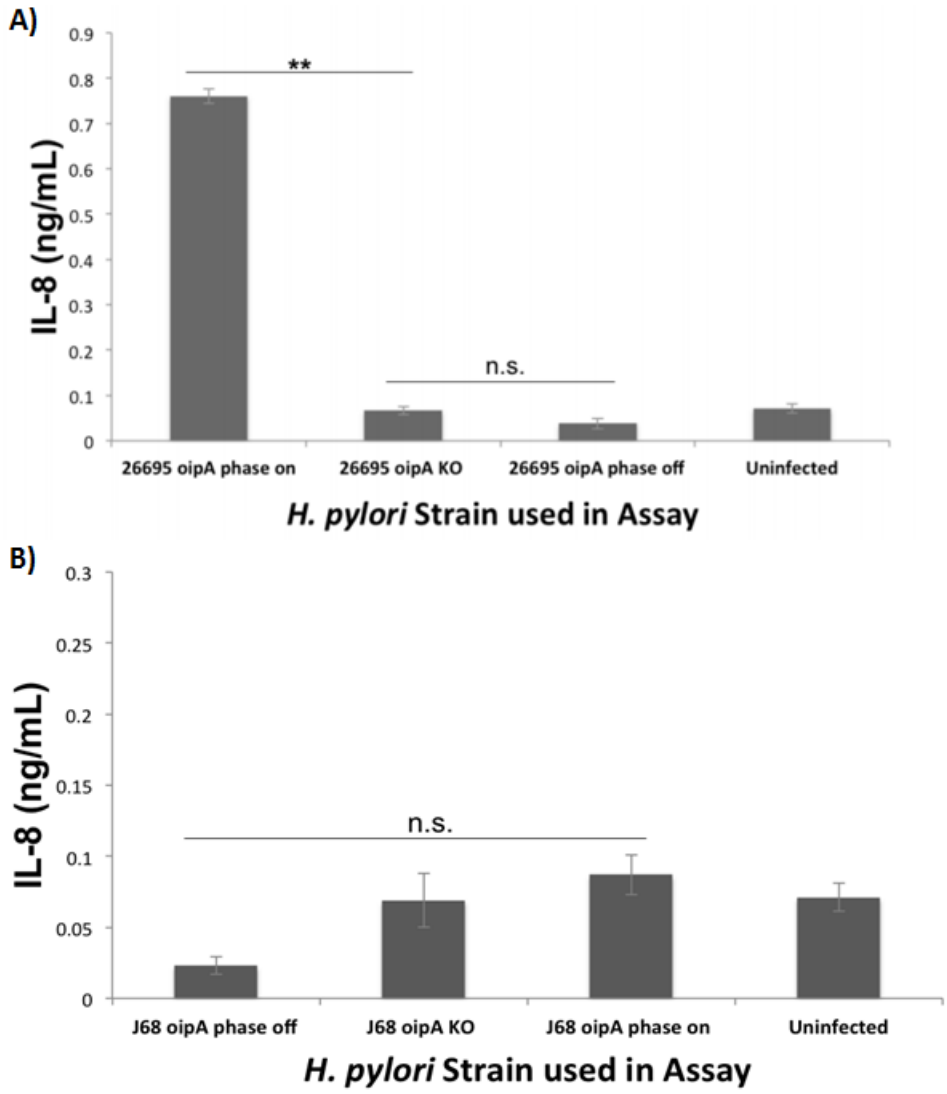


*Figure 3* ***OipA is Necessary but Not Sufficient for AGS Cell Secretion of IL-8. (****A) IL-8 concentrations in the supernatants of attachment assays using* H. pylori *strains 26695* oipA *phase on, KO, phase off, and uninfected AGS cells as a control group were quantified by ELISA.* H. pylori *strain 26695 with* oipA *turned phase off or knocked out (KO) is correlated with decreased adhesion (Fig. 2) and decreased AGS cell IL-8 production. (B) ELISAs revealed that IL-8 concentrations in the supernatants of attachment assays using* H. pylori *strains J68* oipA *phase off, KO, phase on, and uninfected cells were not significantly different. 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 ≤ .05, n.s. = p > .05).*

There was no significant difference in the concentration of IL-8 produced by AGS cells infected with strain 26695 with *oipA* knocked out by the CAT-*rdxA* cassette when compared to the 26695 strain with *oipA* turned phase off. The concentrations produced by these AGS cells were comparable to uninfected AGS cells (Figure 3A). Additionally, ELISA experiments revealed no significant difference in the IL-8 production by AGS cells infected with the *H. pylori* J68 *oipA* phase on mutant strain with the J68 *oipA* phase off strain, as well as the J68 *oipA* knockout strain and the IL-8 production from the uninfected AGS cells (Figure 3B). This suggests that *oipA* is necessary, but not sufficient to induce an inflammatory response.

