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Adaptation of *H pylori* to Changing Environments Based on Allelic Variation of Sensor Histidine Kinase Arss

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Adaptation of *H. pylori* to Changing Environments Based on Allelic Variation of
Sensor Histidine Kinase *arsS*

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A Thesis presented to the Graduate Faculty
of the College of William and Mary in Candidacy for the Degree of
Master of Science

Department of Biology

The College of William and Mary
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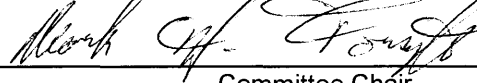
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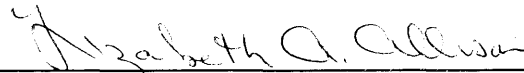
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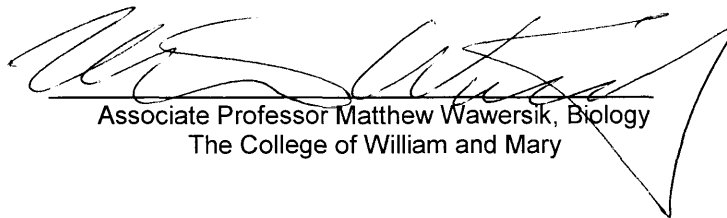


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ABSTRACT

Helicobacter pylori is a Gram-negative bacterium that infects the human stomach and can cause illnesses ranging from gastric or duodenal ulcers to gastric cancer. To survive in the human stomach, *H. pylori* must sense and respond to environmental signals, partially via two-component signal transduction systems that lead to altered gene expression as an adaptive response. *H. pylori* survives the changing acidic environment of the stomach, in part, via the two-component system, ArsRS. The sensory histidine kinase, ArsS, exists as variant isoforms due to mutations in a homopolymeric cytosine tract near the 3' terminus of *arsS*. This unusual location for a repetitive tract allows phase variation near the 3', rather than the 5' end of the gene, resulting in C-terminal variant ArsS isoforms, rather than affecting expression of the protein altogether. We hypothesize that the persistence of *H. pylori* infection may be furthered by its ability to generate variant populations expressing different isoforms of this critical sensory protein. To study the roles of these ArsS isoforms, *arsS* freeze frame mutants of *H. pylori* were created which express one isoform at a time. These mutants were made such that the third position of each proline codon encoded by the poly-C tract was altered to limit slipped strand mispairing, thus reducing the natural mutation rate of *arsS*. Mutants expressing the alternate ArsS isoforms were constructed by adding cytosines to change the reading frame. Amplified Fragment Length Polymorphism (AFLP) analysis of the mutants showed a reduction in, but not elimination of, allelic variation between the wild type *H. pylori* J99 and the three freeze framed *arsS* mutants. Frame shifted mutants demonstrated a change in the dominant reading frame and altered frequency of allelic variation. qRT-PCR was used to quantify expression of members of the ArsRS regulon among the *arsS* allele variants and showed some variation in response to regulon gene members between the *arsS* mutants at pH 7 and pH 5. The ability of *arsS* to mediate adaptation can also be addressed by examining the stratification of *H. pylori* strains in various regions of the stomach. A portion of the *arsS* alleles possessed by such strains that have already been selected for adaptation to different gastric regions were amplified and sequenced to correlate *arsS* alleles with potential adaptive benefits. Gastric region restricted *H. pylori* populations were further characterized via multi-locus sequencing. These results showed a difference between the cardia clonal strains and the antrum and corpus strains at all loci examined. A better characterization of *arsS* expression through such experiments will improve our understanding of how *H. pylori* is able to infect the human stomach for the lifetime of the host.

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This M.S. degree is dedicated to my loving husband, Lynch D. Bennett IV, who loved and supported me throughout this entire journey. I also want to thank him for his accompaniment on all those late night runs to the lab!

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Introduction to *Helicobacter pylori*

Helicobacter pylori is a Gram-negative neutrophilic bacterium that commonly infects the human stomach in individuals worldwide (Marshall & Warren, 1984). *H. pylori* colonizes the mucus layer covering the human gastric epithelium where the environment varies, but is closer to pH neutrality. Only some of the cells that colonize the human stomach will attach to epithelial cells (Hessey et al., 1990). Many of the cells will remain in the mucus layer where they can bind to glycosylated mucins (Lindén et al., 2002, Lindén et al., 2008). Infection with *H. pylori* usually occurs before age ten, typically acquired from a family member, usually the mother (Goodman & Correa, 2000, Perry et al., 2006). Infection by the bacterium is thought to occur through fecal-oral, oral-oral, or gastric-oral contact (Brown, 2000). Because of these routes of infection, areas with poor sanitation and hygiene, or intrafamilial clustering have higher risks of *H. pylori* infections (Mendall et al., 1992, Goodman & Correa, 2000, Bamford et al., 1993). The bacteria could be transmitted from one person to another via contaminated drinking water, or possibly vomit or stool during gastroenteritis (Parsonnet et al., 1999). In developing countries often 80% or more of the population is infected by *H. pylori*, whereas in developed countries the infection rate is closer to 40% (Dunn et al., 1997, Amieva & El-Omar, 2008).

People infected with *H. pylori* often harbor the infection for decades, if not their entire lifetime when untreated (Herrera & Parsonnet, 2009). While 80 to 90% of infected individuals never exhibit symptoms of the infection, others may develop peptic ulcer disease, atrophic gastritis, gastric adenocarcinoma or lymphoma (Makola et al., 2007, Parsonnet et al., 1999). This led to the bacterium being classified as a carcinogen by the

World Health Organization, the first bacterium to be identified as such (IARC Working Group 1994). Individuals that develop gastric or duodenal ulcers can be treated by eradicating the *H. pylori* infection (Makola et al., 2007). For those individuals in which *H. pylori* infection has resulted in gastric adenocarcinoma, the cancer is difficult to treat successfully and eradication of the bacteria does not cure the cancer (Herrera & Parsonnet, 2009). Carcinogenesis may result from the host response to the infection, resulting from chronic inflammation of the gastric epithelial lining, leading to an increased release of free radicals, release of cytokines, and instances of apoptosis in the human host (Herrera & Parsonnet, 2009). The bacterial oncoprotein CagA, discussed below, is considered a key mediator of carcinogenesis as *H. pylori* strains that lack this translocated effector rarely cause gastric neoplasms.

Key Virulence Factors and Adhesins

Other factors that can affect the virulence of *H. pylori* include several virulence proteins and adhesins. The best studied virulence determinants are VacA (vacuolating cytotoxin) and the cagPAI (*cytotoxin-associated gene-pathogenicity island*). Many studies have focused on the VacA mechanisms of modifying endosomal properties and maturation, which can lead to the formation of vacuoles in epithelial cells. Once secreted, VacA is endocytosed and vesicles progress into late endosomes. VacA then forms pores in the vesicles that serve as chloride channels. The altered anion concentration leads to osmotic swelling (Boquet & Ricci, 2012). VacA is also implicated in induction of host-cell apoptosis, which can begin through pore formation in the mitochondria (Willhite & Blanke, 2004). VacA also interferes with the host immune response by interfering with T

cell activation by preventing NF-AT from successfully translocating to the nucleus and by incorrectly activating Rac-specific nucleotide exchange factor (Boncristiano et al., 2003).

The *cagPAI* is a multi-gene locus that encodes a type IV secretion system (T4SS) in *H. pylori*. The T4SS forms a needle-like structure that translocates virulence determinants, specifically CagA and peptidoglycan, from the bacterium into the host epithelial cell upon contact (Rohde et al., 2003, Tanaka et al., 2003). Once CagA is in epithelial cells, it undergoes tyrosine phosphorylation at EPIYA motifs catalyzed by host cell Src family kinases and Abl kinase members c-Abl and Arg (Stein et al., 2002, Tammer et al., 2007, Poppe et al., 2007). These kinases are capable of phosphorylating CagA and have also been implicated as oncoproteins (Backert et al., 2011). Phosphorylated, CagA activates the phosphatases SHP-2 and ERK; the latter is a mitogen-activated protein kinase (MAPK) family member. This results in morphological growth changes to the cells (Higashi et al., 2004). Such changes to signaling pathways in the host cells have led CagA to be referred to as the master key, as it takes over the hosts signaling cascades which can affect membranes, cytoskeletal arrangements, and cell-cell junctions (Backert et al., 2010).

Adhesins, proteins that facilitate adherence of *H. pylori* to the gastric epithelium, are another important part of colonization for *H. pylori*. Attachment to the gastric epithelium can be beneficial to long-term colonization for the bacterium. Because there is secretion of mucins and shedding into the lumen, attachment of *H. pylori* to the epithelium can be important for long-term survival (Schreiber & Scheid, 1997). Two particularly well-studied adhesins are SabA and BabA. BabA is the blood group antigen

binding adhesion, which binds Lewis b (Le^b) and related ABO carbohydrate antigens associated with host epithelial cells (Ilver et al., 1998). Le^b is able to function as a receptor for *H. pylori* and functions as a dominant receptor in individuals who secrete the enzyme α 1,2-fucosyltransferase (Sakamoto et al., 1989). This enzyme functions to fucosylate (addition of a fucose sugar) oligosaccharide chains that ultimately leads to surface exposure (Green, 1989). SabA binds sialylated antigens expressed predominantly in inflamed gastric tissue. This adhesin binds specifically to glycosphingolipids with a specific type of carbohydrate, in this case, sialyl-dimeric Lewis X (sialyl-Le^x) antigens. SabA mediated *H. pylori* binding may contribute to virulence by allowing infection to persist long-term, despite host inflammation and immune response (Mahdavi et al., 2002).

Treatment of *H. pylori* infection

Infection with *H. pylori* is typically treated with antibiotics, however, efficacy of many of these drugs has declined recently. Clarithromycin resistance to *H. pylori* is especially noteworthy, as the antibiotic has been frequently used as a monotherapy (Megraud, 2004). Other antibiotics used in treating *H. pylori* infection include: amoxicillin, metronidazole, tetracycline, tinidazole, rifabutin and fluoroquinolones. Metronidazole resistant *H. pylori* have evolved, but they can frequently be overcome by increasing both the dose and treatment duration of the antibiotic. Amoxicillin resistant *H. pylori* remain quite rare (O'Connor et al., 2010, Mégraud & Lamouliatte, 2003). To overcome antibiotic resistant *H. pylori*, a newer therapeutic regimen referred to as quadruple therapy has recently been introduced, especially in areas of high

clarithromycin resistance. This treatment traditionally uses omeprazole, bismuth subcitrate, metronidazole, and tetracycline to treat infection. This is compared to the previous triple therapy that did not contain bismuth subcitrate (Selgrad & Malfertheiner, 2011). In a phase 3 trial in 2011, triple therapy was compared to quadruple therapy and had a much lower eradication rate of 55% compared to 80% for the quadruple therapy (Malfertheiner et al., 2011).

Life in an Acid Environment

For *H. pylori* to survive in the human stomach for a sufficient length of time to cause disease, it must have adaptations that allow it to sense and respond to the changing environment of the stomach, acclimating before it is eradicated by environmental factors. One of the greatest challenges to survival for the neutrophilic bacterium is the fluctuating acidic environment of the stomach. *In vitro*, *H. pylori* grows between pH 4.5-8 and at the optimum pH of 7.4 (Wen et al., 2003). However, in the human stomach it is exposed to even lower pH levels. Although, the gastric mucus layer where *H. pylori* is often found has a pH between 4.5-6.5, the bacterium must first pass through the low pH of the stomach lumen where the pH can be 3.0 or lower in order to colonize the mucus layer (Baumgartner & Montrose, 2004). Once in the mucus layer, there are changes of pH that occur due to feeding or fasting of the host. As a result, the pH ranges from 1.0 to 5.0 (Figure 1) (Pflock et al., 2006).

One of the ways *H. pylori* handles this acid stress is through use of the enzyme urease, which neutralizes the pH of the immediate area where the bacterium resides by hydrolyzing host urea into NH_3 and CO_2 , thus creating a microniche around itself that is

closer to neutrality. There are seven genes in the *H. pylori* urease gene cluster: *ureA/B/I/E/F/G/H*. *ureA* and *ureB* encode the subunits of the enzyme urease while four genes are involved in the recruitment of nickel to the urease apoenzyme to activate it. The final gene of this cluster, *ureI*, encodes the inner membrane pH channel that imports urea into the *H. pylori* cytoplasm (Wen et al., 2003). UreA and UreB as a holoenzyme hydrolyze urea and release ammonium ions (NH₄) and CO₂. The CO₂ is then converted to bicarbonate (HCO₃) by the periplasmic α -carbonic anhydrase. Bicarbonate buffers the surrounding cytoplasm and periplasm (Krulwich et al., 2011). The cytoplasmic urease is regulated by the pH-gated channel UreI in the inner membrane of the bacterium. This channel controls access of urea to the bacteria during changes in pH.

Urease is not the only system used by *H. pylori* to deal with the changing acidic environment. The bacterium also has a two-component signal transduction system, ArsRS (acid responsive signaling), that has been termed the master regulator for its ability to modulate acid regulation (Müller et al., 2009) and ArsRS also regulates *ureAB* transcription (Pflock et al., 2005, Marcus et al., 2012).

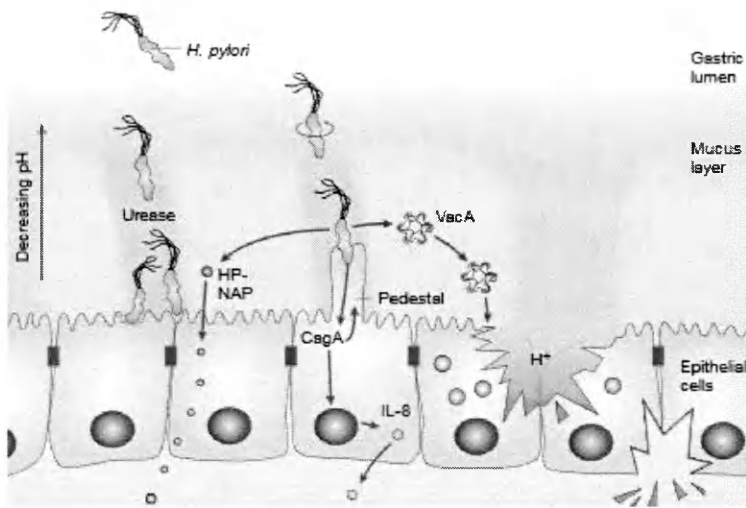


Figure 1: **Human stomach Mucous Layer and Lumen.** The gastric lumen of the stomach maintains a low and inconsistent pH within which, *H. pylori* cannot thrive. The area below the lumen is the mucous layer of the stomach. Here, the pH is more balanced so that the neutrophilic bacteria is able to survive. There is also a complex interaction between the host immune cells and *H. pylori* at the surface of the epithelial cells (Montecucco and Rappuoli 2001).

Two Component Signal Transduction

Two component signal transduction (TCST) is a type of signaling that makes use of a sensory histidine kinase and a cognate response regulator. TCST is found mainly in bacteria, but it is also used by archaea, a few lower eukaryotic organisms, and some plants. Eukaryotic organisms often use kinase cascades of Ser/Thr and Tyr phosphorylation, distinguishing them from the TCST observed in bacteria (Stock et al., 2000). The chemistries differ between these two types of signaling. Phosphorylation of Ser/Thr/Tyr results in phosphoesters while the histidine kinases of TCST create phosphoramidates. Phosphoramidates have a greater negative free energy than

phosphoesters, and this energy is used to drive the phosphoryl transfer in the signaling cascade (Stock et al., 2000). However, even though TCST is frequently observed in bacteria, the extent of use varies greatly among species. *Pseudomonas aeruginosa* possesses the highest number of two component systems identified to date with 64 response regulator and 63 histidine kinases (Rodrigue et al., 2000). In contrast, there are only three complete TCSTs, not including the CheAY chemotaxis system, within *H. pylori*. The focus of this thesis is on one of these systems, ArsRS, a pathway detecting and mediating the response to acid signals in the environment. *H. pylori* also has two other TCST: FlgRS which regulates flagellar gene expression and CrdRS that assists in adaptation to copper (Waidner et al., 2005, Niehus et al., 2004).

