The Acid Response in Helicobacter pylori via the Two Component System ArsRS

Jiajia Chen

Follow this and additional works at: https://scholarworks.wm.edu/honorstheses

Part of the Biology Commons, and the Pathogenic Microbiology Commons

Recommended Citation
https://scholarworks.wm.edu/honorstheses/1178

This Honors Thesis is brought to you for free and open access by the Theses, Dissertations, & Master Projects at W&M ScholarWorks. It has been accepted for inclusion in Undergraduate Honors Theses by an authorized administrator of W&M ScholarWorks. For more information, please contact scholarworks@wm.edu.
The Acid Response in *Helicobacter pylori* via the Two Component System

ArsRS

Jiajia Chen
1- The College of William & Mary, Williamsburg, VA 23187

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

2018

Accepted for Honors – May 1, 2018

Mark H. Forsyth Ph.D.

Shuyin Jiao Ph.D

Matthias Leu Ph.D

Paul D. Heideman Ph.D.
The Acid Response in *Helicobacter pylori* via the Two Component System

**ArsRS**

Jiajia Chen

1- The College of William & Mary, Williamsburg, VA 23187

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

May 2018

**Abstract**

*Helicobacter pylori* is a gram-negative bacterium that colonizes the mucosal layer of the human stomach. Today, nearly half of the world population is infected with *H. pylori*. This infection leads to chronic inflammation, and potentially peptic ulcer disease, or gastric cancer. Developing therapeutics based on the colonization mechanism of this bacterium holds great promise as a therapeutic paradigm to promote human gastric health. To adapt to the hostile acidic environment in human
stomach, *H. pylori* utilizes a Two-Component Signal Transduction system (TCS), ArsRS, to mediate the expression of acid response genes, such as the adhesin gene *sabA* and the urease component gene *ureA*. Here, with Electrophoretic Mobility Shift Assays (EMSA), we further demonstrated the importance of ArsR in adaptation and adherence by showing its binding activities in the promoter regions of other adhesin genes such as *labA, hopZ, and sabB*. Our lab has previously shown that the substitution with a phosphomimetic amino acid, such as glutamic acid (ArsR D52E) and asparagine (ArsR D52N), in the highly conserved 52\textsuperscript{nd} position in ArsR preserves its acid response function, despite its inability to serve as a phosphoacceptor. Here, through site-directed mutagenesis and real-time qPCR, we discovered that the alanine substitution for adjacent aspartic acids at the 47\textsuperscript{th} and 59\textsuperscript{th} positions of mutant D52E retained normal *sabA* repression under pH5, but showed an increase in *ureA* induction under pH5. This study also demonstrates that ArsRS TCS failed to regulate *sabA* and *ureA* in response to pH 5 exposure when the phosphoaccepting histidine (H214) in ArsS was substituted with alanine (ArsS H214A), which disabled its enzymatic function. Furthermore, a kinetics experiment under constant acidity revealed that in ArsR D52E
mutant, the repression of sabA occurred immediately upon exposure to acidity (time 0 min), peaked at time 150 min, and disappeared at time 210 min. The induction of ureA started at time 60 min, maximized at time 120 min, and stopped at 210 min. Together, our results demonstrate the complex nature of this TCS system. We speculate that this complex acclimation to the acid regimen of the stomach helps facilitate the decades long infection of arguably the most extreme environment associated with the human condition.

**Introduction**

*Helicobacter pylori*

*Helicobacter pylori* is a gram-negative pathogenic bacterium that colonizes the mucosal layer of the human stomach and can potentially lead to peptic ulcer disease (ulcers of the stomach or duodenum) and gastric cancer, the second leading cause of death from cancer worldwide (Atherton & Blaser, 2009). Today, *H. pylori* infects more than 50% of the world population and is identified as a class I carcinogen by the International Agency for Research on Cancer (Testerman & Morris, 2014), indicating
that there is sufficient evidence that *H. pylori* has caused human cells to become neoplastic.

*H. pylori* has infected humans and thus co-evolved with humans for more than 58,000 years, and potentially much longer (Linz et al., 2017). Previously, researchers believed that stress and anxiety caused ulcers. In 1982, however, Barry Marshall and Robin Warren isolated *Helicobacter pylori* from a patient with chronic gastritis and gastric ulcers and identified this bacterium as the cause of this disease (Marshall & Warren, 1984). Their joint discovery of *H. pylori* and its role in gastritis and peptic ulcer diseases won these two Australian scientists the Nobel Prize in Physiology or Medicine in 2005. As the prevalence of this bacterium decreases due to effective antibiotic therapy, so does the incidence of associated diseases in western countries. The widespread use of antibiotics in these countries, however, has led to increased antibiotic resistance in the developed world. Despite the decreasing nature of *H. pylori* associated disease in some parts of the world, fatalities from these diseases are still major contributors to mortality in developing countries (Gerritis et al., 2006).

Studies to characterize the biology of *H. pylori* infection have led to effective
diagnostic procedures. For example, the urea breath tests utilize the bacteria’s natural ability to convert urea to ammonia and carbon dioxide. Patients swallow $^{13}$C isotopically labeled urea. The presence of isotopes in carbon dioxide exhaled subsequently by patients indicates the presence of urease, which is produced by *H. pylori* to metabolize urea. Therefore, it is important to study the biology of *H. pylori* infection in order to develop effective antimicrobial treatment in the future. (Savarino et al., 1999)

**H. pylori Persists in the Stomach for Decades**

After researchers linked *Helicobacter pylori* to gastric diseases, many studies focused on the mechanism by which this bacterium colonized the stomach. For decades, researchers believed that the stomach was sterile, or nearly so. This paucity of bacteria recovered was because of technical problems in collecting samples and the lack of reliable diagnostic tests. Indeed, the extreme acidity, proteolytic enzymes, thickness of mucus layer, and gastric peristalsis each impede bacterial homeostasis and colonization. (Nardone & Compare 2015). The discovery of the gram-negative bacterium *H. pylori* shifted the paradigm of gastric microbiota research. However, our
understanding of the role of *H. pylori* and other gastric microbiota in human health and disease, and its mechanisms to colonize gastrointestinal tract is a critical unanswered questions (Yang et al., 2013). Therefore, it is important to understand how *H. pylori* manages to not only reside in the stomach, but also persist there for decades.

Studies have found that the expression of the cytoplasmic protein urease in *H. pylori* is essential for this bacterium’s colonization of the gastric mucosa. *H. pylori* can survive a pH range from 4.0 to 8.0 in the absence of urea, and survive in the pH range from 2.5 to 8.0 in the presence of urea (Dunne et al., 2014). The urease enzyme hydrolyses urea to NH\(_3\) and CO\(_2\), neutralizing the acid in a microenvironment for *H. pylori*, after which the bacterium is able to adhere to epithelial cells using outer membrane proteins as adhesins.