### *oipA* mRNA Transcript Levels in Adherent vs. Non-adherent *H. pylori*

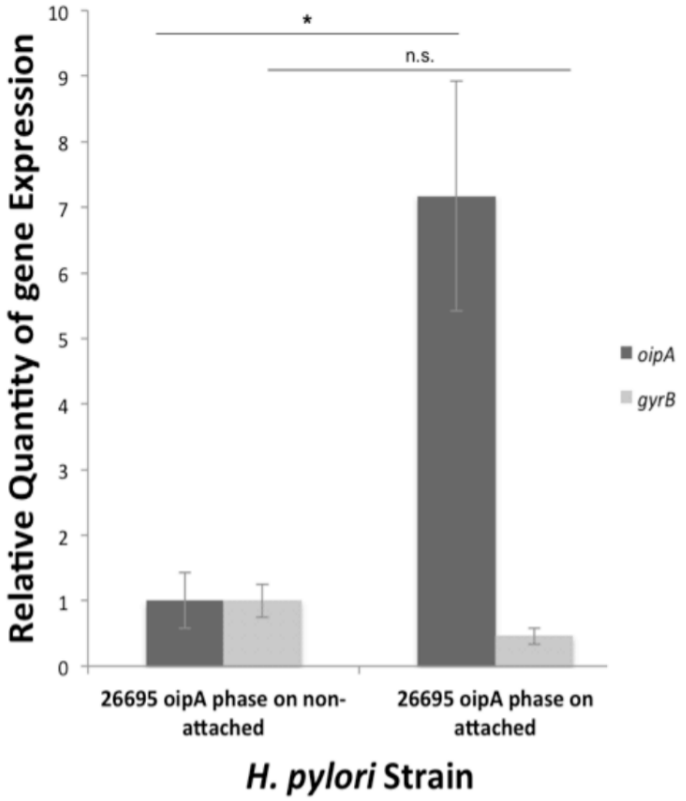


*Figure 4:* ***H. pylori 26695 attached to AGS cells has increased oipA mRNA.*** *AGS cell infection assays using* H. pylori *strain 26695 revealed that* H. pylori *upregulates oipA transcript seven-fold in AGS-attached cells after five*

*hours of infection compared to unattached cells, while gyrB transcript levels remained unchanged. 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 ≤ .05, n.s. = p > .05).*

Next, we asked if there was a difference between the amount of *oipA* mRNA transcript produced by *H. pylori* cells that had been incubated with and attached to AGS cells (adherent *H. pylori*), compared to *H. pylori* cells that were also incubated with host cells but did not adhere (non-adherent) (Figure 4). Using *H. pylori* strain 26695, AGS cell infection assays were performed as described. RNA was extracted from samples and cDNA was synthesized and used for RT-qPCR to quantify relative *oipA* expression, using *ftsZ* as a housekeeping/normalizing gene and *gyrB* as an unregulated control for comparison to *oipA*. This experiment revealed that adherent *H. pylori* cells possessed seven-fold more *oipA* than unattached cells in the same experiments, while *gyr*B transcript levels remained unchanged.

These findings suggest that there is selection occurring within the population of *H. pylori* that grants an adherence advantage to cells that produce higher levels of OipA. *H. pylori* cells naturally producing more OipA may be selected for due to an enhanced adherence ability, adhering to host cells at high rates to protect against harmful effects, such as the periodic shedding of the mucosal layer, while those expressing lower levels of *oipA* are not able to bind to host cells and thus remain free swimming and unattached. Alternatively, we hypothesize that *H. pylori* increases transcription of OipA in response to contact with AGS cells, suggesting a behavioral change dependent on contact with host gastric epithelial cells. This led us to consider potential changes in other outer membrane adhesins based on contact with AGS cells.