The process of signal transduction itself commences when a signal activates the sensory arm of the two component system, the histidine kinase. In the case of ArsRS in *H. pylori*, an external acid signal activates the histidine kinase ArsS. This kinase has its signal input domain exposed in the bacterial periplasm and anchored in the cytoplasmic membrane. The remainder of this membrane protein, including the kinase domain, lies inside the cell. Once activated, the histidine kinase dimerizes and undergoes autophosphorylation at an exposed, cytoplasmic histidine residue. Autophosphorylation occurs between the homodimers of the histidine kinase where one monomer catalyzes phosphorylation of the conserved residue of the second monomer (Stock et al., 2000). The response regulator then catalytically transfers the phosphate group to an aspartic acid residue on the response regulator protein ArsR, also within the cytoplasm. This phosphotransfer is a highly specific process within each two-component system and depends on molecular recognition between a histidine kinase and its cognate response

regulator. In some cases, the histidine kinase also has phosphatase activity which can remove the phosphate group from the response regulator when the need for a cellular response is no longer needed (Podgornaia & Laub, 2013). If each of these components are functioning properly, the response regulator ArsR, can now go on to affect gene transcription through activation or repression of members of the regulon (Loh & Cover, 2006, Panthel et al., 2003, Hallinger et al., 2012)

The domains of histidine kinases and response regulators have areas that are well conserved within these protein families. The histidine kinase superfamily usually contains a cytoplasmic kinase core coupled to a sensory domain. The kinase core can be subdivided into two domains: a well-conserved C-terminal ATP binding domain, and the less well conserved phosphotransfer domain which contains the histidine residue (Gao & Stock, 2009) (Figure 2). Further homology is seen within the kinase core in the form of sequence motifs termed boxes. There are five boxes, the H, N, G1, F, and G2 box, with the majority of boxes located in the ATP-binding domain and only the phosphorylation site containing H box located in the phosphotransfer domain (Marina et al., 2005, Marina et al., 2001). In the case of ArsS in *H. pylori* strain J99, the boxes show significant homology to those of other bacterial histidine kinases as would be expected, but there are significant difference at the carboxy-terminal end of the kinase. These differences include a unique 17-amino acid insertion that shares homology to annotated acetyltransferase proteins and an unusual poly proline tract (Figure 3).

The sensory domain is typically diverse between histidine kinases due to the variety of stimuli to which they must respond, including light, redox potential, acid, and electrochemical gradients (Szurmant et al., 2007). The prototypical response regulator

protein has an N-terminal REC domain (response regulator domain) linked to an effector domain. The REC domain is capable of catalyzing the transfer of the phosphate from the histidine kinase to the response regulator (Gao & Stock, 2009). More detail is known about the DNA-binding domain (DBD) of the ArsR response regulator as it has been analyzed using nuclear magnetic resonance spectroscopy methods. Results show that *H. pylori* ArsR belongs in the OmpR/PhoB subfamily of bacterial regulators. This placement is based on the overall structure of the DBD which consists of an N-terminal β -sheet, a helical core, and C-terminal β -hairpin. A winged helix-turn-helix motif is also present and is predicted to bind DNA (Gupta et al., 2009).

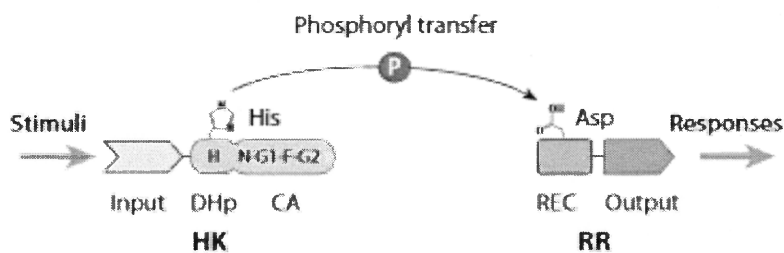


Figure 2. **Two-component system schematic with functional domains.** A typical two-component system is shown where stimuli serve as a signal input that results in an output signal and response. The histidine kinase (HK) is shown with 5 conserved amino acid motifs or boxes, the H, N, G1, F, and G2 box. The H box is part of the DHp or phosphotransfer domain and the other 4 boxes are part of the CA box or ATP-binding domain. Once the histidine kinase undergoes autophosphorylation at the histidine residue, a phosphate transfer occurs to the aspartate residue on the REC domain (response regulator domain) of the response regulator (RR) (Gao & Stock, 2009).

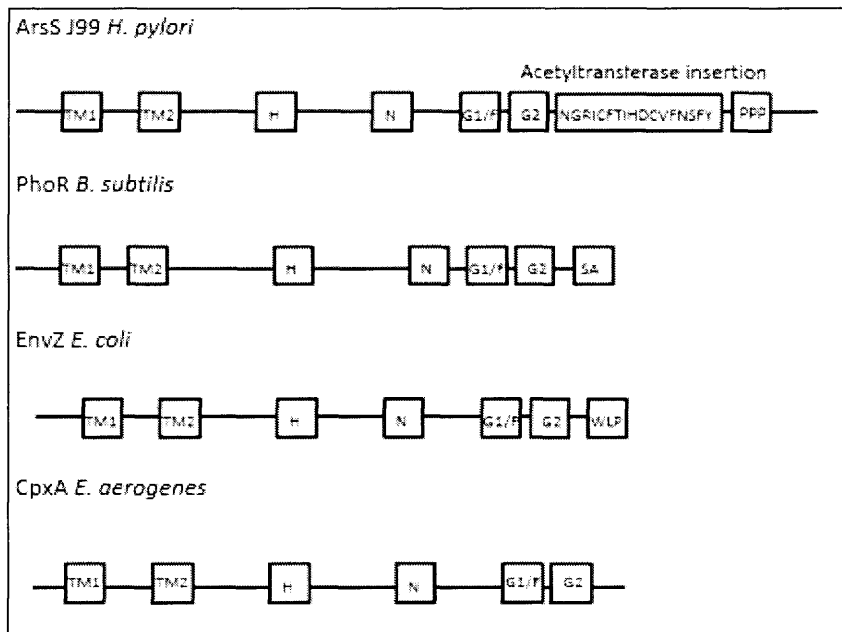


Figure 3. **Protein sequence motifs of histidine kinases in comparison to ArsS.** Three histidine kinases are compared to ArsS of *H. pylori* J99 to highlight the unique insertion of a potential acetyltransferase domain and poly proline tract at the C-terminal end of the kinase. TM1 represents the transmembrane α helix connected to a second transmembrane α helix, TM2. The H box contains the autophosphorylation box and histidine residue. The H box is phosphorylated by the ATP-binding domain which includes the N, G1, F, and G2 boxes.

ArsRS and Acid Acclimation in *H. pylori*

Global transcriptional profiling has shown differential expression of between 100 and ~280 genes when *H. pylori* is exposed to low pH. ArsRS is the master regulator that coordinates the response of all these genes (Merrell et al., 2003, Wen et al., 2003, Bury-Moné et al., 2004, Pflock et al., 2006). ArsS, while dispensable for *in vitro* growth, is necessary for the bacterium to colonize mice. In contrast ArsR is indispensable for *in*

in vitro growth of *H. pylori*, suggesting that ArsR is crucial in both a phosphorylated and non-phosphorylated form (Panthel et al., 2003, Schar et al., 2005). The histidine kinase, ArsS, does not sense the changes in pH based on the protonation of histidine residues of the signal input domain alone, but also in combination with various amino acids changing charge in response to the low pH stimulus. This led to the suggestion that ArsS may also change conformation depending on the protonation state of the signal input domain. This could generate a gradual transcriptional change in response to a pH stimulus as varying numbers of amino acids are protonated depending on the strength of the pH signal (Müller et al., 2009). This suggests a mechanism by which *H. pylori* is able to adapt to the varying stomach environments and respond accordingly.

A 2010 study by Loh *et al.* demonstrated that while ArsRS is sensitive to acid changes, it also regulates expression of proteins that affect acetone metabolism, resistance to oxidative stress, and motility. This study showed that even when *H. pylori* cells are not experiencing low pH conditions that would trigger the need for acid response, ArsRS still regulates other functions of the bacterium. ArsR, even in its non-phosphorylated state plays a key regulatory role in gene expression for the bacterium. Some of the proteins regulated by ArsRS that were identified in that study were AcxABC, the three subunits of acetone carboxylase. Acetone is an alternate carbon source and ArsRS appears to play a role in regulating the expression of these genes. *amiE*, *ureA/B/G/H*, *trxR1/2*, and *flaA/B/E/K/L* were just a few other ArsRS regulon genes that were also identified as playing a role in acid acclimation, oxidative stress, or motility. The arginase encoding gene *rocF* was also identified as a member of the ArsRS

regulon. This is an important enzyme in the urea cycle, and has also been indicated to be repressed in response to acid (Forsyth et al., 2002, Loh et al. 2010, Pflock et al., 2006).

H. pylori uses the enzyme urease (UreAB) to survive the acidic environment. Studies with ArsS knockouts show that urease can be upregulated during acid exposure by a secondary mechanism when ArsS is absent, possibly via FlgS (Marcus et al., 2012). Furthermore, studies show that urease knockouts of *H. pylori* are not able to effectively colonize animal models (Eaton & Krakowka, 1994, Mollenhauer-Rektorschek et al., 2002, Tsuda et al., 1994). These studies underscore the importance of the *ureAB* gene cluster as key members of the ArsRS regulon in adapting to the acidic environment. ArsRS is involved in the transcriptional regulation of urease genes, by binding *ureA* and *ureI* promoters (Wen et al., 2003).

Transcription of the *ureAB* genes is also regulated by another protein, NikR, but it seems to be of secondary importance to ArsR (Pflock et al., 2005). However, NikR is sensitive to increasing Ni²⁺ ions in the cytoplasm, which results from increases in acidity. Therefore, it appears that these proteins (ArsRS & NikR) work together to regulate urease production in *H. pylori* (Pflock et al., 2004). Urease is also dependent on Ni²⁺ ions due to its metalloenzyme nature (Moblely et al., 1988, Dunn et al., 1990). However, an accumulation of Ni²⁺ ions can have a toxic effect on bacteria so it is necessary that there to be strict intracellular control of metal ions (Contreras et al., 2003). NikR helps regulate this balance between Ni²⁺ ion and urease concentration by upregulating urease genes when there are available nickel ions (van Vliet et al., 2001, van Vliet et al., 2002). NikR may also be induced under acid conditions to aid the growth of *H. pylori* (van Vliet et al., 2004).

Ferric Uptake Regulator (Fur), is another metal-dependent regulator that functions along-side NikR and ArsRS to help adapt *H. pylori* to an acidic environment. Fur deficient mutants were hindered in their ability to grow at low pH (Bijlsma et al., 2002). Aside from its involvement in acid acclimation, Fur is a metal-dependent repressor of *H. pylori* genes involved in iron metabolism (Bereswill et al., 2000). Specifically, Fur maintains appropriate levels of Fe²⁺ levels and down-regulated transcription of iron transport systems when the levels increase too high (Andrews et al., 2003). *amiE* is another gene of the ArsRS regulon that encodes an aliphatic amidase, an enzyme that hydrolyzes short chain amides to produce ammonia (Loh et al., 2010, van Vliet et al., 2004). Studies have implicated Fur as regulating AmiE production. Under iron-restricted conditions, Fur has been shown to repress transcription of *amiE*. Although transcription of *amiE* is not controlled by Fur, *amiE* is induced under acid conditions when Fur is inactivated (van Vliet et al., 2002, van Vliet et al., 2004). In sum, ArsRS is an essential two component signal transduction system and it is of critical importance for *H. pylori* to handle the fluctuating acidic conditions of its environment.

Thesis Objectives

Hypothesis #1: The hypermutability of a 3' *arsS* poly-C tract generates variant ArsS carboxy terminal domain isoforms that confer selective advantages on *H. pylori* subpopulations within the human gastric environment.

The role of the *arsS* poly-C tract will be analyzed in my thesis studies by making various freeze frame mutations in the poly-C tract that will then be used to replace the wild-type *arsS* allele of *H. pylori* strain J99. The mutants created for this study will aim

to reduce the hyper-mutable nature of the poly-C tract by reducing its repetitive nature, thus making slipped-strand mutations less likely during chromosomal replication. The *arsS* freeze frame mutations will stably express one isoform, allowing for a comparison of their output under different acidic conditions and for differential expression via quantitative reverse transcription polymerase (qRT-PCR). We hypothesize that the generated ArsS variant isoforms are adaptive in variant stomach locations.

Hypothesis #2: Because different stomach regions contain different acidic profiles, *H. pylori* has preferentially developed isoforms of the histidine kinase ArsS that lead to adaptability in the changing stomach environment.

This hypothesis will be tested by analyzing stomach region-specific isolates of *H. pylori* from the stomachs of human patients. Sequencing of a portion of the *arsS* allele from each patient and from each region of the stomach will be compared to other clonal isolates to determine the level of divergence of *arsS* between the different clonal strains. Multi-Locus Sequence Typing (MLST) will also be performed on seven other housekeeping genes among the divergent strains to determine if *arsS* is the sole source of divergence or if polymorphisms are present at other loci. We hypothesize that the clonal isolates will show region-specific stratification in all loci examined.

Preliminary findings from a related study in our lab:

A recent study done in our laboratory examined the homopolymeric cytosine tract at the 3' end of *arsS* (subsequently referred to as the poly-C tract) from twelve age and gender matched patients with known clinical diagnoses (Hallinger et al., 2012) (Figure 2). Amplified fragment length polymorphism (AFLP) analysis demonstrated that in each

population of *H. pylori* examined, there are six to nine different *arsS* alleles resulting from variations in the length of the poly-C tract. The tract length varied from eight to seventeen tandem cytosine bases. These varying alleles can each produce functional ArsS isoforms, with more than one present in an *H. pylori* population at one time. It was also shown that usually one particular isoform dominates the population, but there are still quantities of the others present (Hallinger et al., 2012).

Histidine Kinase

Poly C tract
stop
ATG... N^{d248} ...CTACCCCCCCCCCCCCCGA.AAATTIGA

Figure 4. **Poly-C tract of *arsS***. The poly-C tract of *H. pylori* strain J99 is illustrated. The tract is ten cytosine bases long and encodes three prolines in the annotation of *H. pylori* strain J99, but the length varies among annotated genomes and within populations of clonal strains (Hallinger et al., 2012).

Chapter 1: ArsRS and the poly-C tract

An Introduction to the Research Problem

Helicobacter pylori is a bacterium that is not new to human biology. Genetic evidence has shown that *H. pylori* has co-evolved with humans for 60,000 years, and possibly longer (Linz et al., 2007). Because *H. pylori* is such a long-term colonizer of the human stomach, it can cause many problems over the lifetime of an individual.

Sometimes infection with *H. pylori* can lead to ulceration (Atherton & Blaser, 2009). *H. pylori* was the first bacterium to be designated a class I carcinogen, as infection may also lead to adenocarcinoma and MALT lymphoma (IARC Working Group 1994). In order to survive long enough to cause disease, the bacterium must successfully survive and thrive in the acidic environment of the stomach. It does this, in part, through two component-signal transduction systems. ArsRS is a two component system that mediates an acid responsive gene expression system. The histidine kinase, ArsS, contains a unique, hypermutable homopolymeric tract that generates different ArsS protein isoforms by altering the open reading frame in the 3' portion of *arsS*. One recent study from our lab (Hallinger et al., 2012) demonstrated that increased or decreased poly-C tract length in *arsS* allows several isoforms to be predicted in a single population. However, it is as yet unknown if variability of these ArsS isoforms confers selective advantages to *H. pylori* cells bearing altered ArsS carboxy-terminal domain proteins. This idea is attractive as an *H. pylori* population living in a variable environment creates sub-populations that make three ArsS isoforms. One isoform may offer adaptive advantage under a specific acid profile and thus a population expressing this isoform may predominate if the population is more optimally adapted to that specific pH environment. However, if the stomach acidity varies, the bacteria expressing the predominant isoform may be killed or outcompeted by cells that express a different carboxy-terminal domain of ArsS. The population of *H. pylori* may be lost if it were not for the presence of cells bearing each of the two alternate ArsS isoforms.