*H. pylori* also utilizes flagellar switch proteins to promote persistent infection. A set of signal transduction proteins comprised of the chemotaxis pathway regulates flagellar motility (Lowenthal et al., 2009). Effective flagellar motility directs the migration of *H. pylori* in response to environmental signals, such that it avoids acidity,
moves up urea gradients, and navigates towards the epithelial cells underneath the mucus. Studies have shown that the deletion of the regulator FlhA, which controls expression of flagellins genes, *flaA, flaB, and flgE*, leads to non-motile mutants with decreased colonization rates (Ottemann & Lowenthal, 2002). Besides motility, FlhA also plays a role in regulating the urease activity in *H. pylori*. After modifying the microenvironment and orienting itself according to environmental cues, *H. pylori* establishes the initial necessary conditions for colonization.


BabA, the blood group antigen binding adhesion, binds to H-type 1 and to Lewis b blood-group antigens on normal gastric mucosa. The expression of *babA* is
regulated via gene conversion with its paralogous genes elsewhere in the genome, the
characteristics of the promoter sequence, and the presence of polymorphisms of the
poly-cytosine-thymine (poly-CT) dinucleotide tract in the 5’ region of the \textit{babA}
sequence. The repetitive nature of the poly-CT dinucleotide tract makes it prone to
slipped strand mispairing, a mechanism resulting in phase variation through the
deletion or insertion of nucleotides in the nucleotide tracts and thus the alterations of
frame length. BabA has two highly divergent paralogous genes \textit{babB} and \textit{babC},
located in at least 3 different chromosomal loci. While the function of BabB and
BabC remains unknown, BabA mediated adherence has been shown to augment the
Type IV Secretion System (T4SS) encoded by the \textit{cag} pathogenicity island by
triggering proinflammatory cytokines production (Mahdavi et al., 2002; Yamaoka et

Upon colonization, the infection induces inflammatory responses, which initiates
the expression sialylated antigens on the gastric epithelial cells. SabA adhesins then
recognize sialyl-Lewis A and sialyl-Lewis X antigens and help with the persistent
adherence to host cells (Magalhães & Reis, 2010). The SabA adhesin is encoded by
gene HP0725, which is predicted to encode an outer membrane protein (Forsyth et al., 2002). The *sabA* locus has a homopolymeric thymine (poly-T) tract in the promoter region, and a dinucleotide cytosine-thymine repeat (CT repeat) in the coding region. Both the varied length of thymidine nucleotide repeat tract adjacent to the -35 promoter region among different strains and the phase variation via slipped-strand misparing which inserts or deletes nucleotides within repetitive DNA tracts regulate the expression of SabA (Goodwin et al., 2008). Phase variation is a mechanism that either turns on or off gene expression at both the transcriptional and the translational levels (van der Woude & Baumier, 2004). In addition to varied length of promoter poly-T tract length and phase variation, studies have shown that the binding of recombinant ArsR of the ArsRS TCS to the promoter region of *sabA* also mediates the expression of *sabA* in vitro (Harvey et al. 2014). Activated ArsRS also regulates the gene transcription of *sabA* paralog *sabB, ureA, hopZ,* and *labA* (Acio et al. 2017)

Overall, *H. pylori* utilizes urease activity, flagellar motility, and outer membrane adherence to establish its chronic colonization. This colonization promotes the functions of a Type IV secretion system encoded by the *cag* pathogenicity island, and
thus is critical to the pathogenesis of *H. pylori* associated diseases.

**Two Component Signal Transduction System**

Two component signal transduction (TCS) systems are signaling pathways that allow bacteria to sense changing stimuli in the environment and adequately acclimate to them. These systems can be found in all domains of life, but they are particularly frequent in bacteria, and rare in eukaryotes and completely absent in multicellular animals. Among the genome of all sequenced bacteria, the majority of species entail dozens, and even hundreds of two-component proteins (Capra & Laub, 2012). A classic system is comprised of two conserved components: a sensor histidine kinase protein and a response regulator protein. This histidine protein kinase is typically a transmembrane protein. Different histidine kinases sense different environmental stimuli, including pH, temperature, and ions. The cytoplasmic kinase domain then autophosphorylates at a conserved histidine residue, which can be reversed and results
in dephosphorylation in the absence of the appropriate extracellular cue. This phosphoryl group is then transferred to an aspartate residue in the cognate response regulator protein and thus leads to a conformational change, which then mediates gene expression of a series of genes, i.e., the regulon, that serve to assist the bacterium to acclimate to the novel exterior environment. In some cases, however, due to the bifunctional nature of histidine kinases, signals promote the phosphatase states in kinases to regulate their cognate response regulator rather than autophosphorylation (Alm et al., 2006). In this process, exquisite specificity is required for the avoidance of cross talk to ensure accurate transmission of information between sensor kinases and their cognate response regulators, which mounts appropriate gene expression responses and cellular physiological changes in response to external signals (Capra et al., 2010).

Compared with some bacteria that have over 150 different two-component pathways, *H. pylori* has a relatively small number of complete TCS systems. This is likely due to the fact that *H. pylori* has a small sized genome (1.66 MB), and a relative scarcity of genes encoding regulators of transcription (Tomb, et al., 1997).
Genes *hp1043* and *hp1021* encodes two orphan response regulators, the cognate sensor kinases of which are missing. Although response regulators HP1043 and HP1021 have non-conserved substitutions in major amino acids of their receiver domains, the ablation of the corresponding genes is lethal for the bacteria unless a second gene copy were integrated elsewhere into the *H. pylori* chromosome (Delany et al., 2002). Studies have also shown that allelic replacement inactivates HP1043, and mutations in HP1021 lead to growth defects, which suggests their important roles in regulating crucial cellular processes (Pelliciari et al., 2017). The HP1043 regulator belongs to the OmpR family. Although with a degenerative sequence, HP1043 cannot be phosphorylated, studies have shown that its symmetric dimer structure allows it to function independent of phosphorylation. The target genes bound and regulated by this regulator, however, still remains unclear (Schär et al., 2005; Müller et al., 2007).