**Effects of *H. pylori* Contact with AGS Cells on Transcription of other Outer Membrane Proteins**

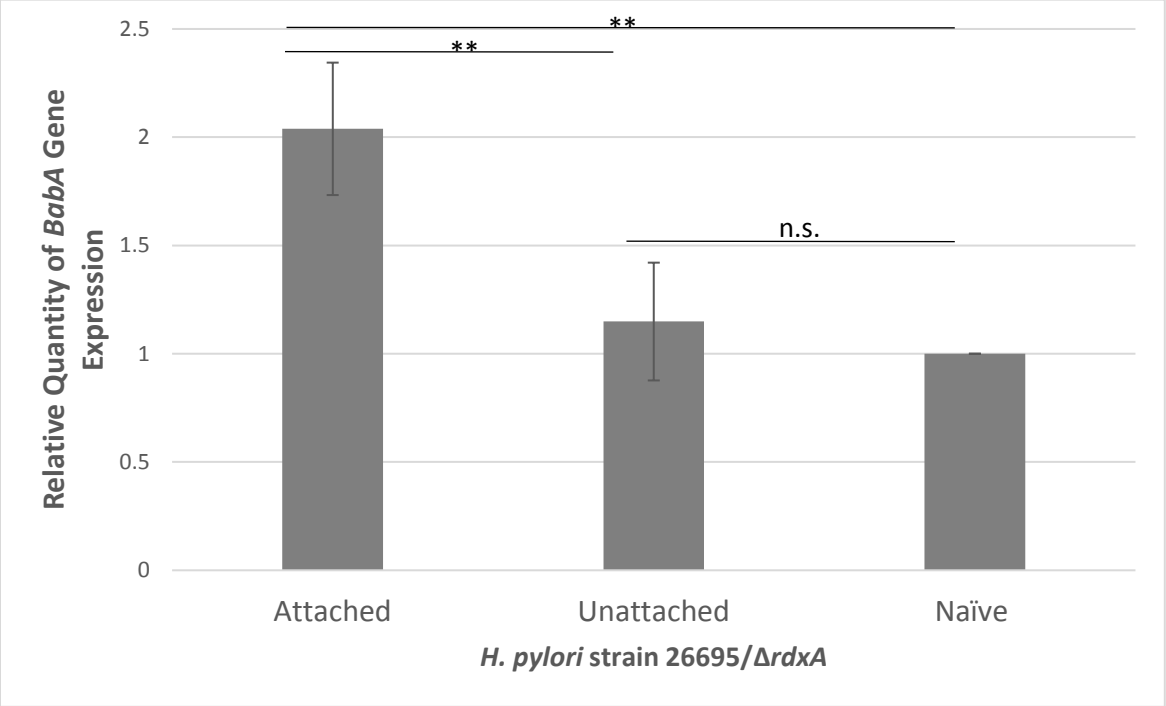


*Figure 5:* **H. pylori *cells attached to AGS cells express* babA *at higher levels than free-swimming* H. pylori *cells.*** *RT-qPCR experiments revealed that* babA *transcription is increased significantly in attached* H. pylori *cells when compared to unattached and naïve cells. The data shown here is representative of the results obtained in three independent experiments. Error bars show standard deviation. Statistics were calculated using a Welch's unpaired t-test of unequal variance (** = p ≤ .01, * = p ≤ .05, n.s. = p > .05).*

After determining that *oipA* mRNA levels are increased in adherent *H. pylori* cells, we next asked if the expression of other outer membrane proteins was affected by contact with AGS cells. Using the *H. pylori* strain 26695/*ΔrdxA*, we performed infection assays as above. RNA extraction was performed on the samples and the mRNA extracted was used to synthesize cDNA. The cDNA was used in RT-qPCR to quantify the relative expression levels of *sabA* and *babA*. RT-qPCR revealed that the expression of babA is increased nearly twofold upon contact with AGS cells (Figure 5). This experiment was run three times independently. Statistical analysis using Welch's unpaired t-test revealed that the difference in the expression of *babA* in the naive *H. pylori* cells and the unattached *H. pylori* cells is statistically insignificant. Our data suggests that *babA* mRNA is increased in bacteria in contact with the AGS cells, and there may

be a fitness advantage in producing more BabA protein in attached cells. Because the transcription levels of *babA* in the unattached and naïve groups were similar, we hypothesize that *babA* transcription is unaffected by any molecules secreted by AGS cells.