Due to the higher mutation rate of repetitive sequences such as the poly-cytosine tract via slipped strand mispairing, the alternate reading frames of *arsS* are continually

generated during replication. Those cells better adapted to the altered acidic environment can prevent the population of *H. pylori* in that region from being extirpated under the new environmental situation. Now the population possessing an alternate allele of *arsS* can multiply as it expresses a more appropriate isoform, taking over the predominant role in the *H. pylori* population. Bacteria utilizing such a population genetics strategy may continue to thrive.

In order to address this first aim of the study, it was necessary to generate *H. pylori* strains expressing a single ArsS isoform, not several. This was accomplished by making freeze frame mutations of the *arsS* 3' poly-C tract in an attempt to isolate the expression of a single ArsS isoform. These mutations changed the poly-C tract from sequential cytosine bases, encoding tandem prolines (CCC CCC CCC), to proline codons that are no longer homopolymeric (CCA CCT CCG). These mutations, along with an inserted antibiotic resistance gene, were used to replace the wild type *arsRS* operon to allow further study.

Materials and Methods:

Generation of Freeze Frame Mutants

The overall creation of the *H. pylori* strain J99 *arsS* freeze frame mutants involved several steps. Initially, a portion of *arsS* that contains the poly-C tract was targeted for PCR amplification, cloning and mutagenesis. This amplicon included the downstream gene, *hemB*, and a portion of the final gene of the *arsRS* operon, HP0162, encoding a putative transcription factor. For subsequent cloning purposes, a unique *Bgl*III restriction site was introduced by site directed mutagenesis (GeneArt, Life Technologies)

into HP0162 and a chloramphenicol acetyltransferase gene from *Campylobacter coli* (Wang & Taylor, 1990) conferring chloramphenicol resistance was cloned into the novel, unique *Bgl*III site. Oligonucleotide directed mutagenesis was then used to create freeze frame mutations in the poly-C tract of *arsS*.

To begin the process of cloning the portion of *arsS*, a primer was designed to anneal 1430 base pairs downstream of the poly-C tract and a forward primer annealing site was 668 base pairs upstream of the poly-C tract (primers MRB-B2 and MRB-A-, Appendix 1). Polymerase chain reaction using these primers created an amplicon of 2098 base pairs from *H. pylori* strain J99, a genome sequenced strain. The amplicon was cloned into an *E. coli* pCR4-TOPO vector (Invitrogen). This plasmid was designated pArsS1. A *Bgl*III site was added into the final gene of the plasmid construct for cloning an antibiotic resistance marker for subsequent screening for homologous recombination and allelic exchange in *H. pylori* strain J99. This was accomplished through site directed mutagenesis (GenArt, Life Technologies). This is a necessary step for the purpose of cloning chloramphenicol resistance into HP0162. The successful addition of the *Bgl*III site into the plasmid created a new plasmid, pArsS2.

In the next step, a *Bgl*III digestion was performed on pArsS2 to linearize the plasmid. The plasmid was then purified to remove any remaining *Bgl*III enzyme and buffer components using PCR purification spin columns (IBI) and treated with Klenow fragment and shrimp alkaline phosphatase (SAP) (New England Biolabs). Klenow enzyme treatments fills in the recessed ends thus creating blunt ends and SAP treatment removes the 5' phosphates so the plasmid is much less likely to ligate with itself. This was done with a Quick Blunting Kit from New England BioLabs. A Quick Ligation Kit

(New England BioLabs) was used with purified chloramphenicol acetyl transferase (CAT) gene, excised from pBSC103 via *SmaI* and *EcoRV* digestion, to ligate into pArsS2. This was successfully completed and CAT was ligated into the blunt ended *BglIII* site of pArsS2, as determined by selection in *E. coli* DH5 α for resistance to 25 μ g/ml chloramphenicol and sequencing confirmation of CAT gene placement and orientation. In one of the resulting constructs, pArsSCATControlA, the CAT gene is transcribed in the same direction as HP0162. In an alternate construct, the CAT gene is transcribed in the opposite direction of the HP0162, pArsSCATControlB. The CAT gene in these constructs possesses its own promoter. One form of the plasmid, designated, pArsSCATControlA, recombined easily with *H. pylori* J99 conferring resistance to 10 μ g/ml chloramphenicol and thus was used for all subsequent mutagenesis experiments (Figure 6).

Once the addition of the CAT gene was completed and verified, subsequent mutagenesis reactions were performed using pArsSCATControlA. This was again accomplished through an *in vitro* process accomplished using the GENART Site-Directed Mutagenesis System (Invitrogen). The plasmid DNA was methylated and specific primers, designed to complement the plasmid and introduce the designed mutations of the poly-C tract, were used to amplify a non-methylated amplicon from the methylated plasmid template in a sequential methylation/mutagenesis reaction. An *in vitro* recombination reaction immediately followed to enhance the probability of colony formation from the mutagenesis reaction. The mutagenesis mixture was then transformed into wild type *E. coli* DH5 α and an endonuclease was used to digest the methylated template. This leaves the unmethylated and thus mutated plasmid product (Figure 7). This

method was used to change every third position of the poly-C tract, encoding a poly-proline tract, to a different nucleotide thus allowing continued translation into a poly-proline tract, but potentially reducing the natural mutation rate by decreasing the repetitive nature of this portion of *arsS*. This reduces the rate of mutation in this region in *H. pylori* because it will no longer be a homopolymeric tract. The sequence will be mutated from CCC CCC CCC to CCT CCA CCG. The plasmid bearing these three silent mutations was designated pArsSFrame0A.

Another mutation was made in which the reading frame downstream of the poly-C tract in *arsS* was altered by adding an additional C to mimic a naturally occurring slipped-strand mispairing event. This change was in addition to the poly-C tract freeze frame mutations. This C addition was repeated so that there was a second frame shifting insertion in the 3' *arsS* poly-C tract mutants, one reading, CCT CCA CCG C and the other CCT CCA CCG CC. The plasmid with one cytosine inserted was designated pArsSFrame+1A, and that with two cytosine bases inserted was designated pArsSFrame+2A. The addition of the cytosines encodes different C-terminal ends and allows for functional investigation of the different ends of the ArsS gene (Figure 5, Table 1).

After mutagenesis, the TOPO vector with the modifications was used in a natural transformation of *H. pylori* strain J99. Allelic replacement of the wild-type *arsS-hemB-HP0162* locus with the mutant version encoded on the plasmid was selected for by the addition of 10 µg/ml chloramphenicol to the Sulfite-Free Brucella Broth Agar plates (SFBB) (1% tryptone, 1% peptamin, 0.2% yeast extract, 0.1 % dextrose, 0.5% NaCl, 7.5% agar) supplemented with 5% newborn calf serum (NCS). Recombination occurs

between the cloned *H. pylori* sequences of the *E. coli* plasmids and the *H. pylori* genome. The *arsS* sequence of *H. pylori* strain J99 was exchanged with the mutated alleles in the plasmid constructs described above in the *E. coli* plasmid, and the mutated poly-C tract (ex. CCACCGCCT) replaced the wild type sequence in *H. pylori* genome. The chloramphenicol resistance gene was also introduced into the *H. pylori* genome during this process. The *E. coli* plasmid is unable to replicate in *H. pylori* and is lost upon *H. pylori* subsequent cell divisions. Screening of the bacteria that have successfully undergone allelic exchange was accomplished by growing cells in the presence of 10 µg/ml chloramphenicol. Those colonies that were able to grow were screened by PCR and sequencing to confirm the presence of the mutated poly-C tract.

Natural transformations involved the growth of six plates of *H. pylori* J99 for use with each mutant plasmid. Cells were grown for 24-48 hours at 37° C and 5% CO₂ on trypticase soy agar plates with 5% sheep blood. Cells were then removed from the blood agar plates and suspended into 1 ml of SFBB with 5% NCS and 20 µg/ml vancomycin using a sterile cotton swab. The cells were harvested in a microcentrifuge for 2 minutes ~ 5000 x g. The supernatant was removed and the cells were resuspended in 100 µl fresh Brucella broth. Approximately 15 µg of the various mutation bearing plasmids, heat treated at 80°C for 20 minutes to reduce contamination levels, were then added to the *H. pylori* J99 cell suspension and mixed gently. The mixture was left to sit for 2 minutes before 30 µl was spotted onto blood agar plates until the mixture was depleted. The plates were incubated agar side down for four hours at 37°C/5% CO₂. The spots were then spread over the entire plate as a lawn and flipped agar side up to grow overnight. All growth from one plate was then transferred to a new SFBB/5% NCS plate with 10 µg/ml

chloramphenicol. Plates were left to grow at 37 °C 5% CO₂ environment for 5-7 days. Individual colonies were then picked with a sterile toothpick and patched onto a fresh blood agar plate about the size of a quarter, with room in between the patches. Patches were grown 48 hours before swabbing onto a new blood agar plate. From here plates could be grown to extract chromosomal DNA and make freezer stocks in SFBB/10% NCS/15% glycerol broth.

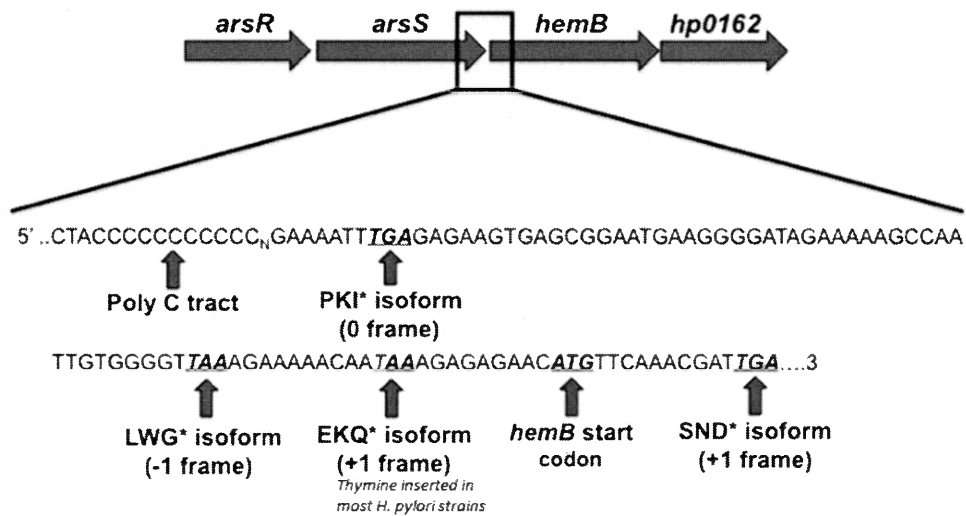


Figure 5. *ArsRS* operon with isoform options. The poly-C tract varies in length by slipped-strand mispairing during DNA replication which alters the downstream reading frames. This results in different *ArsS* isoforms possessing alternate C-terminal end domains.

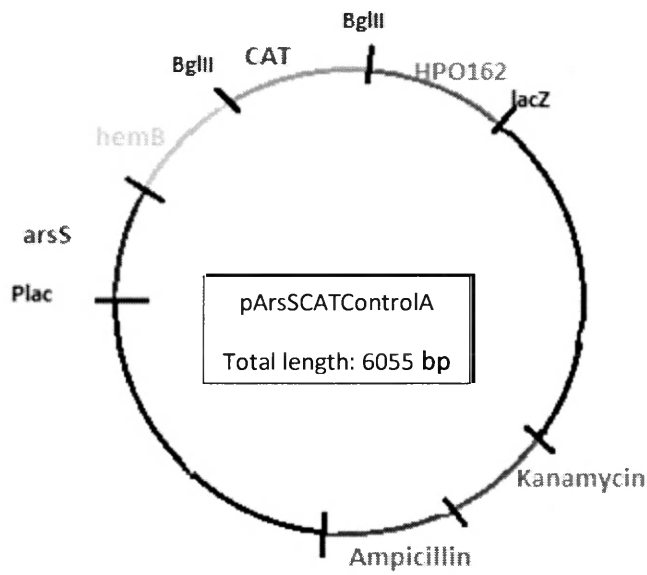


Figure 6. pArsSCATControlA. This shows the TOPO vector (pCR4) with the additional PCR product of '*arsS*, *hemB*, *CAT*, and *HP0162*' which have been cloned. The BglII site is lost upon blunt-ending during cloning of the *CAT* gene into the introduced BglII site.

Plasmid Designation	Description	<i>arsS</i> poly C tract	Protein Sequence
J99 WT	J99 WT	CCCCCCCCC	MRFSI X ₄₀₉ ADNEELPPPRRKFERGEGNEG NRKSQLWG
pArsSCATControl	CAT gene control	CCCCCCCCC...CmR	MRFSI X ₄₀₉ ADNEELPPPRRKFERGEGNEG NRKSQLWG
pArsSFrame0	CAT gene, Freeze frame	CCTCCACCGC... CmR	MRFSI X ₄₀₉ ADNEELPPPRRKFERGEGNEG NRKSQLWG
pArsSFrame+1	CAT gene, Freeze frame +1C	CCTCCACCGCC...CmR	MRFSI X ₄₀₉ ADNEELPPPKI
pArsSFrame+2	CAT gene, Freeze frame, +2C	CCTCCACCGCCC...CmR	MRFSI X ₄₀₉ ADNEELPPPENLREVKGMKGTEKANC GVKEKQK ERTCSND

Table 1. *E. coli* plasmids and *H. pylori* mutants used in this study. This table shows the name, description, and nucleotide and protein sequence of the mutants used.

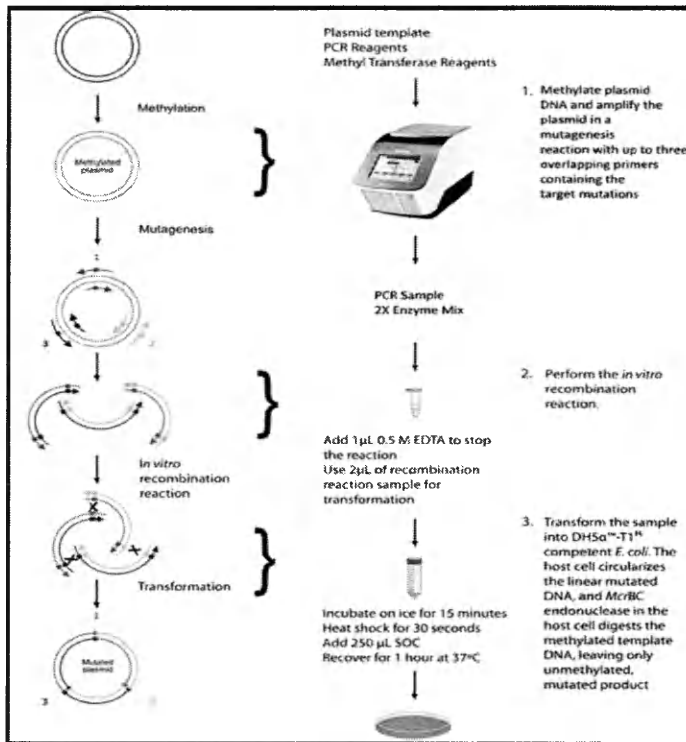


Figure 7. **A Schematic of GeneArt Mutagenesis (Life Technologies).** A brief outline of the site-directed mutagenesis protocol is shown with the methylation and mutagenesis reactions, *in vitro* recombination, and transformation steps highlighted.

