The Unusual Paradigm of the Acid Response Two Component System of Helicobacter pylori

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of Helicobacter pylori

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

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Abstract

Two component signal transduction systems in bacteria are key for environmental adaptation. Signaling via these systems is traditionally considered to be relatively simple, only involving interactions between the sensory protein and its cognate response regulator. The ArsRS two component system is vital for the acid response in *Helicobacter pylori*. This study investigates the mechanism by which ArsRS responds to acid in *H pylori*. Recent studies in our lab have challenged the classic model in which the response regulator ArsR is activated by the phosphorylation of a conserved aspartic acid by the transfer of a phosphoryl group from a histidine within the acid sensing histidine kinase ArsS. We show that only phosphomimetic substitutions for the aspartic acid at the 52nd position on ArsR (D52E and D52N) yield viable *H. pylori* mutants, while substitutions of alanine, glycine or serine at this position fail to yield viable mutants. As deletion of *arsR* is a lethal mutation, the recovery of phosphomimetic amino acid substitutions and failure to recover non-phosphomimics suggests that the crucial activity of ArsR depends upon phosphorylation at D52 and that ArsR D52E and D52N must be at least partially active. We thus examined the extent to which these phosphomimetic mutations affect the expression of two of the genes in the ArsRS regulon, the acid-repressed outer membrane adhesion protein, *sabA* and the acid-induced urease subunit, *ureA*, under acidic conditions. Our findings show a slight but significant decrease in *sabA* transcription in *H. pylori* mutants with the phosphomimetic forms of ArsR but did not show a phenotype of constitutive acid regulation of *ureA* as expected of phosphomimetic response regulators. These mutants do, however, show full acid repression of *sabA* and acid induction of *ureA* at pH 5, indicating another step of activation in the acid response besides phosphorylation of ArsR D52. Our investigation into the role of ArsS in the Acid Response System (ARS), found that the histidine-kinase activity of ArsS is necessary for both acid-induced
repression of sabA and acid-induced activation of ureA in both wild type H. pylori and H. pylori with phosphomimetic ArsR D52E. Thus, a functional ARS is lost without phosphorylation by ArsS. Furthermore, a mutant H. pylori strain with a loss of ArsS kinase ability and a phosphomimetic ArsR failed to show acid-induction of ureA, indicating that the acid induction of ureA seen in the ArsR D52E mutant does not come from an additional activation of ArsR, but from phosphorylation by ArsS of a protein other than ArsR. Lastly, we explore the role of cross talk between ArsRS and CrdRS or FlgR in the acid regulation of ureA and sabA via genetic deletions in the response regulators CrdR and FlgR and the non-cognate histidine kinase CrdS. Our results indicate that cross-talk with these non-cognate TCS proteins is not involved in the acid regulation of sabA and ureA. Our findings challenge the classic model of two component systems and suggest a much more complex interaction between sensory proteins and response regulator proteins in order to regulate gene expression.

**Figure 1. Overview of TCS signaling**
An extracellular stimulus activates the sensory protein. The activated sensory protein changes conformation and binds the response protein, activating it. The active form of the response protein then induces an intracellular response, often through changes in gene transcription.

**Introduction**

**Bacterial Two Component Systems**

In order to colonize their hosts and initiate pathogenesis, pathogenic bacteria must closely regulate their gene expression in order to adapt to hostile environments and conserve energy.
Bacteria are able to quickly adapt to changing environments through Two Component Systems (TCS), which are simple signal transduction systems consisting of a sensory protein and a response regulator (RR) (Figure 1). Sensory proteins are able to detect changes in the environment and subsequently activate their cognate response proteins. The activated response proteins go on to induce cellular changes that allow the bacteria to adapt to the environment.

The sensory protein is most often a transmembrane protein and typically a homodimer of two histidine kinase (HK) proteins (Casino, Rubio, & Marina, 2010). Both monomers have a dimeric helical HK domain on the intracellular side that houses a highly conserved histidine amino acid, which may be phosphorylated (Figure 2). This domain, abbreviated as DHp, is connected to a C-terminal catalytic domain, abbreviated as CA. The CA domain has the ability to bind ATP and phosphorylate the His residue of the DHp domain. On the extracellular or periplasmic side of a typical sensory HK, both monomers have a sensor domain that may be activated by an extracellular stimulus. Activation of the extracellular sensor domain causes a conformational change that brings the CA domain in close contact with the DHp domain His residue.