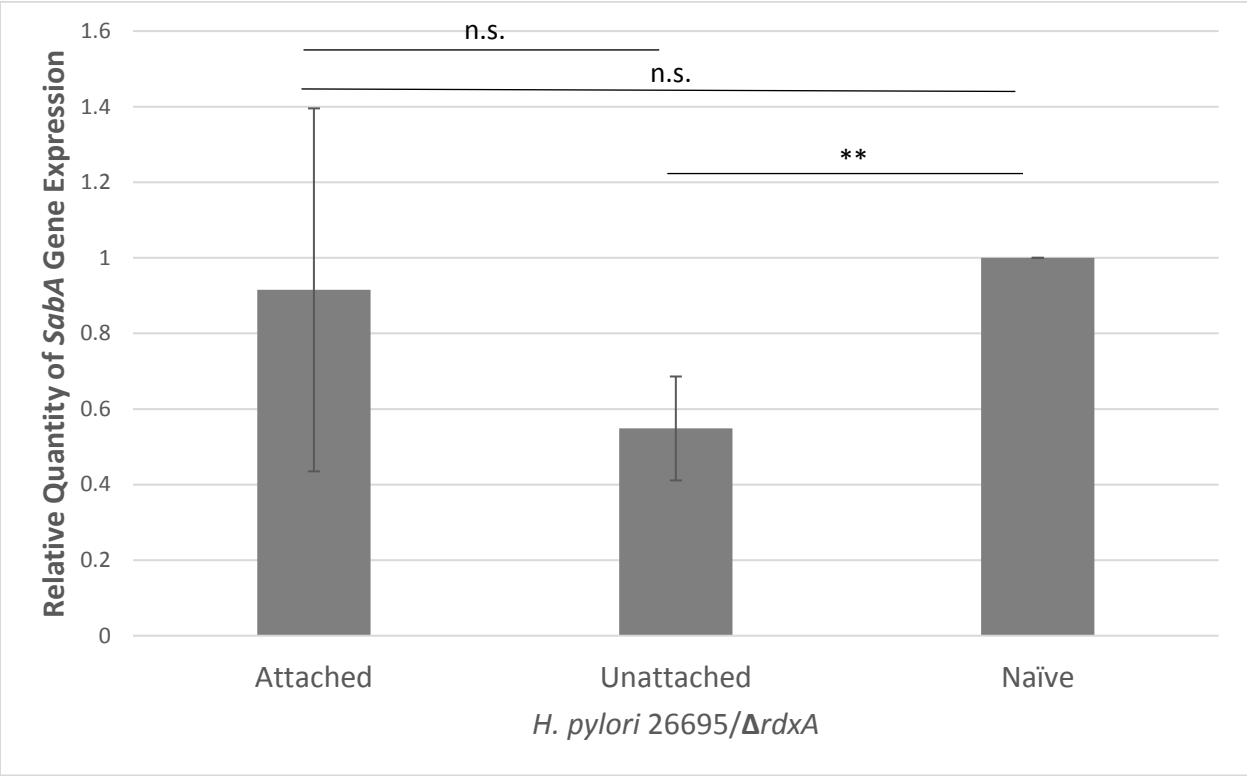


*Figure 6:* ***Unattached (non-adherent)*** **H. pylori** ***express*** **sabA** ***at a lower level than naïve*** **H. pylori** ***cells.*** *Infection assays were performed at a multiplicity of infection of 100:1 (H. pylori cell: AGS cell) using strain 26695/ΔrdxA. Attached, unattached, and naïve H. pylori cells were harvested after 5 hours of infection and analyzed for differences in gene expression of adhesins SabA and BabA. RT-qPCR experiments revealed that sabA* mRNA *is reduced in non-adherent AGS cells. The data shown here is representative of the results obtained in three independent experiments. Error bars show standard deviation. Statistics were calculated using a Welch's unpaired t-test of unequal variance (** = p ≤ .01, * = p ≤ .05, n.s. = p > .05).*

We found that the expression of *sabA* in AGS adjacent, yet unattached *H. pylori* cells is decreased significantly when compared to the expression levels of *sabA* in naive *H. pylori* cells incubated contemporaneously in the same tissue culture medium, but in the absence of AGS cells (Figure 6). We believe that these results suggest that the expression of *sabA* may be repressed in the presence of AGS cells, but independent of contact. We hypothesized that the AGS cells are secreting a molecule(s) that repress the expression of *sabA*. Interestingly, the expression levels of

*sabA* in the attached and naive cells were similar and the difference between these two groups was statistically insignificant. Alternatively, we hypothesized that we may be selecting for cells expressing higher levels of *sabA* in the infection assay experiments comparing attached and unattached 26695/*ΔrdxA* cells. Further experiments using AFLP to quantify differences between the number of poly(T) repeats in the promotor region of *sabA* in attached and unattached *H. pylori* cells may shed light on this.

### *sabA* in Non-Adherent *H. pylori* Cells is Repressed by AGS Cell Secretions

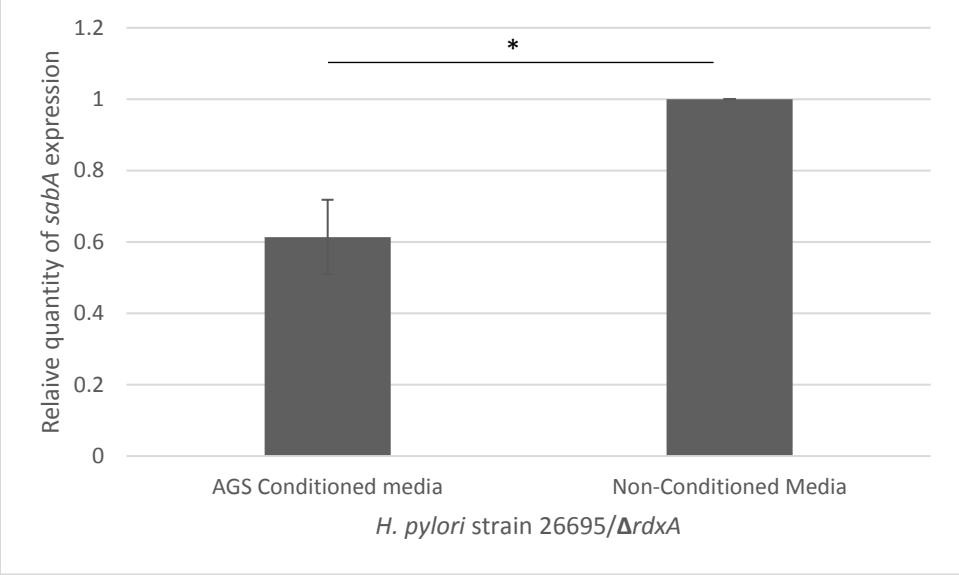


*Figure 7:* **sabA *expression decreases in* H. pylori *strain 26695 in media conditioned with AGS cells.*** *AGS cells were grown in sterile RPMI media with no antibiotics for 24 hours to condition media with AGS cell secretions. Media was filtered and* H. pylori *was incubated for 5 hours in standard conditioned, shaking at 50 rpm. The data shown here is representative of the results obtained in three independent experiments. Error bars show standard deviation. Statistics were calculated using a Welch's unpaired t-test of unequal variance (* = p ≤ .05, n.s. = p > .05).*

Experiments using media conditioned with AGS cells for 24 hours suggest that *sabA* in free-swimming *H. pylori* cells is repressed by molecules secreted by AGS cells (Figure 7). The expression of *sabA* mRNA is significantly decreased in *H. pylori* cells incubated in filtered media conditioned by AGS cell secretions when compared to *H. pylori* cells incubated in non-conditioned, sterile RMPI media. We hypothesize that the repression of this important adhesin

may play a role in maintaining the separation between the two populations (adherent and nonadherent) of *H. pylori* in infected individuals. The separation between these two populations may confer an advantage against events such as the shedding of the gastric epithelial and mucus layers (Arnqvist 2016).