The HP1021 protein belongs to the atypical response regulator family, which influences the expression of approximately 80 *H. pylori* genes. Research has shown that this protein is involved in the initiation of *H. pylori* chromosome replication (McDaniel et al., 2001).
*H. pylori* utilizes three complete TCS: ArsRS, FlgRS, and CrdRS, to respond to different stimuli and modulate expression of appropriate genes. Using the ArsRS TCS, this bacterium responds to alterations in acidity in the environment of mucosal layer. ArsS autophosphorylates upon detection of increased acidity and transfers the phosphoryl group to the OmpR family response regulator ArsR which dimerizes and becomes an active transcription factor of genes of the regulon such as sabA, ureA, and babA. Studies have shown that ArsR not only regulates *H. pylori* gene expression in its phosphorylated state, but also in its unphosphorylated state. This is supported by the observation that ArsR with a mutated phosphorylation site is viable, while the loss of ArsR is not viable as these mutants are not recoverable unless intact copy is placed elsewhere as a second copy (Gupta et al., 2009; Pflock et al., 2006; Loh et al., 2010).

*H. pylori* utilizes the FlgRS system to regulate transcription of σ54-dependent flagellar genes. FlgS is different from typical histidine kinase proteins in that it is cytoplasmic instead of membrane bound. FlgS regulates different gene regulons at different acidities. The regulation of nine genes including flaB, flgK, fla, flgE, is responsible for flagellar gene expression and is independent of pH. In acidic
conditions of pH 4.5 without exogenous urea, twenty-two genes including \textit{ureA}, \textit{ureB}, and \textit{ureI} are regulated. Under pH 2.5, genes including \textit{ureF} are regulated. This fact supports the view that by activating $\sigma^{54}$ to coordinate flagellar biosynthesis, FlgS plays an important role in responding to a drastic change in the medium pH. (Wen et al., 2008; Niehus et al., 2008; Niehus et al., 2004). The deletion of FlgS is viable at pH 5, but not pH 2.5. The deletion of FlgR, corresponding to HP0703, is viable at both pH5 and pH 2.5. This fact suggests the possibility of crosstalk between histidine kinases and response regulators in response of acidity changes. (Loh et al., 2006; Dunne et al., 2014)

The last TCS system of \textit{H. pylori}, CrdRS, regulates the expression of \textit{crdA}, encoding the copper resistance determinant \textit{A} in response to copper ions in the environment. Under strain specific variations of \textit{H. pylori}, CrdS is found to respond to acidic signals in strain J99 but not 26695 (Pflock et al. 2007). Recent studies have also shown that the knockout of \textit{crdS} results in the loss of viability upon exposure to NO, which underscores the importance of CrdRS in the regulation of the expression of metal-homeostasis and transport binding proteins (Loh and Cover, 2006; Waidner
et al., 2005; Huang et al., 2015).

Figure 1: ArsRS Two-Component Signal Transduction system. The Two-Component Signal Transduction system utilizes a transmembrane protein ArsS to sense acidity from the environment. Upon increased acidity, this histidine kinase ArsS autophosphorylates at histidine 214 within the cytoplasmic kinase domain, and transfers its phosphate group to ArsR. The phosphorylation of the 52nd aspartic acid domain causes ArsR to dimerize and bind to the promoter regions of acid response genes sabA and ureA. This regulation leads to repression of adhesins gene sabA and induction of urease component gene ureA. ureA encodes for urease which will hydrolyse urea to NH₃ and CO₂ and neutralize the environment.
**Research Goals**

Adaptation to the highly acidic conditions in the stomach is key to successful and persistent colonization of the pathogen *Helicobacter pylori* in hosts. In the process of persistent infection, *H. pylori* utilizes the ArsRS Two Component System to regulate acid responsive genes such as the adhesin-encoding sabA gene and the urease subunit encoding gene *ureA*. ArsR, the cognate response regulator of the histidine kinase ArsS, is required for the bacterial survival while the presence of ArsS is not. The main goal of this study is to examine the roles of ArsS and ArsR in the TCS and its regulatory mechanism controlling acid responsive genes. Our investigation of this major regulatory mechanism focuses on the determination of direct or indirect mediation from ArsR on the promoter regions of genes such as sabB, hopZ, and labA.

Another focus was on phosphomimetic substitutions in ArsR and potential alternative phosphorylation sites. More specifically, we aim to determine if two nearly adjacent aspartic acids in ArsR relative to the highly conserved 52\(^{nd}\) position, i.e., the 47th and 59\(^{th}\) positions, serve as compensatory activating sites in the presence of
phosphomimetic mutations at 52nd position, the canonical site of phosphorylation.

Another point of emphasis in our studies of this regulatory mechanism is the kinetics of ArsRS response to acidity. By examining the change in mRNA transcript levels of *sabA* and *ureA*, we hope to reveal and document for the first time the hidden characteristics of its time sensitive nature. In addition, we hoped to determine if it were the kinase enzymatic activity of ArsS, or some other activity that regulates corresponding genes expression. Overall, this study characterizes the acid response in *H. pylori* via TCS system in an *in vitro* model.
Methods

Electrophoretic Mobility Shift Assays (EMSAs)

Using PCR, we amplified the hopZ, labA, and sabB promoter regions using gDNA from *H. pylori* strain J99. Then these probes were labeled with biotin using primers hopZ-Bio and hopZ-R3, labA-Bio and labA-R3, sabB-Bio and sabB-R, respectively. Unlabeled but specific DNA competitors were amplified using non-biotin labeled hopZ-Bio, labA-Bio and sabB-Bio, respectively. rArsR was expressed and purified (Harvey et al. 2014, Acio-Pizzarello et al. 2017). The binding of rArsR and probes was done using the LightShift Chemiluminescent EMSA Kit (Thermo). Bioin-labeled probes were used at 9–20 fmol per reaction and 0.2 nmol rArsR per reaction. 175-fold molar excess of specific competitor DNA was used for labA, 500-fold molar excess was used for hopZ, and 300-fold molar excess was used for sabB. 250-500 fold molar excess of EBNA DNA was used in non-specific competition assays relative to the bioin-labeled and specific probes. In 5% TBE native polyacrylamide gels (Bio-Rad), DNA and DNA–protein complexes were resolved. Western blotted to Zeta Probe membranes (Bio-Rad) and UV cross-linked.
Streptavidin-horseradish peroxidase was used to detect biotin DNA and DNA–rArsR complexes.

**H. pylori Natural Transformation**

*H. pylori* strains from freezer stock were spotted at the center of BAPs. After growing for 24-36 hr, cells were spread out evenly across the plates. After 24 hr, cells were 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. 7 to 13 ug plasmid were added to resuspended *H. pylori* cells. 30µl – 50ul of the suspension was spotted onto the center of BAPs. They were then incubated face up for 2-4 hours under 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 were isolated and harvested from their own BAP for genomic DNA extraction.
Plasmid Purification and Isolation

5 mL 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 16-24 hours. Cells were spun down at 5000 g. Cells were lysed and plasmids were isolated using Mini Hi-Speed Plasmid Kit (IBI). Concentrations of plasmid dsDNA were quantified using a NanoPhotometer (Implen).