Amplified Fragment Length Polymorphism (AFLP)

A VIC-labelled forward primer (Applied Biosystems), Oligo1, was designed to amplify the sequence upstream of the *H. pylori* strain J99 *arsS* poly-C tract and used in conjunction with reverse primer hemBR-1 (Appendix 1), to produce an amplicon approximately 300 base pairs long. This amplicon possessed any polymorphisms present due to slipped strand mispairing. Genomic DNA (gDNA) of the mutants was amplified with these primers using standard PCR procedure. The thermocycler was run with a hot start (94°C/5 minutes) and then for 30 cycles with a cycle at 94°C for 30 seconds, 60°C

for 30 seconds, and 72°C for 30 seconds. A final extension cycle ran at 72°C for 7 minutes. PCR products were cleaned up via ExoSAP-IT PCR Cleanup Procedure (Affymetrix). 5 µl of the PCR product was used for every 2 µl of ExoSAP. The reaction was incubated at 37°C for 15 minutes and then 80°C for 15 minutes. A 1:100 dilution of the ExoSAP purified product was made in sterile dH₂O and then run in a Genetic Analyzer 3500 Instrument (Applied Biosystems) with LIZ 300 standard to quantify peak height. Results were analyzed using the program GeneMapper 4.1 (Applied Biosystems).

Design of Polyclonal ArsS Antiserum

A custom polyclonal ArsS antiserum was created for these studies by Pierce Biotechnology for use in immunoblotting. The anti ArsS serum was made to peptide sequence RICFTIHDCVFNSFYDLEAD. This peptide is a portion of the cytoplasmic region of ArsS and is on the amino terminal side of the poly-proline tract encoded by the poly-C tract. The production took approximately 12 weeks, with 2 weeks for peptide synthesis, 1 week for peptide conjugation, and 10 weeks for further work with an animal protocol. Two rabbits were injected and bleed throughout that time period, with no additional boosting or affinity purification steps after day 72. All animal work was done by Pierce Biotechnology.

Sample Preparation and Immunoblot Protocol

Three plates of 36-48 hour *H. pylori* cells for each mutant were grown on blood agar and then swabbed into 1 ml 0.9% sterile saline. A 1:10 dilution was taken to determine O.D.₆₀₀ so that 1.55×10^9 cells could be harvested for each sample from the 1

ml of re-suspended cells. These cells were then centrifuged for 3 minutes at 5,000 x g and the pellet was re-suspended in an equal volume of 37°C warmed PBS (0.9% NaCl in 10 mM phosphate buffer, pH 7.4). This was repeated twice to remove any extra media. The pellet was then brought back up in 200 µl 95°C SDS sample solubilization buffer (1% SDS, 100 mM Tris-HCl, pH 9.5) and vortexed to mix. The samples were incubated at 95°C for 5 minutes and then cooled to room temperature. 250 µl of 2x SDS-PAGE sample buffer (Bio Rad) was added to each sample and then samples were incubated for another 20 minutes at room temperature. Samples were then centrifuged at 20°C for 20 minutes at 14,000 x g. The supernatant was stored at -20°C when not in use.

When ready to use, samples were heated to 95°C for 5 minutes and then cooled to room temperature. Pre-cast 4-15% gradient gels (BioRad) were prepared in the Mini-PROTEAN Tetra System (BioRad) with 10x SDS buffer (250 mM Tris, 1.92 M glycine, 1% SDS, pH 8.3) diluted to 1x SDS buffer in dH₂O. 15 µl of each sample, equivalent to ~ $N \times 10^6$ cells were loaded into the wells and run at 300v for 10-17 minutes or until necessary to reach the black line at the bottom of each gel. Once finished, the gels were washed in Towbin Western Blot Buffer (25 mM Tris, 192 mM glycine, 20% methanol (v/v), 1% SDS, pH 8.3) until the Western blot was set up. The filter paper, scotch pads, and nitrocellulose were all cut to the gel size and soaked in Towbin Buffer before assembly of the Western blotting cassette. The cassette was assembled as follows: scotch pad, filter paper, nitrocellulose, filter paper, with the nitrocellulose side inserted to face the red charge to allow proper transfer of the proteins from the gel. The cassette was assembled into the Mini-PROTEAN Tetra System (BioRad) with an ice pack and run for 1 hour at 100 v. Blots were Ponceau S (Sigma) stained to check for successful protein

transfer and then frozen at -20°C if necessary before immunoblot incubation with antibodies.

Nitrocellulose blots were blocked in 5% Bovine Serum Albumin (BSA) blocking buffer (20mM Tris-HCl, 500 mM NaCl, 0.1% Tween-20, 5% w/v BSA, pH 7.5) for one hour at room temperature, shaking at 20 rpm. The original blocking buffer was discarded and the blots were incubated with the primary antibody for one hour. The ArsS antisera was used for this purpose at a dilution of 1:10,000 in 5% BSA and incubated with shaking at room temperature at 20 rpm. After one hour, the primary antibody was removed and three wash steps of 15 minutes each were performed in Wash Buffer (20mM Tris-HCl, 500 mM NaCl, 0.05% Tween 20, pH 7.5). Blots were then incubated for one hour with a secondary goat anti-rabbit IgG horseradish peroxidase (HRP) antibody (Promega) at a 1:5,000 dilution in 5% BSA. After the secondary antibody was discarded, three more 15-minute washes were performed with fresh wash buffer each time. Blots were then incubated with sufficient ECL western blotting substrate (0.125 ml/cm²) (Thermo Scientific) to cover the blot. The ECL was mixed with an equal volume of detection reagent 1 and detection reagent 2. Once they incubated for one minute at room temperature with no shaking, blots were taken from the ECL and laid between layers of plastic wrap, moved into an autoradiography cassette (Fisher Biotech), and overlain with CL-Xposure Film (Thermo Scientific), and exposed for 1 minutes, 2 minutes, or 5 minutes as necessary in the Konica Minolta srx 101a developer.

Acid Shock Protocol

Three plates of 36-48 hour *H. pylori* cells for each mutant and strain J99 wild-type cells were used to inoculate 50 mls SFBB/10%NCS/vancomycin/pH 7 broth. Cells were grown overnight to an OD₆₀₀ of 0.5- 0.6. Each culture was then split into two equal parts and harvested at 1.9 x1000 g for ten minutes and the supernatant decanted. One pellet was re-suspended in SFBB/10%NCS/vancomycin/pH 7 broth while the other pellet was re-suspended in SFBB/10%NCS/vancomycin/pH 5 broth. The cells then incubated with shaking at 250 rpm at 37°C/5% CO₂ for one hour. Three milliliters from each culture were taken to harvest cells at a concentration of approximately 1.0 x 10⁹. Cells were then harvested by centrifugations at 1,700 x g for five minutes at 4 °C. The supernatant was saved and brought to room temperature in order to check the pH. The pellet for each sample was then re-suspended in 3 ml of Trizol reagent (Applied Biosystems) using trituration. Once re-suspended each sample was divided into 1 ml aliquots and frozen at -80 °C for subsequent RNA isolation and cDNA synthesis.

MagMax RNA Isolation and cDNA synthesis

If isolating RNA from plate grown *H. pylori* cells, three plates of 36-48 hour cells for each mutants were used to inoculate SFBB/10% NCS/vancomycin. Broth cultures were inoculated at a starting OD₆₀₀ of 0.25 and were grown until an OD₆₀₀ of 1.0-1.2. At this point, 1 x 10⁹ cells were harvested from each broth culture and spun down for 5 minutes at 1,700 x g at 4° C. The supernatant was decanted and the pellet resuspended in 1 ml Trizol Reagent per sample. Cells were either frozen at -80 °C or transferred to lysing matrix B bead-ruptor tubes (MP Biomedicals) at this time. Once in well-labeled bead-

ruptor tubes, the samples were loaded in the OMNI bead-ruptor 24 (Omni International) and the machine tightened and locked. The samples shook for 20 seconds. Once finished, the samples were placed back on ice and 100 μ l of BCP reagent (Molecular Research Center) were added to each sample to bind proteins and remove them from the solution. After the BCP reagent was added, the samples were vortexed and then placed back on ice. Once all samples were on ice they were incubated on ice for five additional minutes. Samples were then centrifuged at 4 $^{\circ}$ C for 10 minutes at 10,200 x g. While centrifuging samples, reagents were collected and aliquoted for the MagMAX express. All reagents used were from the 96 Kit for Microarrays (Ambion) and the protocol was adapted from manufacturer's recommendations. In brief, one MagMAX tray was collected for use with the MagMAX express machine (Applied Biosystems). 50 μ l of isopropanol were added to each divet in row A that would correspond with a sample. 150 μ l of Wash Solution #2 were added to rows B and C in each divet that corresponded with a sample. Finally, 30 μ l of Elution Buffer were added to row D in strips. When samples were done centrifuging, they were taken out with care not to disturb the clear organic:aqueous phase interface. 100 μ l of the upper phase from each sample were placed in strips in row A. 10 μ l of RNA binding beads were added to each sample in row A. The tray full of sample and a new, white, plastic comb were taken to the MagMAX express machine. The comb was placed in the instrument on the metal rack underneath magnetic rods. The white shelf was pulled out from the instrument and the tray was inserted with care to make sure the notch on the plate was under the rubber catch. The instrument was run on the AM1839 spin program. Once finished, the sample was eluted to row D and then harvested into RNase free tubes and placed on ice. Samples were then immediately quantified using the Nanophotometer

(Implen), and cDNA was made via an iSCRIPT cDNA synthesis kit (BioRad). The total reaction volume was 25 μ l, using 4 μ l from the master buffer, 1 μ l reverse transcriptase, 1 μ g of RNA, and remaining volume was sterile deionized water. A thermocycler program was run at 25°C for 5 minutes, 42°C for 60 minutes, and then 85°C for 5 minutes with only one cycle. The product was then frozen at -80°C until use.

ArsS Protein Half-Life Determination

Three plates of 36-48 hour blood agar plate grown cells for each of the *H. pylori* J99 wild type, control and *arsS* poly-C tract mutants were used to inoculate SFBB/10%NCS/vancomycin. Broth cultures were inoculated at a starting OD₆₀₀ of 0.25 and were grown until an OD₆₀₀ of 0.9, about 9-12 hours of growth. If necessary, the volume of each remaining culture was adjusted with fresh broth so that all OD₆₀₀ values were consistent. The total volume of each sample was harvested by centrifugation at 1,900 g for ten minutes at 4°C. Each pellet was resuspended in 1 ml of filter sterilized PBS before being added back to the culture flask with 49 ml of PBS. At this time, antibiotics were added to prevent transcription and translation. The cocktail consisted of rifampin, streptomycin and spectinomycin with final concentrations of 200 μ g ml⁻¹, 400 μ g ml⁻¹ and 100 μ g ml⁻¹ respectively (adapted from Hung *et al.*, 2013). Streptomycin and spectinomycin were resuspended in PBS and filter sterilized. Rifampin was re-suspended in methanol. Samples continued to shake for three hours and 1 ml aliquots were taken out every fifteen minutes. Once a sample was taken out it was harvested for 3 minutes at 5,000 x g. The pellet was re-suspended in 1 ml of 1x PBS to wash and remove extracellular proteases and growth media and centrifuged again for 3

minutes at 5,000 x g. The supernatant was decanted and the pellet was re-suspended in 200 μ l of 95°C SDS sample solubilization buffer (1% SDS, 100 mM Tris-HCl, pH 9.5). Each sample was vortexed to mix and incubated at 95°C for 5 minutes. The samples were cooled at room temperature and then 250 μ l 2x SDS-PAGE sample buffer (BioRad) was added to each sample. The samples were then centrifuged for 30 minutes at 14,000 x g at 20°C. The supernatant was harvested and 20 μ l was used to load pre-cast 4-15% gradient SDS-PAGE (Bio-Rad) gels.

Results and Discussion

Effects of *arsS* poly-C tract freeze frame mutations on *arsS* allelic frequency *in vitro*

Amplified Fragment Length Polymorphism (AFLP) was performed on *H. pylori* J99 WT and all three *H. pylori arsS* mutants and the control mutant by making use of VIC (Applied Biosystems) fluorescent labeled amplicons generated from each cell population. We hypothesized that phase variation based on the poly-C tract would generate sub-populations of *H. pylori* with varying length *arsS* alleles in both the WT and *arsSCATcontrol*, but phase variation would no longer be possible once the poly-C tract had been freeze framed by mutagenesis. A graph of the AFLP data compiled for both the WT and mutants is shown in Figure 8. As depicted in Figure 8, both *H. pylori* J99 and *arsSCATcontrol* mutants have AFLP amplicon lengths of 295 base pairs, 296 base pairs, and 297 base pairs, with 296 base pairs being the dominant length. This is the length predicted based upon the annotated sequence of *H. pylori* strain J99 and the amplicon length designed by the primers. This is the *ArsS* primary sequence predicted from the

original annotated genome sequence of *H. pylori* strain J99 (Alm et al., 1999). The results show that while the dominant subpopulation of *H. pylori* (length 296) had one particular poly-C tract and expressed one isoform, in about 40% of the population a cytosine base was either added or subtracted due to slipped strand mispairing acting on the hypermutable poly-C tract. This allelic frequency is similar for both *H. pylori* J99 WT and the *arsSCATcontrol*, as was predicted since the *arsSCATcontrol* possessed no mutation other than the insertion of the CAT gene ~1200 base pairs away. AFLP analysis of the *H. pylori arsS0Frame* mutant, possessing a stabilized (each third C changed to a different nucleotide) poly-C tract, but no manipulation of the tract length, showed a reduction in overall percentage of the amplicon length of 295 base pairs, but it still possessed *arsS* amplicons of 296 base pairs and 297 base pairs. The 296 base pair amplicon remained the dominant amplicon, encoding the ArsS isoform LWG as the dominant isoform. This implies that some variation is still inherent in this region of *arsS*, even without a homopolymeric tract, as about 25% of the population still added or deleted a cytosine to the poly-C tract. The change that is present in percent of the various *arsS* length sub-populations between *H. pylori* J99, *arsSCATcontrol* mutant, and the *arsS0Frame* mutant is expected. Because the *arsS0Frame* represents a freeze framed population of *H. pylori*, it would be expected to see a reduction in the distribution of *arsS* alleles present in a population, due to a reduction in slipped strand mispairing. This result suggests that our strategy of stabilizing the *arsS* poly-C tract length in a mutant population is a measured success. The addition of one cytosine in *arsS+1Frame* is shown in the graph by the peak length 297 and 298, with 296 no longer present. Now the predominant frame is 297 and not 296, and represents the ArsS isoform PKI*.

arsS+2Frame and the addition of the second cytosine leads to 298 and 299 being the only peak lengths present. Once again, the addition of the base has displaced the length of the frames. The dominant population is now frame 298 and not 297, which encodes the ArsS carboxy terminal isoform, SND.

A control VIC-labelled and unlabelled primer pair was used to amplify another portion of *arsS* to identify if the presence of extra amplicon peaks may be an artifact of PCR amplification. The primers amplified a 240 base pair region upstream of the poly-C tract and possessed no homopolymeric tracts greater than five bases. AFLP results show an amplicon with a length of 240 base pairs, which corresponds to the designed length (Figure 9). No other amplicons were generated suggesting that those additional amplicons generated when amplifying the region containing the poly-C tract were not artifacts. This indicates that the AFLP results as seen in Figure 8 are accurate when they show the differences in the populations between the mutant isoforms.