**Two-Component Systems Sense Contact and Regulate *babA***

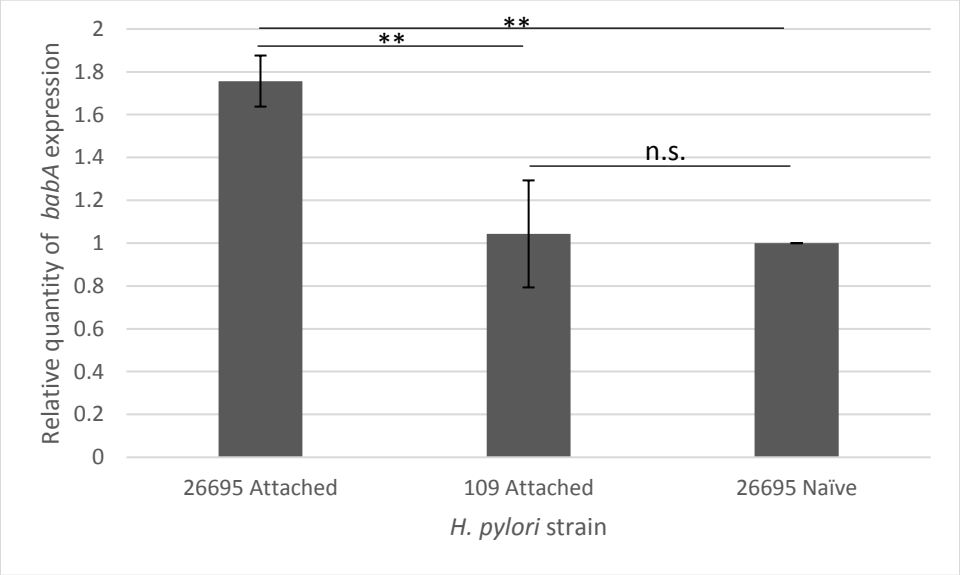


*Figure 8:* **babA *expression is significantly decreased when the histidine kinases of the three known two-component systems are knocked out.*** *Additionally, the expression level of* babA *in the histidine kinase triple knockout strain is not statistically different when compared to* babA *expression in the naïve* H. pylori *cells. The data shown here is representative of the results obtained in three independent experiments. Error bars show standard deviation. Statistics were calculated using a Welch's unpaired t-test of unequal variance (** = p ≤ .01, * = p ≤ .05, n.s. = p > .05).*

RT-qPCR experiments were performed to compare the mRNA levels of outer membrane proteins in adherent *H. pylori* cells from the control strain 26695/Δ*rdxA* with a triple deletion knockout mutation strain of the three known histidine kinases of the two-component systems (ArsS, CrdS, and FlgS) designated 109' $\Delta^3$ (Forsyth, Unpublished), and naïve *H. pylori* 26695/Δ*rdxA* cells. We found that *babA* expression is significantly decreased in the adherent cells of the *H. pylori* strain 109' $\Delta^3$ when compared to the *babA* expression levels in adherent *H. pylori* cells from the strain 26695/Δ*rdxA* (Fig. 8). This data suggests that one of the two-

component systems plays a role regulating *babA* expression. The expression level of *babA* is also significantly lower in the naïve 26695/Δ*rdxA* cells when compared to the adherent 26695/Δ*rdxA* cells, and shows no statistical difference to the adherent 109' $\Delta^3$ cells. We hypothesize that two-component systems, in addition to regulating *babA* expression upon contact with host gastric epithelial cells, have a role in sensing contact with host gastric epithelial cells.