Genomic DNA Extraction

*H. pylori* was grown on BAPs for 24-48 hours in 5% CO₂ 37°C incubator. Cells were harvested and suspended 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).

H. pylori Culture on Plates
*H. pylori* strain 26695 and all mutants were cultured on tryptic soy agar II with 5% sheep’s blood (BBL™) for 24-48 hours at 37°C in an ambient air/5% CO₂ atmosphere.

**H. pylori Broth Culture**

Liquid cultures of *H. pylori* of wild type, ∆arsS, arsR D52E, or arsR D47A D52E D59A mutations were grown in sulfite-free Brucella broth supplemented with 1X cholesterol (Gibco® by Life Technologies™), and 20µg vancomycin/mL at pH 7 shaking at 150 rpm. With calculation and dilution, the OD₆₀₀ was set to 0.2. After 10-12 hours of incubation, OD₆₀₀ was measured and adjusted to OD₆₀₀ = 0.4. After 6-7 hours of incubation under same conditions, cells grew into their mid-logarithmic phase (OD₆₀₀ from 0.7 to 1.2). The aliquots harvested by centrifugation at 1800 x g for 10 minutes. Cell pellets resuspended in the same broth medium, at either pH 7 or pH 5 for 1 hour. Cells were then spun down by centrifugation at 1800 x g for 10 minutes and re-suspended in RNAzol RT (Molecular Technologies). They were preserved at -80 freezer before RNA extraction.
For the kinetics experiments, liquid cultures of *H. pylori* wild type were grown in sulfite-free Brucella broth supplemented with 1X cholesterol (Gibco® by Life Technologies™), and 20µg vancomycin/mL at pH 7 shaking at 150 rpm. With calculation and dilution, the OD$_{600}$ was set to 0.2. After 10-12 hours of incubation, OD$_{600}$ was measured and adjusted to 0.4. After 6-7 hours of incubation under same conditions, cells grew into their mid-logarithmic phase (OD$_{600}$ from 0.7 to 1.2). Aliquots (10$^9$ cells) harvested by centrifugation at 1800 x g for 10 minutes. Cells pellets were resuspended in SFBB broth with the addition of 50 mM potassium phosphate buffer to maintain pH. Immediately after the re-suspension at pH 5 and pH 7 broth, the cells from group time = 0 were collected and harvested by centrifugation at 1800 x g for 10 minutes, and then resuspended in RNAzol RT. The supernatants of all time points were collected and their pH was recorded. Repeats of this cells collection occurred at a 30 min time interval until after 210min. Harvested cells were kept in 1 ml RNAzol at -80°C for RNA extraction.
RNA Extraction

1 mL of cells with RNAzol RT were transferred to screw caps and 2-7µl polyacrylamide carrier (Molecular Technologies) added to assist in precipitation and visualization of the RNA pellets. The RNAzol-RT mixture were beadrupted, with no glass beads, for 45 seconds. Molecular grade water was added to each sample and allowed to incubate for 15 minutes on ice. Samples were centrifuged for 15 minutes at 12,000 x g. 400µL of 75% ethanol were added to 1mL samples and incubated for 10 minutes. Samples were centrifuged at 10,000 xg for 8 minutes and supernatant was discarded. Two additional ethanol washes were used and RNA pellets were allowed to dry for 20 minutes. Pellets were resuspended in 20-70 µL of PCR grade H2O. Samples were then held on ice and RNA concentrations quantified using a NanoDrop. RNA samples were used in cDNA synthesis.
**cDNA Synthesis**

With calculation, 1 µg of purified RNA was combined with 4µg of iScript Reverse Transcription Supermix, together with H2O, making the total volume 20µg, 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)**

We used *ftsZ*, which encodes for the cell division protein FitZ, as the house keeping gene, and compared the expression level of the *H. pylori* gene *sabA* and *ureA* relative to the housekeeping gene. With TaqMan Gene Expression assay (Life Technologies) performed on the Applied Biosystems StepOne apparatus, which were performed in both biological triplicates and technical triplicates for each gene and each strain according to the manufacturer’s protocol using custom TaqMan Custom Gene Expression assays (Thermo-Fisher). *sabA*.Taq, *ureA*.Taq, and *ftsZ*.Taq, probes were used. Mastermix were added. cDNA of each sample was added to each well on a
96-well plate. Relative expression of genes among the various mutants was then calculated using DataAssist software (Applied Biosystems).

Results

ArsS H214A mutant Fails to Regulate mRNA Levels of sabA and ureA under pH5

Here we sought to determine if it were the histidine kinase enzymatic activity of ArsS that regulates the expression of acid responsive genes such as sabA and ureA. With site-directed mutagenesis, we replaced the histidine on position H214 of ArsS, the site of auto-phosphorylation upon receiving a periplasmic acid signal. This mutation thus disabled the kinase activity of ArsS. qPCR quantification of the mRNA transcripts levels of sabA shows that the ArsS H214A mutant lost its ability to regulate sabA and ureA under pH5 (Figures 2 & 3).
Figure 2: sabA Transcripts Expression Levels in ArsS H14A mutant at pH 5 and pH 7
Control strain 89' with a wild type ArsS displayed sabA repression under pH 5 (p=0.007). The ArsS H214A mutant with an alanine substitution at position 214 lost its ability to repress sabA (p = 0.51). Compared to the expression level of sabA in H. pylori arsS wild type (89') at pH7 and pH 5, sabA mRNA in the ArsS H214A mutant is not statistically significant different (p = 0.87).

Figure 3: ureA Transcripts Expression Levels in ArsS mutants at pH5 and pH7
Control H. pylori strain 89' with a wild type ArsS displayed ureA induction under pH5 conditions (p=0.006). The ArsS H214A mutant with an alanine substitution on position 214 lost its ability to induce ureA (p = 0.47). Compared to the
expression level of \textit{ureA} on 89' at pH7 and that on ArsS H214A mutant, there is no statistically significant difference (p = 0.19).

The TCS Response Regulator ArsR binds to promoting regions of genes \textit{labA}, \textit{hopZ}, and \textit{sabB} encoding Outer Membrane Protein Adhesins.

Here, we sought to determine if ArsR also regulates the mRNA levels of other important outer membrane adhesin genes such as \textit{labA}, \textit{hopZ}, and \textit{sabB} through similar DNA-protein specificity mechanism.