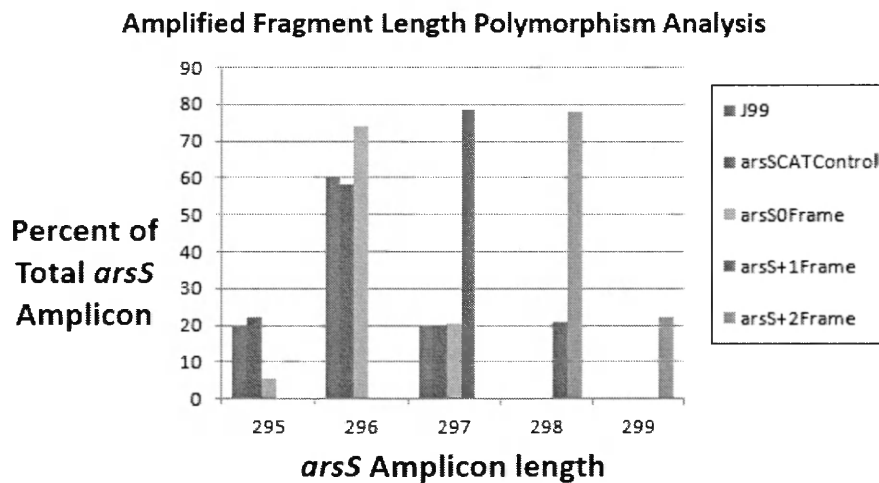


Figure 8. Amplified Fragment Length Polymorphism (AFLP) analysis comparing percent area of *arsS* poly-C containing amplicon length of *arsS* mutants in one AFLP run. *H. pylori* J99 and the *arsSCATcontrol* mutant each possess three subpopulations with similar levels of *arsS* poly-C tract lengths. The *arsS0Frame*, freeze-

framed mutant also possesses three subpopulations of cells with varying poly-C tract lengths as well with a similar dominant population to WT. The *H. pylori arsS+1Frame*, freeze-framed mutant has a dominant population amplicon length one base pair greater and the *arsS+2Frame* mutant has a dominant population that has been displaced by one whose poly-C tract is two base pairs longer.

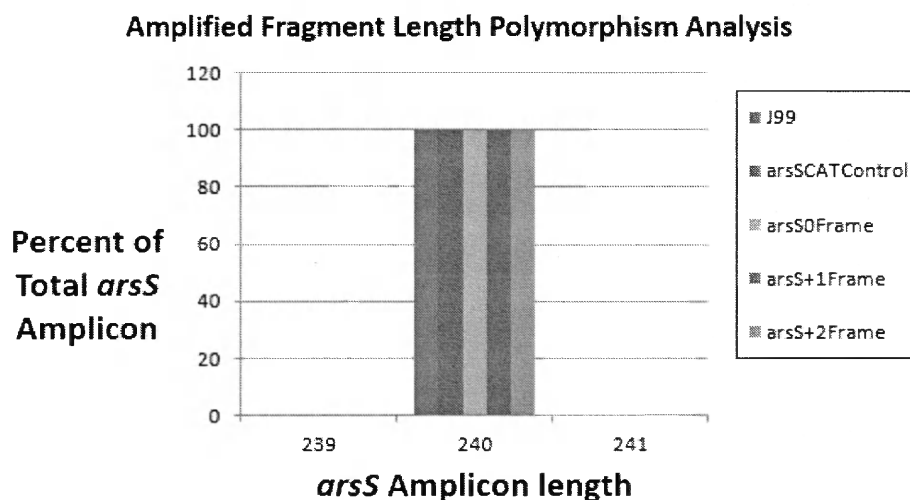


Figure 9. Control Amplified Fragment Length Polymorphism (AFLP) analysis. A control VIC-labeled primer was designed that amplified an amplicon 240 base pairs long. All mutant isoforms show an equal population with 240 base pairs being the only length present.

ArsS Protein Expression Analysis among *H. pylori arsS* poly-C mutants.

Once our AFLP analyses established differences at the population level, an important question to investigate was the continued expression of each ArsS isoform at the appropriate levels. Western blot analysis of ArsS protein expression was accomplished using custom ArsS polyclonal rabbit serum to determine the ArsS protein expression levels among the different *arsS* poly-C tract mutants. Pierce Custom Services designed a custom synthetic peptide based on a portion of ArsS and then went on to develop a polyclonal antibody against that peptide sequence (RICFTIHDCVFNSFYDLEAD). As shown in a representative immunoblot in Figure 10, there is an increase in ArsS expression with *arsS+1Frame* in the carboxy terminal domain

relative to the other *H. pylori arsS* mutants and wild-type *H. pylori* strain J99. This result is in contrast to the qRT-PCR transcription data (Figures 11-16) for this mutant, where there is no apparent increase in the *arsS* mRNA expression in the *arsS*+1Frame mutant that would parallel the increase in protein expression for the *arsS*+1Frame. The increased levels of *arsS*+1Frame carboxy terminal domain protein in the absence of increased transcription of this allele implies that there may be a difference in protein stability between ArsS C-terminal *arsS*+1Frame and other isoforms (*arsS*+2Frame). We hypothesize the *arsS*+1Frame isoform has a longer half-life due to greater stability. Therefore it is detected in greater amounts in immunoblot assays, but is not transcriptionally more highly expressed as evidenced in qRT-PCR assays for *arsS* mRNA. This led us to hypothesize that the different mutants have different protein half-lives and would degrade at different rates.

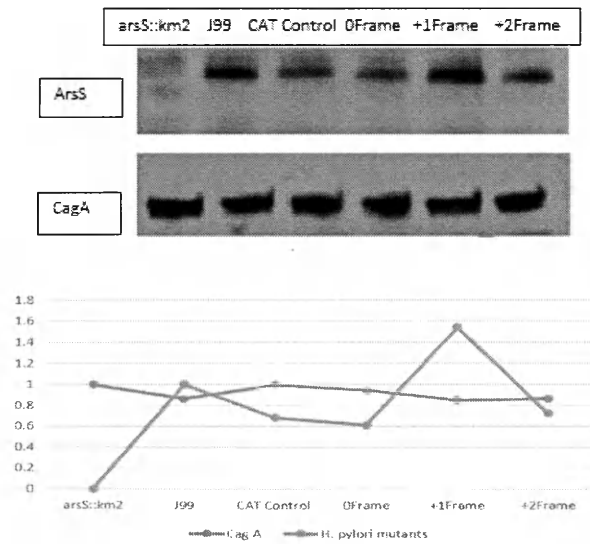


Figure 10. Differential ArsS protein expression among *H. pylori arsS* poly-C tract mutants. A representative immunoblot is shown after ArsS detection with polyclonal.

monospecific rabbit anti-ArsS peptide. The graph illustrates arbitrary intensity units to show the difference in protein expression among mutants. CagA was detected in the same western blot with a monoclonal antibody to CagA (Abnova) and served as a loading control.

Difference in Transcription of ArsRS Regulon Member Genes among *arsS* poly-C tract mutants

Another approach to dissect the roles of the various ArsS sensory histidine kinase isoforms is to compare their effects on the transcription of known target genes of the ArsRS signal transduction pathway. We hypothesized that for a given target or regulon gene, there would be a difference in the expression level from one *arsS* mutant compared to another. Comparisons of regulon gene mRNA levels were made between the *arsS* poly-C tract mutants expressing different isoforms, to see if there was altered expression of regulon genes from that occurring in the presence of alternate ArsS isoforms. Such differences arising among the ArsS isoforms mutants created for this study would indicate that ArsS C-terminal variants play a role in adaptation of *H. pylori* to changes in the environment. To test our hypothesis qRT-PCR was run using a StepOne Real-Time PCR System (Life Technologies). TaqMan primer/probe sets were made to a variety of genes including: *arsS*, *sabA*, *rocF*, *ureA*, *trxR1*, *amiE*, *acxA*, each of which are documented members of the ArsRS regulon (Forsyth et al., 2002, Loh et al., 2010). As controls, mRNA of *hemB*, *cagA* and *gyrB* were also quantified. A Welch's unpaired t-test was used to test for statistical significance between the technical replicates in one biological replicate. Statistically significant results are indicated by asterisks. A p-value \leq

0.05 is represented by *. A p-value of ≤ 0.01 is represented by ** and a p-value of ≤ 0.005 is represented by *** in Figures 11-16.

Representative qRT-PCR graphs are shown in Figures 11 through 16. Each graph represents at least three technical runs with error bars showing standard error between runs. Figure 11 is included as an endogenous housekeeping control. *gyrB* is a gene that encodes one subunit of DNA gyrase, an important part of the DNA replication process which cuts and supercoils DNA in bacteria. *gyrB* is not predicted to be regulated by ArsRS or affected by pH within the normal range of *H. pylori* growth, so the mutants were predicted to show similar expression of *gyrB* at pH 7 and 5. As predicted, the different mutants show similar expression of *gyrB* at pH 7. However, at pH 5 there is a significant difference between *arsS+2Frame* expression at pH 7 and pH 5. The difference is not even a two-fold and so it may not be reproducible. Another control, *hemB*, is shown in Figure 12. *hemB* is the gene immediately downstream of *arsS* and is co-transcribed as part of the operon *arsR/arsS/hemB/HP0162*. We were concerned that alterations in the *arsS* poly-C tract may affect the transcription of *hemB*. *hemB* is important in heme biosynthesis and respiration in the bacteria, so alterations in the poly-C tract might have negatively affected the bacterium. While there is not even a two-fold difference in expression, a t-test showed the difference between the *arsSCATcontrol* and *arsS+1Frame* at pH 7 to be significant. There was also a significant difference between the *arsS+1Frame* and *arsS+2Frame* mutants at pH 7 and pH 5 (Figure 12 A and B). Despite the apparent statistical differences, the lack of two-fold induction or repression of *hemB* expression indicated that there may not be an effect on the mutant's ability to grow or produce heme.

sabA gene expression was also explored as *sabA* is known to be an important outer membrane adhesion gene and expression is reduced when ArsRS is active (Mahdavi et al., 2002, Goodwin et al., 2008, Forsyth et al., 2002). The most notable difference in expression between the mutants at pH 7 can be seen when comparing the *H. pylori arsS+2Frame* to any of the other mutants, or to *arsSCATcontrol*. *sabA* expression of *arsS+2Frame* was more than two-fold repressed in comparison to the *arsSCATcontrol*. There was also a significant repression in *arsS+1Frame* compared to *arsSCATcontrol*. Both of these differences in expression were statistically significant. (Figure 13 A). There were no striking differences between the different *arsS* mutants at pH 5, however, there is a significant difference between expression of the mutants at pH 7 and pH 5 (Figure 13 B). This also indicates that experiment itself was successful and corresponds with the known biological relationship between *arsS* and *sabA*. In agreement with previous studies, this experiment shows that when *arsS* is induced due to acidic conditions, *sabA* expression is repressed (Goodwin et al., 2008, Forsyth et al., 2002). The structural changes made to the poly-C tract are small but the apparent functional differences they cause are seen in the differential transcriptional response of the *arsS* mutants to the *sabA* probe at pH 7. This differential expression of the protein isoforms appears to also be consistent when looking at probe *ureA* in Figure 14. Expression of *arsS+2Frame* is also differentially regulated at pH 7 in response to *ureA*. There is a more than 2.5-fold up-regulation of expression of *arsS+2Frame* in comparison to *arsSCATcontrol* (Figure 14 A). Even under standard pH conditions, there appears to be a difference between the mutants and the wild type. Furthermore, as shown in published studies on urease expression in *H. pylori* (Pflock et al., 2005), our studies clearly indicate transcriptional

induction of *ureA* upon acid shock at pH 5 (Figure 14 B). Though transcription of *ureA* is induced at pH 5 relative to transcription levels at pH 7 for WT and most mutants (Clyne et al., 1995), the *arsS+1Frame* shows a noticeable lack of induction to acid conditions. This would support a hypothesis that different *H. pylori* subpopulations expressing ArsS isoforms differ in regulon gene expression profiles. In this way, each subpopulation of *H. pylori* expressing a different isoform of ArsS may each be able to respond to host acidity in a specific way. *ureA* induction under acidic conditions is necessary due to the critical role of urease in acclimation to acid conditions. Hydrolysis of host or bacterial urea into CO₂ and NH₃ is a key component of the acid acclimation response of *H. pylori* as these molecules buffer the environment immediately surrounding the bacterium (Scott et al., 2007, Scott et al., 2010, Weeks et al., 2000). Ammonium ions produced by hydrolysis of urea are likely important to the overall nitrogen budget of *H. pylori* and enter into pathways for the biosynthesis of nucleic and amino acids.

rocF, encoding an arginase, is another member of the ArsRS regulon (Forsyth et al., 2002, Loh et al., 2010). RocF is an important member in the urea cycle and hydrolyzes arginine to urea and ornithine. However, the host supplies of urea may often be sufficient for urease to create sufficient buffering capacity, as *rocF* mutants are still able to colonize a mouse model of infection successfully (McGee et al., 1999). Though it may not be necessary for colonization, transcription of *rocF* is increased under low pH conditions (Wen et al., 2003). This is in apparent disagreement with results from a 2006 study by Pflock et al. That study demonstrated *rocF* is transcriptionally repressed by ArsR-P, the form of the response regulator predicted to predominate under acidic conditions. Our examination of *rocF* transcription yields results that are consistent with

those of Pflock et al. as we find that *rocF* transcription is repressed under pH 5 conditions when ArsRS is activated (Figure 15 B). This is especially noticeable between J99 WT and the *arsSCAT* control at pH 7 and 5, as the difference in expression between the pH levels is significant. There is an interesting exception to this acid repression of *rocF* in the presence of the *arsS+2* isoform. The qRT-PCR results of *rocF* transcription among our *arsS* mutants are particularly striking as in the *H. pylori arsS+2Frame* mutant, *rocF* is induced at pH 5 whereas in each of the other mutants and wild-type *H. pylori* strain J99, *rocF* is transcriptionally repressed at pH 5. There may be a trend among the ArsRS regulon members for the mutant ArsS isoforms to mediate different levels of regulon gene transcription compared to one another and to wild type *H. pylori* strain J99. While there may be subtle changes in transcription of regulon members under the influence of different ArsS isoforms, *ureA* and *rocF* are the most striking alterations as yet discovered (Figure 14 and 15). At pH 7 alone when the ArsRS system is at its lower activity and thus ArsS-P is minimal, there is not even a two-fold difference in induction of *rocF* of one mutant compared to another, however, some of these differences were shown to be significant using a t-test (Figure 15 A).

In one study examining the transcriptional control by ArsRS in *H. pylori*, active kinase domains of the three isoforms of ArsS were expressed recombinantly in *E. coli* to test whether differences in the open reading frames would change the ability of ArsS to ArsR phosphotransfer (Müller et al., 2006). Each of the ArsS isoforms were able to successfully autophosphorylate and transfer the phosphate group from ArsS to ArsR, with no significant difference at pH 7 or 5. Despite this uniformity in the ability to phosphorylate ArsR, our results suggest that different isoforms have functional

transcriptional differences even though they are each capable phosphorylation. The different *H. pylori* mutant isoforms created affect a slightly different transcriptional response in members of the ArsRS regulon.

Finally in Figure 16, *arsS* transcription itself was explored as a function of the various ArsS isoforms produced. This was examined as ArsRS is autoregulatory (Pflock et al., 2006), therefore we questioned whether the various isoforms of ArsS could differentially express *arsS* itself. Results show a significant difference between J99 WT and the *arsS*SCATcontrol. Because of this difference, these results may not be trustworthy or reproducible. The results show a small difference in *arsS* mRNA levels between the *H. pylori arsS*+2Frame mutant and *H. pylori* J99 wild type. At pH 7, there was an induction of *arsS*+1Frame mutant and not *arsS*+2Frame mutant, indicating the two mutants show differences in transcriptional regulation depending on the pH (Figure 16 A). Notably, not all mutants are induced to the same extent as may be expected under acidic conditions, based on published studies (Pflock et al., 2006) showing ArsRS operon induced by acid or autoregulated. It seems that there are subtle differences in *arsS* transcription among the individual *arsS* poly-C tract mutants and WT that suggest different ArsS carboxy terminal domains do affect *arsS* gene expression.

Based on the results thus far, it would seem that *H. pylori* J99 *arsS*SCATControl and J99 WT are comparable in controlling transcription of the regulon gene members, with no major changes in transcription being seen between the two strains. This indicates that the addition of the CAT gene disrupting the hypothetical protein HP0162 does not alter ArsRS system functionally. In general, *arsS*0Frame does not mediate large inductions in transcription up or down when compared to *arsS*SCATControl among the

regulon members. However, *arsS*+1Frame and *arsS*+2Frame show differences in transcription when compared to *arsS*SCATControl in several regulon members, most notably *rocF* and *ureA*. These responses imply differences in the carboxy terminal ends to effectively adapt to acid and respond to members of the ArsRS regulon.