**Two-component systems do not mediate contact dependent alterations in *sabA* and *oipA***

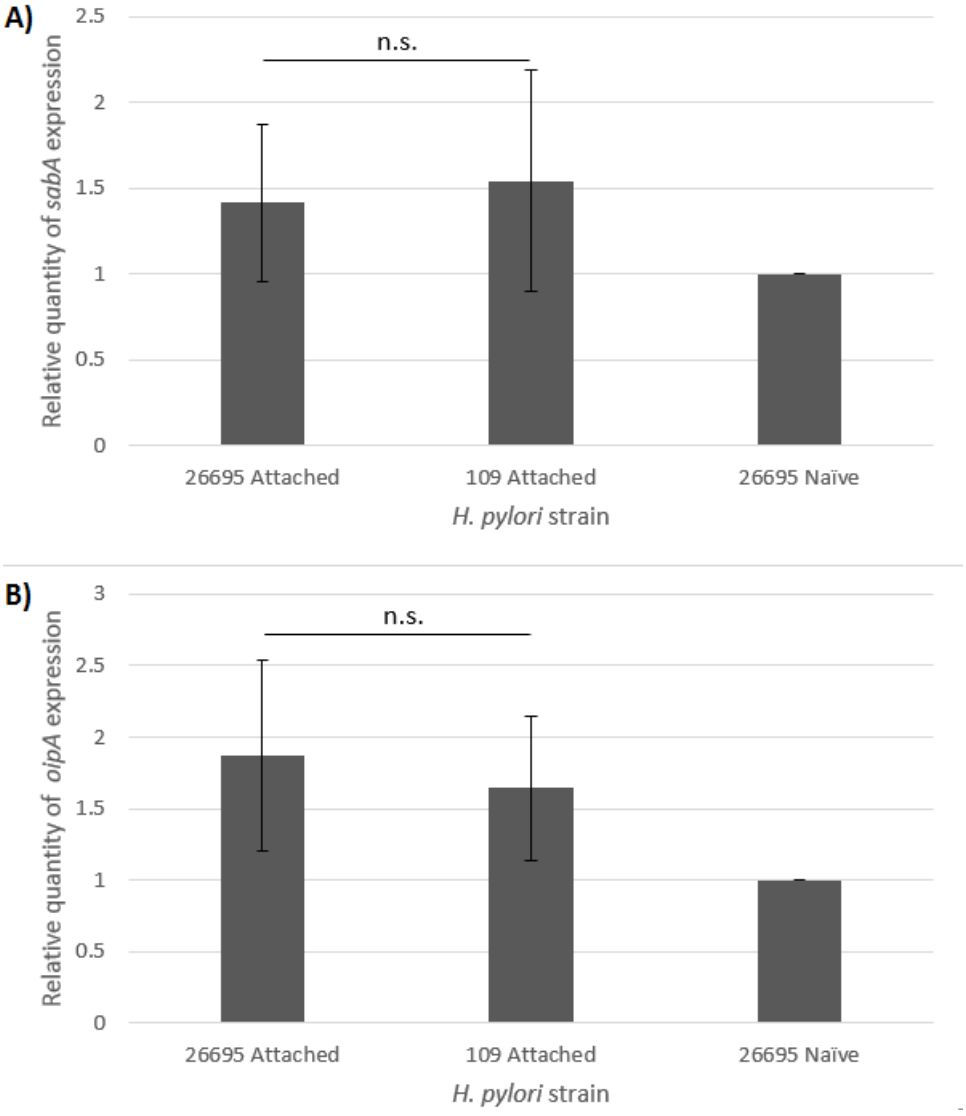


*Figure 9* ***Two-component systems do not regulate contact dependent alterations in the expression of* sabA *and* oipA.** *The difference in* sabA *(A) and* oipA *(B) expression levels between the control strain 26695/Δ*rdxA *and the triple deletion mutation strain 109 $\Delta^3$ are statistically insignificant. 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 ≤ .05, n.s. = p > .05).*

RT-qPCR experiments revealed no significant difference in the expression of both *sabA* and *oipA* between the control *H. pylori* strain 26695/Δ*rdxA* and the triple deletion mutation strain 109 $\Delta^3$. This suggests that two-component systems do not play a role in regulating gene expression for these two outer membrane proteins. We hypothesize that the two-component system that regulates contact dependent *babA* expression is able to bind to BabA receptors on the host cells, but not SabA and OipA receptors.

## Discussion

My honors thesis study characterized the differences in gene expression in three outer membrane proteins of *H. pylori* between attached and unattached populations of *H. pylori* and the role of Two-Component Systems. To the best of our knowledge, changes in gene expression of *H. pylori* adhesins based upon contact with host cells have not yet been characterized. The proteins of interest in this project were SabA, BabA, and OipA, each a carefully studied *H. pylori* adhesin (Odenbreit et al. 2009).

The first part of my project studied the characteristics of OipA and its capability as an adhesin. Attachment assays revealed that strains producing full length OipA proteins are granted an adherence advantage compared to strains that have been turned phase off (26695) or naturally do not produce functional OipA proteins (J68). Attachment of *H. pylori* cells to AGS cells significantly decreased in the absence of a functional OipA protein. These results suggest that OipA is able to mediate attachment to host gastric epithelial cells, even when turned phase on in strains that do not normally produce OipA.

As well as a decrease in attachment, ELISA experiments revealed that turning *oipA* phase off in the *cag*PAI strain 26695 leads to a decrease in production of IL-8, the pro-inflammatory cytokine. Furthermore, ELISA experiments showed that turning *oipA* phase on in the *cag*PAI negative strain J68 do not correlate with higher levels of IL-8 production. These findings suggest that OipA requires interaction with the *cag*PAI in order to induce IL-8 production of the host cell. We hypothesize that OipA requires interaction with the *cag*PAI to illicit a host inflammatory response and pro-inflammatory cytokine production. Another student in our lab, Beau Hawkins, is investigating the nature of OipA and its role as a pro-inflammatory determinant by examining the role of an indel sequence in the *oipA* gene of the strain J75. Although Beau is graduating this May, he will pass his project to another student in the lab.

Post-infection RT-qPCR experiments revealed that *oipA* transcript levels are significantly increased in *H. pylori* strain 26695 that were attached to host AGS cells after 5 hours of infection when compared to the unattached *H. pylori* cells. The increase in the transcription levels of *oipA* in found in the attached cells, but not in non-adherent (unattached) *H. pylori* cells led us to two alternative hypotheses. First, we propose that selection occurs within a population of *H. pylori* that grants an adherence advantage to host gastric epithelial cells. Additional experiments using AFLP on attached and unattached *H. pylori* cells to quantify the number of CT repeats in the *oipA* region to determine the phase status of *oipA* could confirm this hypothesis. Significant differences in the number of CT dinucleotide repeats between the attached and unattached *H. pylori* populations would indicate a genetic difference between the two populations, suggesting that selection is occurring. Alternatively, we hypothesized that contact with gastric epithelial cells could stimulate increased transcription of *oipA* as a response to the presence of the gastric epithelium (Oleastro & Ménard 2013). This is supported by the data showing an increase in *oipA*

transcript levels in attached cells compared to non-attached cells. Either hypothesis supports *H. pylori* persistence in the stomach in the face of stresses caused by the regular mucus and epithelial cell shedding, which favors mechanisms that allow *H. pylori* cells to both adhere to the gastric epithelium and exist free in the mucus. This prompted me to investigate whether contact with AGS cells altered the expression other outer membrane proteins. We selected SabA and BabA as our proteins of interest, two well-characterized outer membrane proteins due to their roles in adhesion to host gastric epithelial cells.