Electrophoretic Mobility Shift Assays (EMSA's) was performed using recombinant ArsR and DNA probes comprised of the promoter regions of \textit{labA}, \textit{hopZ} and \textit{sabB} (Figure 4). The observation of gel showed that rArsR bound to each of these promoter regions. This result underscores the importance of ArsR in direct mediation in not only \textit{sabA}, but also in \textit{labA}, \textit{hopZ} and \textit{sabB}. 
Figure 4. Recombinant ArsR (rArsR) binds labA, hopZ and sabB promoter regions.

Electrophoretic Mobility Shift Assays were performed using rArsR (Harvey et al. 2014) and biotin end labeled labA (A), hopZ (B), and sabB (C) promoter regions. Lanes 1; biotin labeled probe only. Lanes 2; biotin labeled probe & rArsR. Lanes 3; biotin labeled probe, rArsR, & 175 to 500-fold excess unlabeled probe. Lanes 4; biotin labeled probe, rArsR, & 500 fold molar excess non-specific competitor. See Materials and Methods for details on probes, rArsR, specific and non-specific competitors (Acio et al., 2017).
The Substitution of Glutamic Acid, Asparagine, and Alternate Aspartic Acid on

52nd are Viable

The aspartic acid on the 52nd position of ArsR is conserved on *H. pylori* strains. This aspartic acid receives a phosphoryl group from ArsS, which leads to ArsR dimerization and regulation of acid responsive genes. Here, we sought to determine if the substitution of this aspartic is viable. With site-directed mutagenesis, we introduced different amino acids into 52nd position through cross over from *E.Coli* plasmid to *H.pylori* genome. We inserted chloramphenicol resistance gene into HPO167, so that we could select chloramphenicol resistant *H.pylori* colonies from antibiotic plates. HPO167 is used as a location marker, since it locates upstream of *arsS* and *arsR* genes. Aspartic acid with an alternative codon (GAC) was used to ensure that the cross over containing *arsS* and *arsR* genes occur frequently enough. We then confirmed their insertion with sequencing. For each experiment, we collected 10 colonies. Our results show that the aspartic acid with a different codon went into 6 out of 8 colonies; asparagine went into 9 out of 10 colonies; glutamic acid went into 8 out of 10 colonies. Alanine, serine, and glycine, however, were not viable (Figure 5).
Figure 5: Transformation of *H. pylori* 26695 with phosphomimetic and non-phosphomimetic ArsR D52 mutations

(A) A schematic of the plasmid construct used to generate *arsR* codon 52 mutations via site-directed mutagenesis. The Cm-R gene inserted in the gene HP0167 allowed for selection for chloramphenicol resistance if allelic replacement took place in *H. pylori*. (B) Crossover at the D52 codon was only successful with the control plasmid and plasmids containing a codon for a phosphomimetic amino acid at that site or the control D52D* mutation where the wild type D codon (GAT) was replaced by the alternate D codon, GAC. In each experiment, between 8 and 10 chloramphenicol resistant *H. pylori*
**ArsR D47A D52E D59E mutant Represses mRNA Levels of *sabA* under pH5**

Here, we sought to determine if there are alternative activation sites in ArsR. Two aspartic acids in ArsR adjacent to the highly conserved 52^{nd} position are located at positions 47 and 59, D47 and D59, respectively. We therefore hypothesized that the phosphorylation of 47^{th} or 59^{th} positions’ aspartic acids may serve as compensatory activating sites in the presence of phosphomimetic mutations at 52^{nd} position. Through site-directed mutagenesis, we created *H. pylori* mutants *DarsS*, ArsR D52E, and ArsR D47A D52E D59A. Using qRT-PCR, we demonstrated that the *H. pylori* ΔarsS mutant loses the ability to regulate the transcription levels of *sabA* in response to pH 5 conditions while the ArsR D52E mutant represses *sabA* normally. The ArsR D52E mutant displays a lower level of *sabA* repression than the ArsR D47A D52E D59A mutant. In mutant ArsR D47A D52E D59A, the alanine substitutions at positions 47^{th} and 59^{th} disables these two positions as phospho-accepting sites in ArsR. The lack of constitutive activity, together with the observation that it shows an even stronger acid repression function than ArsR D52E (Figure 7), suggests that the phosphorylation of aspartic acids at 47^{th} or 59^{th} positions were not involved in the
regulation of sabA expression. Therefore, there is no evidence that shows 47th or 59th positions are alternative activation sites to the highly conserved 52nd position.

Figure 6: sabA Transcripts Expression Levels in ArR mutants at pH5 and pH7

The ΔarsS mutant, 16’, loses its acid response function to induce the expression of urease component gene ureA (p = 0.73). The wild type 89’ strain, ArsR D52 E mutant 90’ produces functional responses to pH5 by repressing the expression of sabA (p = 0.02 < 0.05). The ArsR D47A D52E D59A mutant also shows sabA repression ability under pH5(p = 0.03). The triple substitution did not interfere with the normal function of ArsR, suggesting that the 47th and 59th might not serve as alternative phosphorylation sites that compensate for the loss of phosphorylation on 52nd position.
Figure 7: *sabA* Transcripts Levels on Different *ArsR* mutants at pH5

89’ is wild type *H.pylori* with a cat gene inserted, which does not change *ArsR*. 90’ is the strain with a glutamic acid phosphometics substitution on the 52\textsuperscript{nd} position aspartic acid. AEA is strain with alanine substitution on 47\textsuperscript{th} and 59\textsuperscript{th} positions, and a
glutamic acid on 52\textsuperscript{nd} position.

(A) There is no statistically significant difference between the sabA repression levels on 89’ strain with wild type ArsR, and that on 90’ strain with ArsR D52E (p = 0.34).

(B) There is no significant difference between the sabA repression levels on 89’ strain with wild type ArsR, and that on 90’ strain with ArsR D52E (p = 0.23 > 0.05).

(C) At pH5, the sabA transcript levels on strain 90’ with ArsR D52E mutant is statistically smaller than that of AEA mutant, suggesting not only a sabA repression ability of ArsR D47A D52E D59A, but also an increased repression.

**ArsR D47A D52E D59E mutant Induces mRNA Levels of ureA under pH5**

Here, with qRT-PCR, we demonstrated that the ΔarsS mutant loses the ability to regulate the transcription levels of ureA in response to pH 5 conditions while the ArsR D52E mutant and the ArsR D47A D52E D59A mutant induces ureA normally. Two-sample t test was performed and determined that the ureA induction levels were significantly higher in the ArsR D52E mutant compared with wild type ArsR, suggesting not only the preservation of ureA induction ability in the substitution of phosphometics glutamic acid, but also its ability to enhance the acid regulation at pH5. The ArsR D47A D52E D59A maintained ureA induction at pH5 but did not promote the acid induction (Figure 8, 9). This observation suggests that the phosphorylation of aspartic acids at 47\textsuperscript{th} or 59\textsuperscript{th} positions were not involved in the
regulation of ureA induction. Therefore, there is no evidence that shows 47th or 59th positions are alternative activation sites to the highly conserved 52nd position.