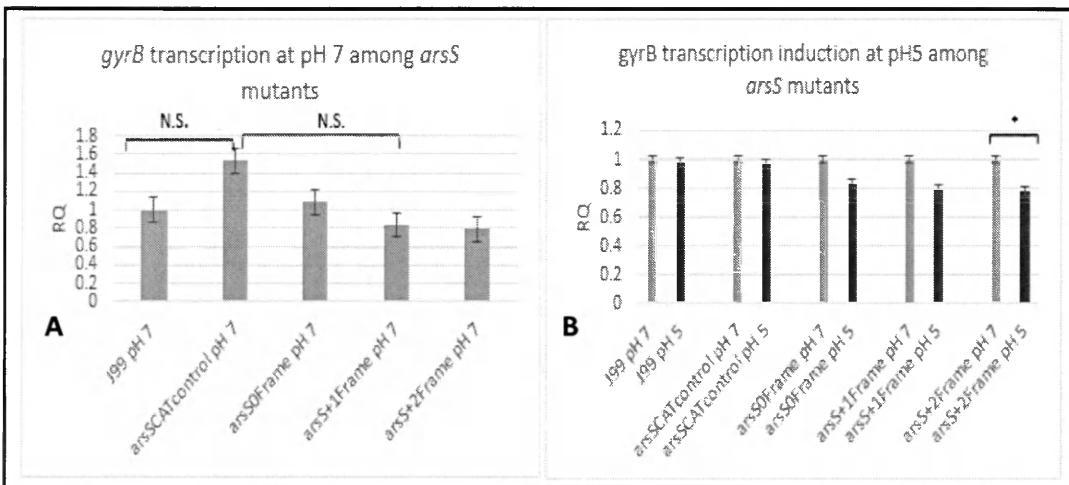


Figure 11. Differential gene expression and induction among *H. pylori arsS* poly-C mutants and *gyrB* control at pH 7 and 5. *gyrB* relative transcription (RO) levels were measured among the various *H. pylori arsS* poly-C tract mutants. In graph A, the *H. pylori* WT strain J99 was set to 1. Expression of different mutants at pH 7 is between 0.8 and 1.5 for all mutants measured. In graph B, each pH 7 mutant is set to 1 to show induction at pH 5. Expression of mutants is between 0.6 and 1.0. There is a significant difference between expression of *arsS*+2Frame at pH 7 and pH 5. A p-value that is not significant is represented by N.S. A p-value < 0.05 is represented by *.

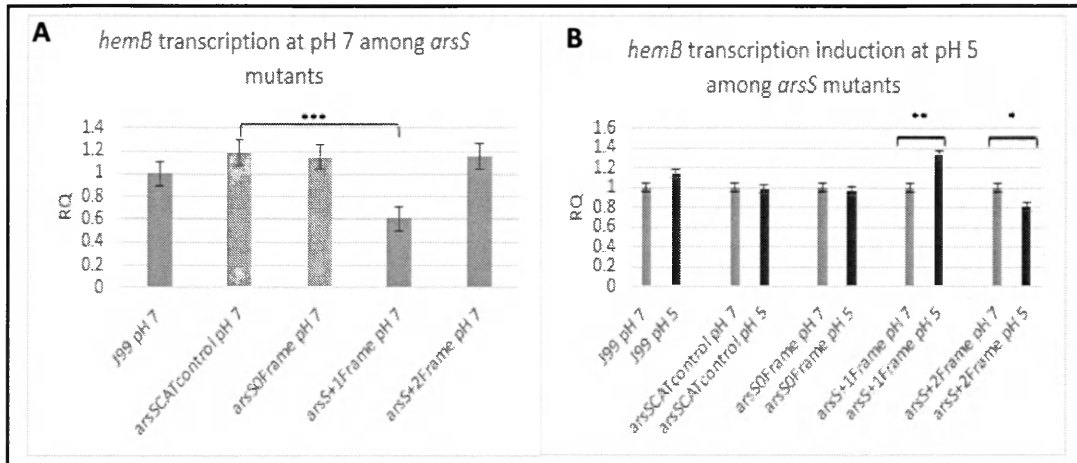


Figure 12. Differential gene expression and induction among *H. pylori arsS* poly-C mutants and *hemB* control at pH 7 and 5. *hemB* relative transcription (RO) levels were measured among the various *H. pylori arsS* poly-C tract mutants. In graph A, the *H. pylori* WT strain J99 was set to 1. Expression of different mutants at pH 7 is between 0.6 and 1.2 for all mutants measured. There is a statistical difference between *arsSCAT*control expression and *arsS+1*Frame expression. In graph B, each pH 7 mutant is set to 1 to show induction at pH 5. Expression of mutants is between 0.8 and 1.3. There is a statistical difference between *arsS+1*Frame and *arsS+2*Frame expression at pH 7 compared to pH 5. A p-value < 0.05 is represented by *. A p-value of ≤ 0.01 is represented by ** and a p-value of ≤ 0.005 is represented by ***.

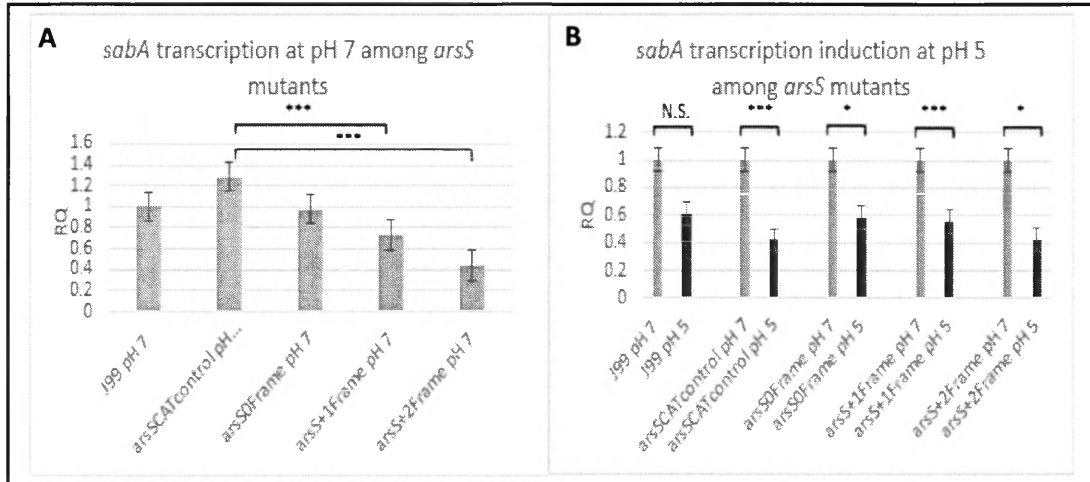


Figure 13. Differential gene expression and induction among *H. pylori arsS* poly-C mutants and *sabA* at pH 7 and 5. *sabA* relative transcription (RO) levels were measured among the various *H. pylori arsS* poly-C tract mutants. In graph A, the *H. pylori* WT strain J99 was set to 1. Expression of different mutants at pH 7 is between 0.44 and 1.3 for all mutants measured. There is a statistical difference between *arsSCAT*control and *arsS+1*Frame, as well as *arsS+2*Frame. In graph B, each pH 7 mutant is set to 1 to show induction at pH 5. Expression of mutants is between 0.42 and 0.61. There is a statistical difference between *arsSCAT*control, *arsS0*Frame, *arsS+1*Frame and *arsS+2*Frame expression at pH 7 compared to pH 5. A p-value that is not significant is represented by N.S. A p-value ≤ 0.05 is represented by *. A p-value of ≤ 0.01 is represented by ** and a p-value of ≤ 0.005 is represented by ***.

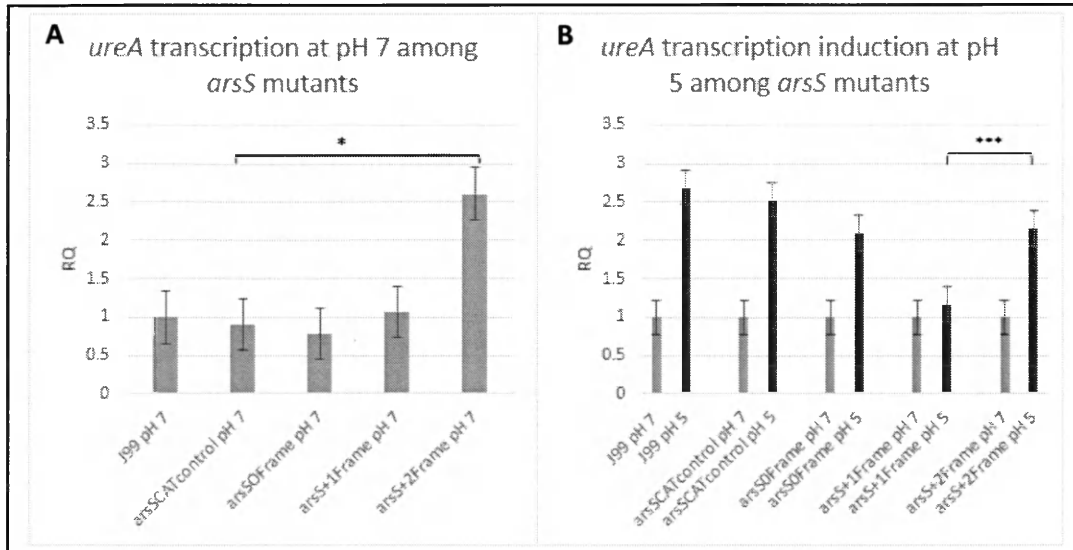


Figure 14. **Differential gene expression and induction among *H. pylori arsS* poly-C mutants and *ureA* at pH 7 and 5.** *ureA* relative transcription (RO) levels were measured among the various *H. pylori arsS* poly-C tract mutants. In graph A, the *H. pylori* WT strain J99 was set to 1. Expression of different mutants at pH 7 is between 0.8 and 2.6 for all mutants measured. There is a statistical difference between *arsS*SCATcontrol and *arsS*+2Frame. In graph B, each pH 7 mutant is set to 1 to show induction at pH 5. Expression of mutants is between 1.2 and 2.7. There is a statistical difference between *arsS*+1Frame expression and *arsS*+2Frame expression. A p-value < 0.05 is represented by *. A p-value of < 0.01 is represented by ** and a p-value of < 0.005 is represented by ***.

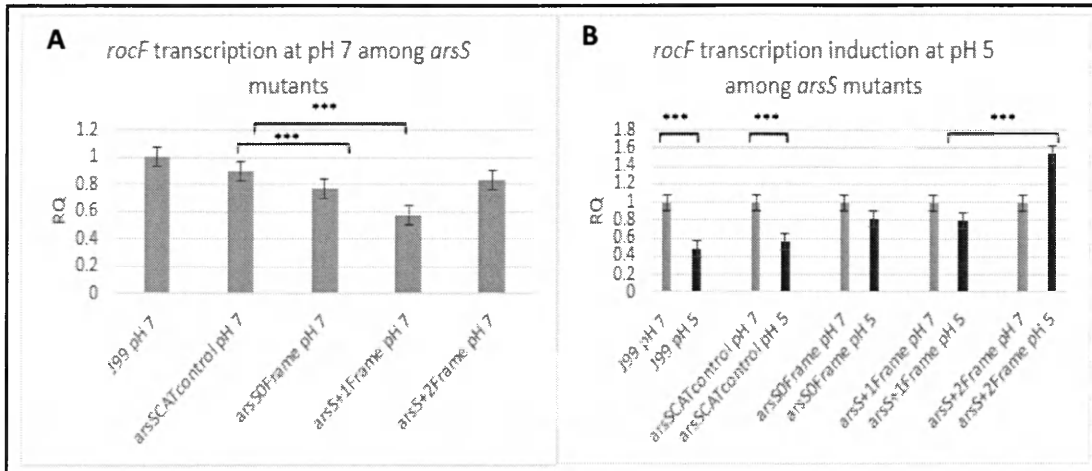


Figure 15. Differential gene expression and induction among *H. pylori arsS* poly-C mutants and *rocF* at pH 7 and 5. *rocF* relative transcription (RO) levels were measured among the various *H. pylori arsS* poly-C tract mutants. In graph A, the *H. pylori* WT strain J99 was set to 1. Expression of different mutants at pH 7 is between 0.6 and 0.9 for all mutants measured. There is a statistical difference between *arsSCATcontrol* and *arsS0Frame*, as well as *arsS+1Frame*. In graph B, each pH 7 mutant is set to 1 to show induction at pH 5. Expression of mutants is between 0.5 and 1.5. There is a statistical difference between *arsS+1Frame* expression and *arsS+2Frame* expression. There is also a statistical significant difference between J99 WT and *arsSCATcontrol* expression at pH 5 compared to pH 7. A p-value of < 0.01 is represented by ** and a p-value of < 0.005 is represented by ***.

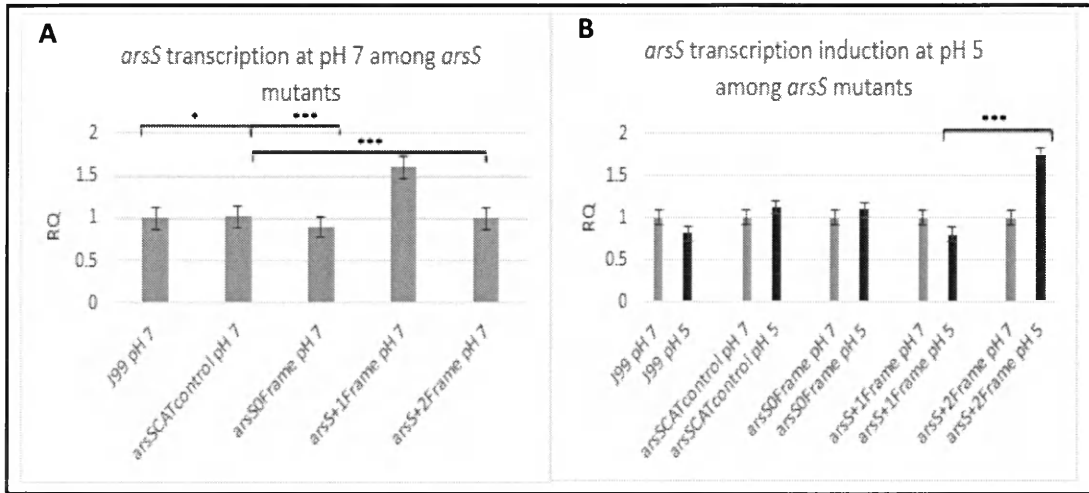


Figure 16. **Differential gene expression and induction among *H. pylori arsS* poly-C mutants and *arsS* at pH 7 and 5.** *arsS* relative transcription (RO) levels were measured among the various *H. pylori arsS* poly-C tract mutants. In graph A, the *H. pylori* WT strain J99 was set to 1. Expression of different mutants at pH 7 is between 0.9 and 1.6 for all mutants measured. There is a statistical difference between *arsS*SCATcontrol and J99 WT. There is also a statistical difference between *arsS*SCATcontrol and *arsS*0Frame, as well as *arsS*+2Frame. In graph B, each pH 7 mutant is set to 1 to show induction at pH 5. Expression of mutants is between 0.8 and 1.8. There is a statistical difference between *arsS*+1Frame expression and *arsS*+2Frame expression. A p-value < 0.05 is represented by *. A p-value of < 0.01 is represented by ** and a p-value of ≤ 0.005 is represented by ***.

ArsS protein stability as a function of the various carboxy terminal domains dictated by variations in the poly-C tract.

H. pylori ArsS isoform half-life studies were used to analyze and compare the stability of the various isoforms to normal protein turnover *in vitro*. We hypothesized that there would be a difference in protein degradation and protein half-life between the different *H. pylori* mutants. Using the polyclonal anti-ArsS antiserum, immunoblot experiments were used to determine the half-life of the varying isoforms and compare their stabilities. It would be interesting to determine if the different amino acid sequences of the various carboxy-terminal domain ArsS domains affected changes in the protein half-life and if these isoforms are the ones that usually predominate in *H. pylori* populations. It is possible that the *H. pylori* ArsS isoforms with the shortest half-lives will tend to predominate because a high turnover is preferable in the changing stomach environment. Current progress on this experiment is shown in Figure 17, however, currently there is no real change shown between the mutants. ArsS appears to be a stable protein overall and difficult to degrade via current experimental methods. More time is needed to troubleshoot this process before a successful protein stability assay could succeed.