Post-infection RT-qPCR experiments revealed that *babA* is upregulated twofold in attached *H. pylori* cells when compared to unattached and naïve cells. This upregulation in the transcription of *babA* suggests that contact with gastric epithelial cells triggers an increase in the expression of this adhesin. We hypothesize that upregulating *babA* shortly after contact with gastric epithelial cells grants an adhesion advantage. Interestingly, Hansen et al. (2017) found that *babA* expression is high for about two weeks after initial infection, but decreases over time as early as two weeks post infection, and is lost completely after 20 weeks in rhesus monkey models. They suggest that *babA* is disrupted by phase variation, and/or is replaced by the *babB* paralog via nonreciprocal gene conversion, although the reason for selection against *babA* is still unknown. Moreover, *babB* was found to be overexpressed while *babA* expression decreased. It is speculated that the glycan receptor for BabB may be induced by inflammation, and expression of *babB* rather than *babA* confers an adhesion advantage to adherent *H. pylori* cells. Additional studies of the role of *babA* and *babB*, as well as other OMPs during infection, will help inform research about *H. pylori* persistence and pathogenicity in humans (Hansen et. al 2017).

RT-qPCR also revealed that *sabA* expression in attached *H. pylori* cells is not statistically different from naïve cells, but interestingly, *sabA* expression is significantly reduced in

unattached, free-swimming *H. pylori* cells. We hypothesized that AGS cells may secrete molecules that repress the expression of *sabA* in the non-adherent *H. pylori* cells. To test this hypothesis, we grew AGS cells in RPMI media (supplemented with 10% FBS or NCS and HEPES, no penicillin or streptomycin) for 24 hours under standard conditions and incubated *H. pylori* strain 26695/Δ*rdxA* for 5 hours in standard conditions, shaking. We found that the expression of *sabA* in *H. pylori* cells incubated in AGS conditioned media was significantly lower than in *H. pylori* cells incubated in non-conditioned media. We hypothesize that soluble molecules secreted by AGS cells are able to suppress the expression of *sabA* in free-swimming, unattached *H. pylori* cells. Further experiments to isolate AGS cell secretions and performing binding assays may shed further light on the specific molecules that suppress *sabA*. The suppression of this significant adhesin may confer an advantage to *H. pylori* populations in the gastric mucosa of infected individuals by promoting the separation of the adherent and nonadherent, free-swimming populations. The presence of the adherent and non-adherent populations is vital for *H. pylori* persistence and survivability in the harsh gastric environment because bacteria adherent to host gastric epithelial cells are regularly cleared by the shedding of the gastric mucosal layer (Mobley 2001). Therefore, a population of *H. pylori* always exists in a non-adherent, free-swimming state alongside the adherent population in order to successfully persist in its harsh gastric niche.

Lastly, RT-qPCR experiments were performed to determine if two-component systems play a role in sensing contact with host gastric epithelial cells and regulating gene expression of OMPs. We used a strain with deletions of all known sensory histidine kinases (ArsS, CrdS, and FlgS) designated 109' $\Delta^3$ (Forsyth, Unpublished) and compared adherent 109' $\Delta^3$ cells to adherent 26695/Δ*rdxA* cells in order to determine if the absence of the histidine kinases affects

the expression of OMPs. Furthermore, we compared the expression levels of these groups to naïve 26695/Δ*rdxA* cells. We found that *babA* expression is significantly lower in the attached 109' $\Delta^3$ cells when compared to the attached 26695/Δ*rdxA* cells. This suggests that one of the histidine kinases mediates *babA* expression. We also found that the difference in the *babA* expression in naïve 26695/Δ*rdxA* cells and adherent 109' $\Delta^3$ cells is statistically insignificant. We hypothesize that *babA* is only expressed through contact, and that contact is indeed sensed by one of the three histidine kinases. Skoog et al. (2017) found that deletion of the histidine kinase ArsS results in a significant reduction of *babA* expression, suggesting that ArsS mediates the regulation of *babA*. Based on their data and our findings, it is likely that ArsS has a role in regulating *babA* expression upon contact with host gastric epithelial cells. Additional experiments using various histidine kinase knockout mutant strains to deduce which two-component system(s) has/have roles in mediating and sensing contact with host gastric epithelial cells would clarify this. Furthermore, the nature of the ArsS interaction with the AGS cell environment that elicits changes in *babA* expression is unknown. Our data also suggests that two-component systems do not mediate contact dependent alterations in the gene expression of *oipA* and *sabA*. Further studies investigating other genes that are mediated in a contact dependent manner via two-component systems may help better characterize the mechanisms that *H. pylori* uses to regulate gene expression during colonization and pathogenesis. Students in our lab are attempting to isolate components of the ArsRS two-component system and determine its binding partners. Another student in our lab, Ryan Shipman, will be using various individual histidine kinase knock out mutant strains, as well as the triple deletion, to investigate the role of two-component systems in *H. pylori* on inflammatory response by gastric epithelial cells. He will also

continue studying the role of two-component systems and their role in regulating other genes by analyzing RNA transcriptomes via RNA-Seq and performing RT-qPCR experiments.