**Figure 8: ureA Transcripts Expression Levels in ArsR mutants at pH5 and pH7**

The ΔarsS mutant, 16’, loses its acid response function to induce the expression of urease component gene ureA (p = 0.73). The wild type 89’ strain, ArsR D52 E mutant 90’ produces functional responses to pH5 by inducing the expression of ureA.
(p = 0.03). The ArsR D47A D52E D59A mutant also shows **ureA** induction ability under pH5 (p = 0.02). The triple substitution did not interfere with the normal function of ArsR, suggesting that the 47th and 59th might not serve as alternative phosphorylation sites that compensate for the loss of phosphorylation on 52nd position.
89’ is wild type H. pylori with a cat gene inserted, which does not change ArsR. 90’ is the strain with a glutamic acid phosphometics substitution on the 52\textsuperscript{nd} position aspartic acid. AEA is strain with alanine substitution on 47\textsuperscript{th} and 59\textsuperscript{th} positions, and a glutamic acid on 52\textsuperscript{nd} position.

(A) The ureA transcripts level regulated by ArsR on wild type 89’ is lower than that of 90’ strain with a ArsR D52E mutant in a statistically significant manner (p = 0.05)

(B) The ureA transcripts level regulated by ArsR on wild type 89’ is lower than that of AEA strain with a ArsR D47A D52E D59A mutant in a statistically significant manner (p =0.04)

(C) There is no significant difference between the ureA induction levels on 90’ strain with ArsR D52E, and that on AEA strain with (p = 0.78).

**sabA mRNA transcript levels in H. pylori maintained in a constant pH 5 Environment**

Since the acid regulation of *sabA* and *ureA* expression levels mediated by ArsR are well documented, we asked the question; "How does the acid response behave when the pH does not respond to the altered gene expression?" We hypothesized that this ArsRS mediated change in *H. pylori* gene expression would persist as long as the pH remains unchanged. However, the kinetics experiments using *H. pylori* 26695 possessing a wild type *arsRS* locus have shown that while the *sabA* repression started immediately upon exposure to acidity, this repression only lasted for 150 minutes under constant pH 5 conditions. In retrospect, we were surprised at the immediacy of the acid repression of *sabA* mRNA transcript levels. Future iterations of these kinetics experiments will include *sabA* mRNA quantification of *H. pylori* cells just prior to the pH 5 exposure. *H. pylori* 26695 cells was incubated at pH 5 and pH 7. After every 30 min time interval, 10\textsuperscript{9} cells were harvested and the pH of its supernatant was recorded and found to have maintained constant. After exposure to pH 5 conditions for 30 min, *H. pylori* showed the maximal acid repression of *sabA*; a 20-fold repression compared to control cells incubated under constant pH 7 conditions.
At 120 minutes, the acid repression had decreased and was no longer significant. At 210 minutes, the acid repression of sabA was lost, suggesting the ArsRS system functions in the relative short term and the gene expression changes mediated in response to acid must acclimate the bacteria to the acidity quickly. This might be due to the loss of cell viability at this point. When *H. pylori* is no longer able to neutralize the acidity in its environment any longer, it may succumb to the effects of the acidity (Figure 10). However, pH 5 is not known to be a lethal pH for *H. pylori* so future experiments using serial viable cell counts, as colony forming units, on agar plates can be performed to determine cell vitality at different time points.
E  

F  

G
Figure 10: sabA Expression Levels at Different Time Points.

89’ mutant is strain *H. pylori* 26695 with ArsR D52 E mutation.

(A) Relative sabA gene expression levels of 89’ strain grown at pH5 for 0 min shows a 5-fold decrease of sabA in comparison to 89’ strain grown at pH7 for 0 min. Mutant control strain 89’ grown at pH7 for 0 min is set to 1 (p=0.027).

(B) Relative sabA gene expression levels of 89’ strain grown at pH5 for 30 min shows a 20-fold decrease of sabA in comparison to 89’ strain grown at pH7 for 30 min. Mutant control strain 89’ grown at pH7 for 30 min is set to 1 (p=0.018).

(C) Relative sabA A gene expression levels of 89’ strain grown at pH5 for 60 min shows a 5-fold decrease of sabA in comparison to 89’ strain grown at pH7 for 60 min. Mutant control strain 89’ grown at pH7 for 60 min is set to 1 (p = 0.031).

(D) Relative sabA gene expression levels of 89’ strain grown at pH5 for 90 min shows a 5-fold decrease of sabA in comparison to 89’ strain grown at pH7 for 90 min. Mutant control strain 89’ grown at pH7 for 90 min is set to 1 (p=0.479).

(E) Relative sabA gene expression levels of 89’ strain grown at pH5 for 120 min shows a 5-fold decrease of sabA in comparison to 89’ strain grown at pH7 for 120 min. Mutant control strain 89’ grown at pH7 for 120 min is set to 1 (p=0.88).

(F) Relative sabA gene expression levels of 89’ strain grown at pH5 for 150 min shows a 2-fold decrease of sabA Mutant control strain 89’ grown at pH7 for 150 min is set to 1 (p=0.007).

(G) Relative sabA gene expression levels of 89’ strain grown at pH5 for 180 min shows a 7-fold increase of sabA in comparison to 89’ strain grown at pH7 for 180 min. This suggests that 89’ strain grown at pH5 for 180 min loses the sabA repression ability. Mutant control strain 89’ grown at pH7 for 180 min is set to 1(p=0.248).