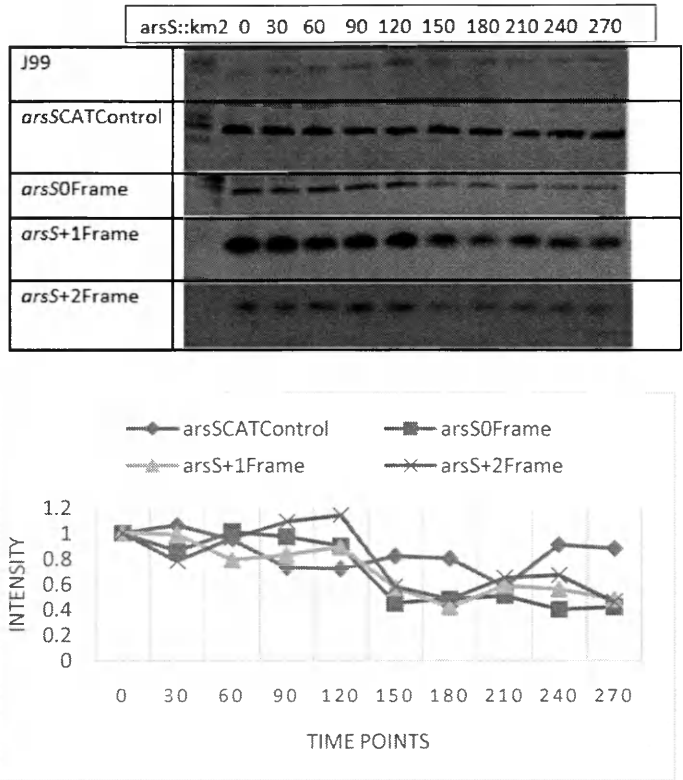


Figure 17. **Protein stability among *H. pylori* *arsS* poly-C mutants.** Differential protein stability is shown between the *arsS* poly-C mutants. The *arsS* protein of all mutants is shown to degrade at about 120 minutes.

Chapter 2: Stomach Specific Stratification in the Cardia, Corpus, and Antrum

Introduction

In a recent study in our lab using non-clonal populations of *H. pylori* from the cardia, corpus and antrum, we generated preliminary evidence that there are stomach region specific *H. pylori* *arsS* polymorphisms in three out of twelve patient samples examined (Hallinger et al., 2012). An amplicon from within the *arsS* coding sequence of each patient was generated from the total *H. pylori* population from each of three regions

of the stomach: antrum, cardia, and the corpus. The amplicons were cloned into a plasmid vector and ten plasmids from each region were sequenced and analyzed. Region specific sequence polymorphisms of *arsS* were identified in this process (Figure 18). We hypothesize that there has been selection for specific *H. pylori* strains in one region to the exclusion of the strains found in the other regions of the stomach. These strains may be better able to colonize and out-compete other *H. pylori* strains that are less fit due to local variations in the gastric environment. Mutations may be selected for by the environment explaining the polymorphisms in these particular strains, making them more fit in that particular stomach location. One *H. pylori* locus that may be subject to selection is *arsS* as it plays a crucial role in acid adaptation. In addition to polymorphisms in the *arsS* coding sequence, phase variation in the carboxy terminal region may allow *H. pylori* strains expressing a specific ArsS isoform to adapt better to a specific gastric acid profile. We hypothesize such *arsS* polymorphisms may help them colonize specific stomach niches.

CLUSTAL W Alignment of internal *arsS* from co-resident

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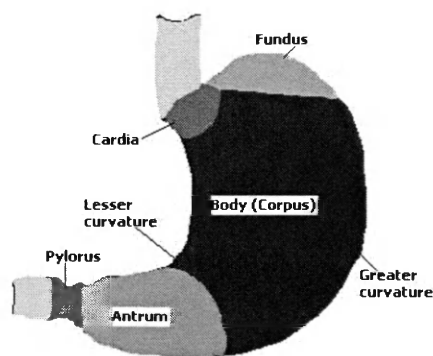
                10                20                30
284 Antrum.1  C T T C T A A A C C C C A G C C A A G C C C A T G G G T T T
284 Antrum.2  C T T C T A A A C C C C A G C C A A G C C C A T G G G T T T
284 Antrum.3  C T T C T A A A C C C C A G C C A A G C C C A T G G G T T T
284 Antrum.4  C T T C T A A A C C C C A G C C A A G C C C A T G G G T T T
284 Antrum.5  C T T C T A A A C C C C A G C C A A G C C C A T G G G T T T
284 Antrum.6  C T T C T A A A C C C C A G C C A A G C C C A T G G G T T T
284 Antrum.7  C T T C T A A A C C C C A G C C A A G C C C A T G G G T T T
284 Antrum.8  C T T C T A A A C C C C A G C C A A G C C C A T G G G T T T
284 Antrum.9  C T T C T A A A C C C C A G C C A A G C C C A T G G G T T T
284 Antrum.10 C T T C T A A A C C C C A G C C A A G C C C A T G G G T T T
284 Cardia.1  C T T C T A A A C C C C A G C C A A G C C C A T G G T T T T
284 Cardia.2  C T T C T A A A C C C C A G C C A A G C C C A T G G T T T T
284 Cardia.3  C T T C T A A A C C C C A G C C A A G C C C A T G G T T T T
284 Cardia.4  C T T C T A A A C C C C A G C C A A G C C C A T G G T T T T
284 Cardia.5  C T T C T A A A C C C C A G C C A A G C C C A T G G T T T T
284 Cardia.6  C T T C T A A A C C C C A G C C A A G C C C A T G G T T T T
284 Cardia.7  C T T C T A A A C C C C A G C C A A G C C C A T G G T T T T
284 Cardia.8  C T T T A A A C C C C A G C C A A G C C C A T G G T T T T
284 Cardia.9  C T T C T A A A C C C C A G C C A A G C C C A T G G T T T T
284 Cardia.10 C T T C T A A A C C C C A G C C A A G C C C A T G G T T T T
284 Corpus.1  C T T C T A A A C C C C A G C C A A G C C C A T G G G T T T
284 Corpus.2  C T T C T A A A C C C C A G C C A A G C C C A T G G G T T T
284 Corpus.3  C T T C T A A A C C C C A G C C A A G C C C A T G G G T T T
284 Corpus.4  C T T C T A A A C C C C A G C C A A G C C C A T G G G T T T
284 Corpus.5  C T T C T A A A C C C C A G C C A A G C C C A T G G G T T T
284 Corpus.6  C T T C T A A A C C C C A G C C A A G C C C A T G G G T T T
284 Corpus.7  C T T C T A A A C C C C A G C C A A G C C C A T G G G T T T
284 Corpus.8  C T T C T A A A C C C C A G C C A A G C C C A T G G G T T T
284 Corpus.9  C T T C T A A A C C C C A G C C A A G C C C A T G G G T T T
284 Corpus.10 C T T C T A A A C C C C A G C C A A G C C C A T G G G T T T
C T T C T A A A C C C C A G C C A A G C C C A T G G G T T T

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Figure 18. *H. pylori arsS* Gastric Region Specific DNA Polymorphisms. Total *H. pylori* populations from the stomach antrum, cardia, and antrum of patient 284 were subjected to *arsS* specific PCR amplification. This multiple sequence alignment shows a G/T polymorphism present in the *arsS* of *H. pylori* of cardia population of patient 284 . This T₂₇ polymorphism was not detected in the *arsS* allele of *H. pylori* populations from the corpus or antrum of the same patient stomach. This is one of 15 single nucleotide polymorphisms (SNPs) specific for the cardia population of this patient within a 307 base pair internal *arsS* PCR amplicon (data not shown).

Stomach Regions

The two largest stomach regions are the corpus and antrum. These regions are distinct from one another and their physiology predicts a particular pathology that is related to *H. pylori* infection. *H. pylori*-induced gastritis in the antrum is correlated with duodenal ulcers but does not typically lead to gastric cancer. However, when gastritis is predominantly in the corpus, this predisposes the patient to gastric ulcer and more commonly leads to intestinal metaplasia (Atherton, 2006). These differences in *H. pylori*-associated pathology may be a result of the types of cells found in these regions of the stomach. Both the antrum and the corpus have high numbers of mucous cells, but the corpus possesses many more parietal cells which secrete HCl, and results in more acid exposure to cells in this region of the stomach (Rolig et al., 2012). This seems to result in a higher colonization rate of *H. pylori* in the antrum, where the environment is less consistently acidic. Even where growth is possible, however, *H. pylori* is typically present in isolated populations subject to specific environmental pressures (Hua et al., 1999, Engstrand et al., 1992)



<http://www.histopathology-india.net/Stomach.htm>

Figure 19. **Structure of the human stomach.** The major anatomical regions of the human stomach are shown, including the cardia, corpus and antrum.

Mutability, Recombination and Mixed *H. pylori* Infections

Studies done on *H. pylori* recombination and mutability often show differences in the mutation frequency and rate of recombination between strains. One study analyzing five sets of strains of *H. pylori* from five different patients showed that imports of foreign DNA were taken up and then non-randomly distributed into the chromosome at lengths of 261-629 base pairs. This suggests the DNA imports were often grouped together on the chromosome, indicating that the bacterium takes longer pieces of DNA and then breaks them apart into smaller pieces during the recombination process. The same study also showed a higher average genome-wide rate of recombination between the sequential isolates of the chronically infected individuals than that calculated based on housekeeping genes, approximately 122-fold higher. However, the fifth set of strains were from a patient who was voluntarily infected with a strain for three months and there was no evidence of recombination and no mixed infection present. This indicated that mixed infection may be necessary for high recombination events to occur (Kennemann et al., 2011, Lundin et al., 2005).

Another study done showed that the *H. pylori* mutation frequency was approximately 10^{-8} (rifampin resistance used as mutation indicator) when following two asymptomatic adult patients. This study showed the patients had a single strain with clonal variants but few polymorphisms were seen at 10 different loci examined, with the exception of the divergent gene *amiA*. *amiE* encodes an N-acetylmuramyl-L-alanine amidase homologue which is part of *H. pylori* cell wall synthesis (Lundin et al., 2005, Aras et al., 2003). Another study used PCR amplification and DNA-sequence evidence to analyze a *cag*⁺ and *cag*⁻ strain in one patient. Most *H. pylori* cells in the patient had

descended from a *cag*⁺ parental strain but later become *cag*⁻ through recombination. *H. pylori* isolated from this patient yielded many recombinant clones which may indicate that frequent recombination between *H. pylori* strains may be a significant advantage for this pathogen. Perhaps natural mixed infection of *H. pylori* and the accompanying genetic exchange between unrelated strains can produce new genotypes faster than mutation could alone (Kersulyte et al., 1999). In another case that looked at mixed infections, stomach biopsies from 12 patients were examined via molecular fingerprinting techniques to determine the incidence of genetic exchange between strains in mixed infection. Multiple biopsies were taken from each patient and single colony isolates from each biopsy were examined for recombination. Eleven out of the 12 patients contained identical clonal strains where >95% of gene loci were conserved within that patient, but one patient had two separate and distinct strain populations. These two strains differed at 113 loci and did not exhibit much genetic exchange. The study also analyzed isolates from a human challenge study on volunteers 15 and 90 days after infection and saw no genetic divergence, indicating rapid genetic divergence of *H. pylori* is not necessary for colonization and transmission does not induce these changes (Salama et al., 2007).

Methods and Materials

Isolation of Clonal Strains

Total populations of *H. pylori* from cardia, corpus and antrum from patients attending a Veterans Administration/Vanderbilt University Medical Center clinic were kind gifts of Dr. Richard Peek Jr. Single colony, clonal *H. pylori* strains were isolated in

our laboratory from the *H. pylori* populations present in each of the stomach regions. This was done through a dilution of the original stock population in SFBB/10%NCS. The dilution series was plated out on blood agar plates and grown in a 5% CO₂ incubator until colonies were visible. Because these are not lab-adapted populations, this growth took between four and seven days. Between 10 and 20 colonies were then patched onto a new blood agar plate and incubated until growth was visible. Next each of these patches were put onto a new plate and streaked for a lawn. A portion of the growth from this plate was taken to make a new plate that was used in making a freezer stock. The other half of the growth was used for a new plate to generate genomic DNA from that clonal strain. This process was used for each strain and each region, for example, Strain B284 Cardia, Strain B284 Corpus, and Strain B284 Antrum.

Amplification and Sequencing of Clonal Strains

Genomic DNA was then diluted with sterile deionized water to 100 ng/ul. The diluted form was used as a template in PCR with the primers arsSF-1 and hemBR (Hallinger et al., 2012) (Appendix I). The resulting PCR yielded one amplicon of approximately 300 base pairs. Some of the *H. pylori* clones failed to yield *arsS* amplicons in PCR with the first set of primers and new primers were designed and synthesized that produced a larger amplicon. Four primers (arsSFor1 & 2 and arsSR1 & 2, Appendix I) were designed from a consensus sequences generated from five different sequenced strains of *H. pylori* available in the NCBI/NLM public database. Once the *arsS* sequences from selected clones from regions of patient 284 were successfully amplified through

PCR, select clonal strains were also sequenced via Sanger Sequencing. Multiple sequence analyses were done by CLUSTAL-W through the MacVector Software package.

Big Dye Sequencing Reaction

Sequencing reactions were done using BigDye Terminator sequencing (Applied Biosystems). Samples to sequence were prepared for PCR using 5 µl of 5x Big Dye buffer, 2 µl of BigDye Reaction mix, 3 µl of water, 1 µl primer, and 10 µl of the sample to be amplified. The PCR sequencing program used a hot start of 94° C for 5 minutes and then 26 cycles of 94° C for 45 seconds, 50° C for 30 seconds, and 60° C for 4 minutes. Once the sequencing PCR was finished, the PCR samples were purified via PERFORMA DTR Gel Filtration Cartridges (Edge BioSystems). Before use, the filtration cartridges were spun down for 2 minutes at 800 x g to dry. The filter was then moved into a special Eppendorf tube, and the sequencing reaction product was transferred directly above the filter. The tubes were spun down for 2 minutes at 800 x g. The filter was then discarded and the contents of the Eppendorf tube were kept. Tubes were dried in a SpeedVac concentrator (Thermo Electron Corporation) for approximately 45 minutes or until no liquid remained. Samples were processed further by the core lab by fragment analysis using the ABI 3500 Genetic Analyzer (Applied Biosystems).

Multi-locus sequence typing (MLST)

The representative clonal *H. pylori* strain from each of various stomach regions of patient 284 were also compared by multi-locus sequence typing (MLST). In MLST, DNA sequence variations in portions of seven housekeeping genes are determined after PCR

amplification and sequencing. The seven genes chosen for *H. pylori* for MLST analysis were *trp*, *ypbC*, *atpA*, *mutY*, *efp*, *ureI*, and *ppa*. The primers used were from those designed previously for use with *H. pylori* as described on the MLST database (<http://pubmlst.org/helicobacter/>) (Appendix I). The sequences were concatenated to form a continual sequence that was then compared against seven housekeeping genes. The clonal isolates from different stomach regions were also compared against each other. Overall, MLST indicates the degree of relatedness of isolates, and allowed us to better characterize the gastric population structure of *H. pylori* in the human stomach.