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## Tables

Table 1: Primers used in this study

| Primer name | 5’ to 3’ sequence |
|---|---|
| HP0955 Fwd | TGTAGGCATTCGTGGGATGAGC [Anneal: 56°C, 2min ext.] |
| HP0953 Rev | GCTCGGACTCATGGAATTGCTCCAT |
| iPCR rdxA Fwd | $PO_4$-CCTAAAATCGCATGCTTGATCGC [Anneal: 55°C, 6min ext.] |
| iPCR rdxA Rev | $PO_4$-GCGTGTTGTAAGAGCTTGGCG |
| oipA universal Fwd | CCATAAGGCATAAGACCTGAC [Anneal: 49°C, 3min ext.] |
| oipA universal Rev | GCAAGACAGCACCACTTTAGCCG |
| oipA.fwd.1 | GGCACATTCGCCCCACAAGCGCT [Anneal: 57°C, 2min ext.] |
| oipA.rev.1 | TCCATGCCAATCACAAGCCCTGA |
| oipA BamHI FWD mut. | AGCATCGGCAAAAAAGGATCCGCAGAAAACGCCTTAAAT |
| oipA BamHI REV mut. | ATTTAAGGCGTTTTCTGCGGATCCTTTTTTGCCGATGCT |
| 26695 oipA OFF mut. | GCTCTCTTACTAACTCTCTCTCTCGTTCTGGCTCCACGCTG |
| J68 oipA ON mut. | ACTCTCTTACTCTCTCTCTCTCTCGTTTTGGCTCCACGCTG |
| oipA.AFLP.fwd | ATTCATTAAGCTTTTGTGGCT [Anneal: 45°C, 30sec ext.] |
| oipA.AFLP.rev | VICAAGGCGTTTTCTGCTGAAGCT |
| oipA.AFLP.control.fwd | AGGGCTTGTGATTGGCATGGAAC [Anneal: 45°C, 30sec ext.] |
| oipA.AFLP.control.rev | VICATATGTATAGTTAAGATAAACGC |
| oipA Consensus.Taq. Fwd | GGCAGAACGCTAGACGCTAA |
| oipA Consensus.Taq. Rev | CCCCTAGTTCCATGCCAATCAC |
| oipA Consensus.Taq | FAM—AAGCCCTGAAGATTTT – MGB-NFQ |
| ftsZ.Taq. Forward | TGAGCGGCATTTCTACGATTATCAC |
| ftsZ.Taq. Reverse | CGCTCTTTAAATCGGCAAAATCAAC |
| ftsZ.Taq | FAM – CAAACCCGGTAATATC – MGB-NFQ |
| gyrB Consensus.Taq. Fwd | AAAGCCAGAGAGCTTACAAGGAAAA |
| gyrB Consensus.Taq. Rev | CGCCCTCCACTAAAAAGATTTCACT |
| gyrB Consensus.Taq | FAM -- TTGCCTGGAAAATTAG– MGB-NFQ |

Table 2: Plasmids used or created for this study

| Name | Description |
|---|---|
| p*rdxA* | TOPO TA cloning vector pCR4 containing a1556 bp amplicon of the *H. pylori* strain 26695 *rdxA* gene |
| pΔ*rdxA* | p*rdxA* with a 390bp deletion in the *rdxA* gene |
| pOipA (26695 and J68) | TOPO TA cloning vector pCR4 containing a 2300bp amplicon including the entire oipA gene, as well as the untranslated regions both upstream and downstream |
| pOipA.*Bam*HI (26695 and J68) | pOipA containing a *Bam*HI site in the cloned allele of *oipA* |
| pMM672 | *H. pylori* 26695 plasmid in which the coding region of *rdxA* is deleted (Loh et al. 2011) |
| pOipA::CAT-*rdxA* (26695 and J68) | pOipA.*Bam*HI with CAT-*rdxA* cassette cloned into the *Bam*HI restriction site |
| p26695.*oipA*OFF | pOipA 26695 with CT repeat tract consisting of 5 CT repeats |
| pJ68.*oipA*ON | pOipA J68 with CT repeat tract consisting of 11 CT repeats |

Table 3: *H. pylori* strains used or created for this study

| Name | Description |
|---|---|
| 26695 Wild Type | $Mtz^S$ and $Cm^S$, contains *oipA* with a CT repeat tract consisting of 6 CT repeats (phase on) |
| J68 Wild Type | $Mtz^S$ and $Cm^S$, contains *oipA* with a CT repeat tract consisting of 10 CT repeats (phase off) |
| Δ*rdxA* (26695 and J68) | 26695 and J68 WT containing a 390bp deletion in the *rdxA* gene. $Mtz^R$ and $Cm^S$ |
| Δ*rdxA*/ *oipA*::CAT-*rdxA* (26695 and J68) | 26695 and J68 Δ*rdxA* containing the *oipA*::CAT-*rdxA* construct. $Mtz^S$ and $Cm^R$ |

| | |
|---|---|
| 26695 Δ*rdxA*/*oipA*OFF | 26695 Δ*rdxA* containing mutant *oipA* with a CT repeat tract consisting of 5 CT repeats (phase off). $Mtz^R$ and $Cm^S$ |
| J68 Δ*rdxA*/*oipA*ON | J68 Δ*rdxA* containing mutant *oipA* with a CT repeat tract consisting of 11 CT repeats (phase on). $Mtz^R$ and $Cm^S$ |