(H) Relative sabA gene expression levels of 89’ strain grown at pH5 for 210 min shows no significant difference in comparison to 89’ strain grown at pH7 for 210 min. This suggests that 89’ strain grown at pH5 for 210 min loses the sabA repression ability. Mutant control strain 89’ grown at pH7 for 210 min is set to 1(p=0.873).
Figure 11. sabA Expression Level Change during Kinetics Experiments. The pH of broth, both pH 5 and pH 7, was nearly constant over the course of kinetics experiments due to the addition of 50 mM potassium phosphate buffer to the culture medium. The arsRS wild type H. pylori cells were cultured in pH 5 and pH 7 broth over time under standard culture conditions, with cell samples harvested at 30-minute intervals. H. pylori showed sabA repression immediately upon acid exposure. Between time points 0 and 150 min, the strain responded to pH 5 acid conditions by repressing sabA, in a statistically significant manner compared with the sabA expression level at pH7 (p<0.05). Beginning at 120 min, the H. pylori's acid repression of sabA began to degrade and was inapparent at 210 minutes. The observation suggests that the acid response in ArsR mutant is time sensitive.

ureA mRNA transcript levels in H. pylori maintained in a constant pH 5 Environment

H. pylori 26695, possessing a wild type arsRS locus, was incubated under
conditions of constant acidity at pH 5 and pH 7 using a 50 mM potassium phosphate buffer. At 30 min time interval, $10^9$ cells were harvested and the pH of its supernatant was recorded and to confirm the constancy of pH. The significant increase in ureA mRNA was not achieved until after 60min pH 5 exposure. Although the graph of transcripts level seemed to show an induction of ureA, the performance of a two-sample t test showed that there were not statistically significantly different from each other. This might be due to the large variances among the technical replicates of each of the samples. Compared to the pH 5 repression in sabA, this acid reponse displayed a delay for approximately an hour (Figure 12).

Starting to respond to acid at 60 minutes, ureA showed a 1.5-fold increase at pH 5 at 60 minutes, 2.8-fold increase at 90 min, 3-fold increase at 120 min, and peaked at an 8-fold increase at 150min (Figure 13).

At 210 minutes, the acid induction of ureA was lost, suggesting the ArsRS system functions in the relative short term and the gene expression changes mediated in response to acid must acclimate the bacteria to the acidity quickly.
Figure 12: ureA Expression Levels at Different Time Points.
89’ mutant is strain *H. pylori* 26695 strain 89’ is wild type with CAT gene upstream in HP0167.

(B) Relative *ureA* gene expression levels of 89’ strain grown at pH5 for 0 min shows no significant difference in the expression level of *ureA* in comparison to 89’ strain grown at pH7 for 0 min. Mutant control strain 89’ grown at pH7 for 0 min is set to 1 \( (p=0.83) \).

(B) Relative *ureA* gene expression levels of 89’ strain grown at pH5 for 30 min shows a 6-fold increase of *ureA* in comparison to 89’ strain grown at pH7 for 30 min. Mutant control strain 89’ grown at pH7 for 30 min is set to 1 \( (p=0.08) \).

(C) Relative *ureA* gene expression levels of 89’ strain grown at pH5 for 60 min shows a 1.5-fold increase of *ureA* in comparison to 89’ strain grown at pH7 for 60 min. Mutant control strain 89’ grown at pH7 for 60 min is set to 1 \( (p = 0.05) \).

(D) Relative *ureA* gene expression levels of 89’ strain grown at pH5 for 90 min shows a 2.8-fold increase of *ureA* in comparison to 89’ strain grown at pH7 for 90 min. Mutant control strain 89’ grown at pH7 for 90 min is set to 1 \( (p = 0.04) \).

(E) Relative *ureA* gene expression levels of 89’ strain grown at pH5 for 120 min shows an approximately 3-fold increase of *ureA* in comparison to 89’ strain grown at pH7 for 120 min. Mutant control strain 89’ grown at pH7 for 120 min is set to 1 \( (p = 0.01) \).

(F) Relative *ureA* gene expression levels of 89’ strain grown at pH5 for 150 min shows an approximately 8-fold increase of *ureA* Mutant control strain 89’ grown at pH7 for 150 min is set to 1 \( (p = 0.03) \).

(G) Relative *ureA* gene expression levels of 89’ strain grown at pH5 for 210 min shows no significant difference in comparison to 89’ strain grown at pH7 for 210 min. This suggests that 89’ strain grown at pH5 for 210 min loses the *ureA* induction ability. Mutant control strain 89’ grown at pH7 for 210 min is set to 1 \( (p = 0.09) \).
**Figure 13. ureA Expression Level Change during Kinetics Experiments.**

The pH of broth remains constant in the course of kinetics experiment due to the addition of potassium phosphate buffer. The wild type *H. pylori* 26695 cells were cultured in pH5 and pH7 broth for time different time length, with 30 minutes as an interval. *H. pylori* showed ureA induction starting from time 60 min upon acid exposure. From time 60 min to 150 min, the strain responded to pH5 acid conditions by inducing ureA, in a statistical significant manner compared with the expression level at pH7 (p<0.05). The induction level increases during the acid response time. At 210 min, the strain failed to regulate ureA (p>0.05). The observation suggests that the acid response in *H. pylori* is time sensitive.

**Discussion**

*Helicobacter pylori* is a gram-negative bacterium that colonizes the human stomach mucosal layer and can potentially lead to chronic inflammation and gastric
cancer. In the process of colonizing the most hostile environment associated with its human host, the stomach, *H. pylori* utilizes Two-Component Signal Transduction system (TCS) to mediate necessary gene expression changes.

With three complete TCS, *H. pylori* develops effective mechanisms to respond to different signals from its environment. Specifically, it utilizes an acid response system ArsRS to adapt to daily and long-term alterations in the acidic environment of mucosal layer in human stomach. ArsS is a histidine kinase that autophosphorylates in response to periplasmic pH change. ArsR is the cognate response regulator that receives the phosphoryl group from the phospho-histidine in ArsS. ArsR-Phosphate dimerizes and subsequently regulates the transcription levels of acid response genes, i.e., the ArsRS regulon. Experiments have demonstrated that the loss of the ArsS protein results in a viable mutant *in vitro*, but the loss of ArsR is lethal.

Since the classic paradigm of ArsRS system points out that histidine kinase activity from the H214 position in ArsS is key to its regulatory function of acid responsive gene expression, we wanted to determine if this enzymatic activity is required for acid gene regulation. We therefore created a histidine kinase minus
mutant by substituting the aspartic acid on 214 position of ArsS to alanine and found that this mutant lost its ability to repress \textit{SabA} or induce \textit{ureA} under acidic conditions. This loss of regulatory activity suggests that it is the enzymatic activity in ArsS that regulates acid responsive genes such as \textit{SabA} and \textit{ureA}. For a complete ArsRS system to function, the presence of ArsS alone is not sufficient. The phosphorylation of ArsS is necessary in the mediation of regulatory events affecting regulon genes.

It is well documented that ArsRS TCS regulates the expression level of the adhesion gene \textit{SabA} via phase variation (Forsyth et al. 2002, Goodwin, et al., 2008). The binding of ArsR in the promoter regions of \textit{SabA} results in acid repression \textit{in vitro}. In this study, we noticed similar DNA-protein specificity mechanisms of ArsR on the promoter regions of other important outer membrane adhesin genes such as \textit{labA}, \textit{hopZ}, and \textit{SabB}. This interaction was quite avid in nature, as unlabeled DNA containing the promoter regions could exhibit some competition, albeit only upon the addition of between 175-500 fold excess of the unlabeled probe.