Genomic DNA extraction for sequencing

Strains will be sent to UCLA for sequencing by Illumina MySeq technology. In order to be sequenced successfully, high quality genomic DNA was needed. This was harvested via the Pure Link Genomic DNA Extraction Kit (Life Technologies). Three plates of each *H. pylori* clonal isolate were grown for 24-48 hours at 37° C and 5% CO₂. Cells were then removed from the blood agar plates and re-suspended in 1 ml of sterile 0.9 % NaCl using a sterile cotton swab. The O.D. ₆₀₀ was read using a 1:10 dilution to ensure an accurate reading. Using the O.D. reading, 2x10⁹ cells were harvested by centrifugation for five minutes at 5000 x g. The cell pellet was then resuspended in 180 µl PureLink genomic digestion buffer. 20 µl proteinase K was added to lyse the cell and vortexed to re-suspend the remaining pellet. The cell lysate was incubated at 55° C for two hours with occasional vortexing and 20 µl RNase A was added. The solution was then incubated at room temperature for another two minutes. Next, 200 µl PureLink genomic lysis/binding buffer was added and mixed. 200 µl 100% ethanol was added and mixed well by vortex

for five seconds to yield a homogenous solution. The mixture was then added to a PureLink spin column and collection tube and centrifuged at 10,000 x g for 1 minute at room temperature. The collection tube was discarded and the spin column was placed into a new collection tube. 500 µl of wash buffer 1 with ethanol was then added and spun down again for the same amount of time. The collection tube was again discarded and a new one is replaced. 500 µl of wash buffer 2 with ethanol was added and the tubes were spun down at 3 minutes at maximum speed. The collection tube was discarded and the spin column was placed in a sterile 1.5 ml microcentrifuge tube. 50 µl of elution buffer was added and the tubes incubated at room temperature for one minute before being spun at maximum speed for 1.5 minutes. A second elution buffer step was performed in which another 50 µl of elution buffer was added and the tubes incubated at room temperature another minute before being spun at maximum speed for 1.5 minutes. The recovered DNA was stored at 20° C before being run on a gel to ensure quality and being sent off for sequencing.

Results and Discussion

Amplification and *arsS* Sequencing of Clonal *H. pylori* Strains co-colonizing the stomach of Patient 284

Amplification and sequencing from the clonal *H. pylori* strains revealed that there was a difference between the strains colonizing different regions of the stomach. When approximately 300-500 base pairs of *arsS* sequences (depending on which set of consensus primers was used) from the clonal strains derived from the antrum and corpus

were aligned with themselves, they matched as expected (Figure 20-21). This sequence was not a perfect match for any sequenced strain in the database (data not shown) and so we hypothesized that the same strain of *H. pylori* infected these two adjoining regions of the stomach in patient 284. When *arsS* from the cardia derived clonal *H. pylori* sequences were aligned to orthologous sequences determined for the isolates from the corpus or antrum, there were several single nucleotide polymorphisms (SNPs) noted (Figure 22). None of these *arsS* polymorphisms were found in any antrum or corpus cloned *H. pylori* isolate. These results imply there is strain specific stratification between the different stomach regions and different clonal strains may exist in seclusion from one another. Others in our lab have repeated this procedure and found concordant results indicating *H. pylori* strain stratification among anatomic regions of the stomachs of two other patients from this same cohort of 12 patients (D. Hallinger, A.Bennett, and R. Bennett, unpublished data).

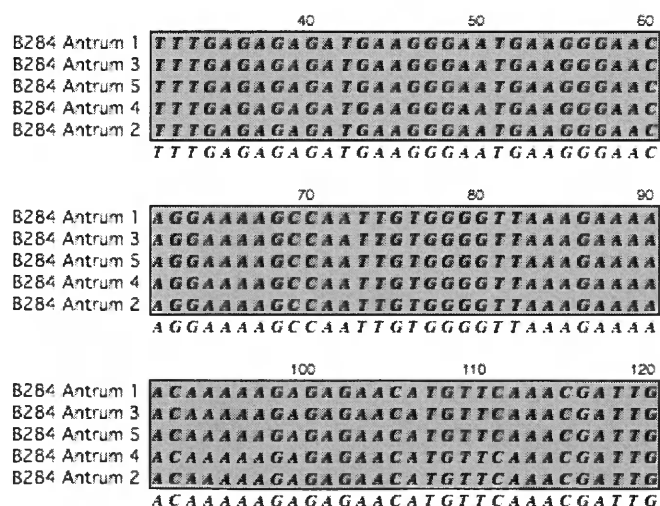


Figure 20. Clustal W arrangement *arsS* of *H. pylori* antrum clonal strains. Five *arsS* sequences from clonal antrum-derived strains from Patient 284 are aligned with a

consensus sequences on the bottom. The alignment of the clonal strains shows no polymorphisms between the strains.

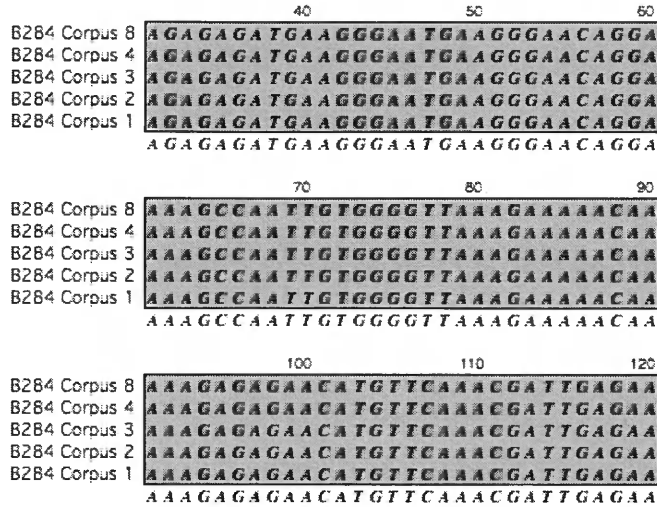


Figure 21. Clustal W arrangement of *arsS* *H. pylori* corpus clonal strains. Five clonal corpus strains from Patient 284 are aligned with a consensus sequences on the bottom.

The alignment of the clonal strains show no polymorphisms between the strains.

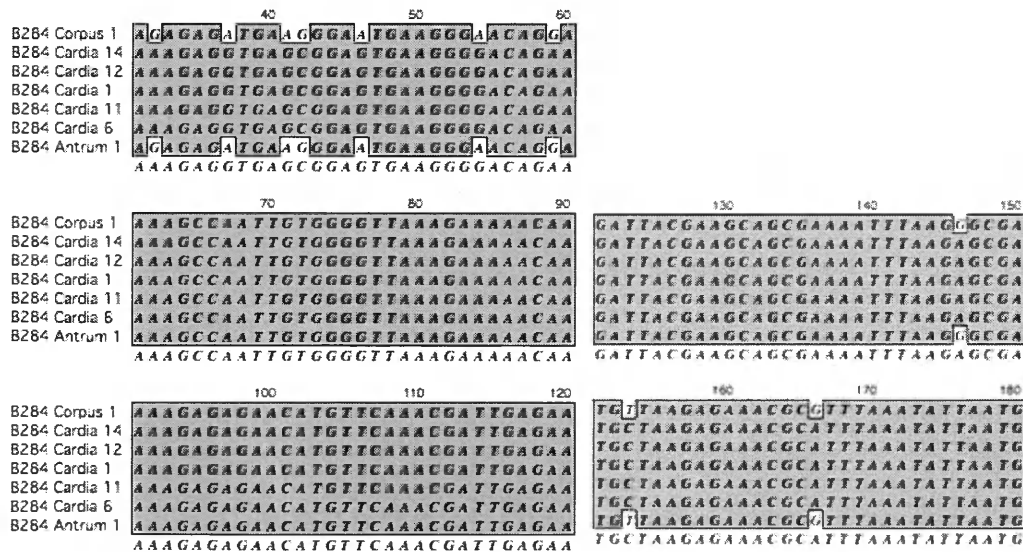


Figure 22. Clustal W arrangement of *arsS* of cardia clonal strains compared with antral and corpus-derived *H. pylori* clones. Ten clonal cardia strains from Patient 284

are aligned with a corpus strain above and antrum strain below to highlight differences present in the cardia when compared to the corpus and antrum.

Multi-locus sequence typing (MLST) shows region specific polymorphisms in the cardia

The results of our analyses of *arsS* sequencing suggested that the isolates of the cardia of patient 284 were distinct from those in the other portions of the same stomach. But to test if the diversity seen was restricted to the acid sensing histidine kinase, *arsS*, or if the diversity at that locus reflected overall genome diversity, we undertook a multi-locus sequence typing (MLST) study of these *H. pylori* clones. MLST was used to determine the relatedness of the *H. pylori* strains isolated from each region and the relatedness of strains found in different stomach regions. Additionally, combining the MLST analysis with the analysis of *arsS* sequence divergence between the isolates, we hoped to determine if the variation among these strains lay only within the *arsS* allele or in one region of these clonal isolates. If the housekeeping genes have a strong consensus between the different clonal isolates but *arsS* is more varied, this may indicate that *arsS* is divergent as an adaptation to a specific set of environmental conditions associated with a gastric region. This could also indicate stratification that has occurred in a region specific way due to *arsS* and its involvement with acid acclimation.

Our MLST analysis did not indicate that there is a difference between the *arsS* allele and the MLST sequences. In *H. pylori* cloned isolates from patient 284, there are nucleotide polymorphisms present in the cardia region of the stomach in all loci examined that are found in this region only and not in any *H. pylori* isolate from the

corpus or antrum (Figure 23). This is true for all seven housekeeping genes amplified, although only three representative genes are shown in Figure 23.

We hypothesize that some environmental factor associated with a gastric region may have acted to select for the strain best adapted to its location. These changes in the segregated clonal strains may be a result of the difference stomach locations, as the environment in each location is home to different types of cells and signals. They may also be a result of recombination with other nearby strains. A similar number of SNPs are also present in *arsS* among these segregated *H. pylori* strains as found among the MLST loci suggesting that *arsS* variations are not necessarily the source of the observed segregation of strains within this stomach. In actuality, the stomach regions are probably responsible for variation between distinct strains independent of *arsS*.

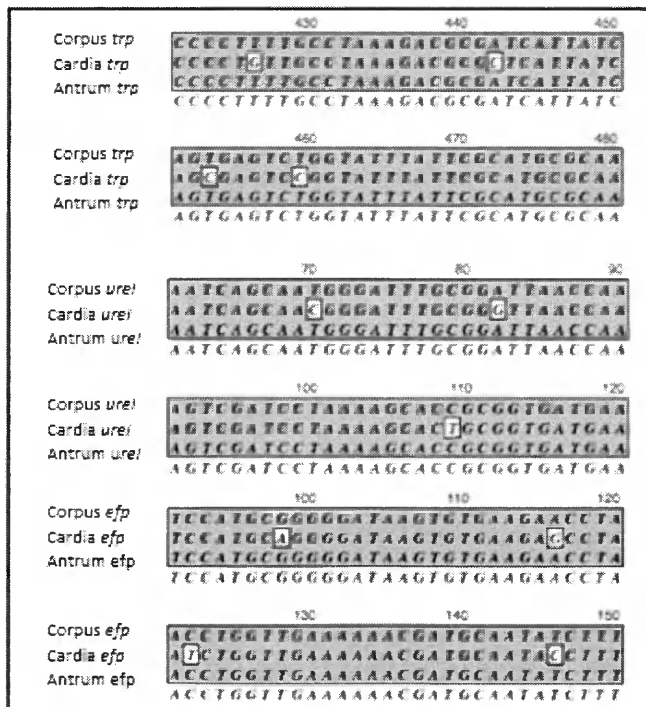


Figure 23. MLST amplicons in the corpus, cardia and antrum using genes *trp*, *ureI*, and *efp*. Aligned sequences are shown for the three regions on the stomach, with the

cardia in the middle, corpus on top and antrum on the bottom (one example clone chosen from each region). An excerpt from *trp* is shown first, and then *ureI*, followed lastly by *efp*. Polymorphisms are shown in white with a box around them to highlight.

qRT-PCR to investigate variations in *arsS* response in different stomach regions

qRT-PCR can also be performed for the *arsS* allele of each strain from each region. We hypothesize that expression levels of this acid sensory and response locus may differ with each gastric region due to altered environments. The different expression levels of the *arsRS* locus may be the subject of selection that leads to geographic stratification in the stomach. It is possible that when each region in a strain is compared via qRT-PCR that there is no difference in expression level. This could be because the difference is not from transcriptional regulation and therefore no induction of expression will be seen via qRT-PCR. The difference may be from a post-transcriptional modification such as phosphorylation, not detected by the qRT-PCR assay.

Appendix 1

Oligonucleotide Name	Oligonucleotide Sequence, 5' to 3'	Melting Temperature
MRB-A2	GTGAAACGCTTCGCTCAAGGGGAT	61.7
MRB-A-	GGCACGATGGTCGCCCCCTGCGTT	68.6
MRB-B2	CAATGCGATGAATGAATCTCGGGT	57.7
MRB-B-	GGCAATCTCAAGCTTTCAAACCCC	58.9
MRB-C2	TTACGCACCCCTATCACTAAGGGC	60.4
MRB-C-	TCAATGGGCGTCGTGGCGATG	62.7
Bgl2A	CAAAGGAATTGAATGGGACGAGA TCTTGAATACAGAAGAGCGGCTA	65.8
Bgl2A-	TAGCCGCTCTTCTGTATTCAAGAT CTCGTCCCATTC AATTCCTTTG	65.8
Oligo1	VICCTTCTAACCCAGCCAAGCCCATGG	-f
PYCA	AAGCGGATAATGAAGAGCTACCTC CACCGCGAAAATTTGAGAGAGGTGAA	68.4
PYCA-	TTCACCTCTCTCAAATTTTCGCGGT GGAGGTAGCTCTTCATTATCCGCTT	68.4
PYCB	AAGCGGATAATGAAGAGCTACCTC CACCGCCGAAAATTTGAGAGAGGTGAA	69.0
PYCB-	TTCACCTCTCTCAAATTTTCGCGGG TGGAGGTAGCTCTTCATTATCCGCTT	69.0
PYC	AAGCGGATAATGAAGAGCTACCTC CAXCGCCGAAAATTTGAGAGAGGTGAA	69.5
PYC-	TTCACCTCTCTCAAATTTTCGCGGT GGAGGTAGCTCTTCATTATCCGCTT	69.5
arsSseqmutF	GACGCCACCAGTTGCACCACAG	63.2
hemBseqmutR	GAGCGATGGTGAGAGAAACACG	57.9
hemBseqmutF	GAGTGGGGAATACGCCATGCTC	60.1
0162seqmutR	GACGATGATGATGTGGTCGCGC	60.7
arsSF-1	CTTCTAACCCAGCCAAGCCCATGG	63.7
hemBR-1	CGCTGCTTCGTAATCTTCTCAATCG	57.9
arsSFor1	CCCGCAATGCCCATGGTCGGTG	65.7
arsSR1	CTAACCCAGCCAAGCCCATGG	62.7
arsSFor2	CGCATGGCTTCCTGTAGCGTC	60.8
arsSR2	ACAGCGATGACAAACAGGTG	55.7
arsRFconsensus	GATCATTGGCCGTTTGCGATC	57.1
hemBRconsensus	GCTGCTTAAATGCACGGTGAG	57.0
ppa-for5	GGAGATTGCAATGAATTTAGA	48.4
ppa-rev5	GTGGGGTTAARATCGTTAAATTG	51.3
urelfor	AGGTTATTCGTAAGGTGCG	52.2
urel-rev2	GTTTAAATCCCTTAGATTGCC	49.2
efp_for1	GGCAATTTGGATGAGCGAGCTC	58.9
efp_rev1	CTTCACCTTTTCAAGATACTC	48.5
mutY_for2	GTGGTTGTAGYTGAAACTTTACAC	55.3
mutY_rev3	CTTAAGCGTGTGYTTTCTAGG	52.5
atpA_for2	GGA CTAGCGTTAAACGCACG	56.3
atpA_rev2	CTTGAAACCGACAAGCCAC	56.7
yphC_for2	CACGCCTATTTTTTTGACTAAAAAC	51.8

yphC_rev3	CATTYACCCTCCCAATGATGC	55.1
HptrpC6_for	TAGAATGCAAAAAGCATCGCCCTC	58.2
HptrpC7_rev	TAAGCCCGCACACTTTATTTTCGCC	60.1

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