Thus, we contend that this acid responsive system is a major control mechanism for the expression of outer membrane adhesins, and thus a major contributor to the
ability of this pathogen to colonize the human host. This observation further confirmed the importance of ArsR in regulating acid responsive genes.

Different than ArsS, the deletion of which is not lethal but the phosphorylation activity is essential for acidic responsive gene regulation, the deletion of ArsR is lethal. According to the TCS paradigm, the 52\textsuperscript{nd} position of ArsR receives the phosphoryl group from ArsS. This led us to become interested in this question: is the substitution of other amino acids on position 52\textsuperscript{nd} ArsR viable?

Our result shows that the substitution of glutamic acid, asparagine, and aspartic acid with an alternative codon were viable, while the substitution with alanine, glycine, and serine were not. This might be due to the fact that these substitutions interfere with essential regulation activities in the survival of \textit{H.pylori}. Because these amino acid cannot be phosphorylated, yet they maintain the vitality of \textit{H.pylori} cells, we hypothesized that the structure of glutamic acid and asparagine were similar to that of a phosphorylated aspartic acid. If this hypothesis were true, then the substitution of phosphomimetics amino acids should constitutively regulate acid responsive genes independent of pH.
Our data on D52E mutant, however, shows normal functionality in regulating *sabA* repression and *ureA* induction. A recent study on *Streptomyces coelicolor* has demonstrated the possibility of a phosphoryl group being transferred to alternative sites (Amin et al., 2016). According to this study, a response regulator, GlnM, involved in nitrogen metabolism is modified by both serine/threonine phosphorylation and leucine acetylation. This led us to hypothesize that this apparent normal function of ArsR D52E was due to the phosphorylation at a compensatory site. We further hypothesized that nearby aspartic acids, D47 and D59 may be phosphorylated to allow the normal acid repression of *sabA*. Using qRT-PCR, we demonstrated that the \( \Delta \text{arsS} \) mutant loses the ability to regulate the transcription levels of *sabA* and *ureA* in response to acidity. This loss of proper activity was due to the specific activity of the histidine kinase and not some other enzymatic function of ArsS as a ArsS H214A mutant (H214 is the site of phosphorylation) also fails to regulate *sabA* or *ureA* in response to acidity. The results show that wild type ArsR, ArsR D52E, and ArsR D47A D52E D59A all repress the transcription levels of *sabA* under acidic conditions (pH5). This observation suggests that the alanine substitutions did not affect the
functionality of ArsR, and that its non-phosphorylated phase regulates the transcription levels of corresponding genes. The same pattern was observed in ureA transcripts, suggesting that 47th and 59th positions were not alternative phosphorylation sites, and that the mechanisms through which the ArsR D52E did not constitutively activated genes transcriptions remained unknown.

In addition to the substitutions of amino acid in ArsR, the functionality of ArsRS TCS is also susceptible to time. Typically, H. pylori senses acid and can ameliorate this challenge by chemotaxis away from acid and using urease to buffer the local environment toward a more hospitable pH. We sought to examine the kinetics of ArsRS repression and induction of gene expression under constant acidity. After demonstrating the effective functioning of mutant ArsR D52E, we found that its repression of sabA under pH5 is evident immediately after pH 5 exposure and is maintained until approximately the 210 minutes point in a constant pH 5 environment.

A similar pattern is observed in experiments measuring the kinetics of ureA mRNA, despite the fact that ureA is induced under pH 5, rather than repressed as sabA is. In addition, the induction of ureA did not start immediately upon acid exposure. Instead,
there was a 60-min delay in its acid response. This delay might be due to the fact that when phosphorylated ArsR regulates the expression of \( \text{sabA} \), it binds to the operator region of \( \text{sabA} \) to repress \( \text{sabA} \) transcription. The synthesis of \( \text{sabA} \) mRNA transcripts therefore, decreases immediately. We speculate however that to induce the expression of \( \text{ureA} \) upon receiving acidity signals, primary transcripts of \( \text{ureA} \) have to be synthesized after ArsR binds to promoter regions and to activate transcription. As this is a processive phenomenon, it may take increased time to achieve measurable increases in \( \text{sabA} \) mRNA. Furthermore, \( \text{ureA} \) induction was observed to increase with time. Starting to respond to acid at 60 minutes, \( \text{ureA} \) showed a 1.5-fold increase at pH 5 at 60 minutes, 2.8-fold increase at 90 min, 3-fold increase at 120 min, and peaked at an 8-fold increase at 150 min. A longer time period allows more \( \text{ureA} \) transcripts to be produced. Therefore, the difference in time needed prior to appropriate acid response may be explained by the difference between two regulatory mechanisms; repression and induction.

Since the measurement of pH at different time points shows that pH remains constant with the addition of 0 mM potassium phosphate buffer in broth culture, we
eliminated the possibility that *H. pylori* neutralizes the environmental pH with the ammonia-producing enzyme urease.

Taken together, our data indicate the complex nature of the functionality of ArsRS TCS and underscores the importance of this TCS system to facilitate the decades long infection of arguably the most extreme environment associated with the human stomach condition.

**Funding**

Research was supported by grants to MHF from The National Institutes of Health, NAIAD; R-15 AI053062 as well as The College of William & Mary Roy R. Charles Center Honors Fellowship program, the Howard Hughes Medical Institute Undergraduate Research Grant to the College's Biology Department.

**Acknowledgements**

I would like to thank advisor, Dr. Mark Forsyth for his endless patience,
support, guidance, and encouragement throughout my research experiences at the College of William and Mary. I would also like to thank my collaborators Clara Sartor who helped with making ArsS H214A mutant, and Anna Kenan who helped with qrt-PCR on sabA and ureA expression levels on ArsS H214A mutant. Thank you to our Molecular Core Lab technician Lidia Epp who has helped me tremendously with RNA extraction and qRT-PCR training. Lastly, thank you to the committee members Dr. Paul Heideman, Dr. Matthias Leu, and Dr. Shuyin Jiao for your support.
References


unctional analysis of the *Helicobacter pylori* flagellar switch proteins


http://doi.org/10.1128/JB.00848-09


11. Ines Yang, Sandra Nell, Sebastian Suerbaum; Survival in hostile territory: the microbiota of the stomach, *FEMS Microbiology Reviews, Volume 37, Issue 5, 1 September 2013, Pages 736–761*


19. Tomb JF, White O, Kerlavage AR, Clayton RA, Sutton GG, Fleischmann RD,