The CA domain possesses a classical Bergerat fold, which houses a single ATP molecule between the ATP lid and a central helix. The γ-phosphate of the ATP is exposed, allowing for transfer of this phosphate to the His residue of the DHp domain. HK phosphorylation may either be cis- or trans-autophosphorylation between the monomers (Zschiedrich, Keidel, & Szurmant, 2016). Activation of the extracellular sensor domain may bring together the CA and DHp domains of the same subunit or of partner subunits. The structural characteristics that determine cis- versus trans-autophosphorylation are still unknown, but it appears that both the handedness
of the connector between the two helices of the DHp domain and the length of the mobile hinge between the DHp and CA domains may be contributing factors.

Phosphorylation of the DHp domain’s conserved His residue induces a further conformational change in the HK that allows the receiver domain (REC) of the RR to bind to helix 1 of the HK DHp domain (Casino, Rubio, & Marina, 2010). Helix 1 is the helix directly attached to the extracellular sensor domain, and it contains the phosphorylated His residue. The \( \beta_5-\alpha_5 \) loop in the REC domain of the RR binds below the His residue positioning an exposed Asp residue in the REC domain of the RR close to the His phosphodonor of the HK. Both subunits of the HK are able to bind a RR, creating a 6 helix bundle on the cytosolic side composed of helix 1 of both RRs and the DHp domains of both HK subunits. Structural elements on the cytosolic side of the HK and the \( \beta_5-\alpha_5 \) loop and helix 1 of the RR provide a great degree of partner specificity to TCSs, thus ensuring highly fidelitous communication between HK and RR cognate partners.

**Figure 2. Overview of TCS protein structure**
The TCS sensory protein is a histidine kinase. The sensory domain is located extracellularly or in the periplasmic space in the case of gram negative bacteria. The histidine residue involved in the autophosphorylation reaction is located in the dimeric helical (DHp) domain, and the ATP molecule required for phosphorylation is bound by the catalytic ATP-binding (CA) domain. The recognition (REC) domain of the response regulator binds to the sensory protein and possesses an exposed aspartic acid residue that can receive the phosphate from the histidine of the DHp domain. The effector domain of the response regulator is responsible for binding DNA and inducing changes in gene expression. (Adapted from Casino *et al* 2010).
When the RR is bound to the HK, the distance between the HK His residue and the RR Asp residue is approximately 5Å (Casino, Rubio, & Marina, 2010). A BeF$_3^-$ cofactor is positioned between these residues with all residues contacting the BeF$_3^-$ cofactor belong to the RR except for the His residue of the HK. The RR also has a central Mg$^{2+}$ ion that likely acts to stabilize the high degree of negative charge involved in the phosphotransfer reaction. The active site of the RR also contains conserved Thr/Ser and Lys residues that contact the phosphoryl oxygen atoms and are involved in the catalysis of the phosphotransfer reaction. Interactions between the RR and the CA domain of the HK further facilitate the transfer of the phosphate group.

Phosphorylation of the Asp residue in the REC domain of the RR causes a conformational change in the effector domain (Casino, Rubio, & Marina, 2010). This conformational change alters the affinity of the effector domain for its targets, which are most often regulon gene promoter regions. 65% of all RRs are transcriptional regulators (TRs) that help the cell adapt by altering the transcription or genes in the TCS regulon (Kalantari, Derouiche, Shi, & Mijakovic, 2015).

Phosphorylation of the RR causes a conformational change in the REC domain that changes the interactions between the RR and the HK (Casino, Rubio, & Marina, 2010). In some cases, the HK of the TCS may also possess phosphatase activities, and the change of conformation in the RR REC domain allows the phosphorylated RR to bind to the HK in a way that facilitates the dephosphorylation reaction. In many cases the phosphatase capability of the HK is dependent upon the presence of additional domains that are not involved in the phosphotransfer reaction. The interactions in the phosphatase reaction follow a similar route as
those in the phosphotransfer reaction, however the distance between the HK His residue and the RR Asp residue is increased to 7Å to facilitate the presence of a H₂O molecule involved in the phosphatase reaction. The H₂O molecule is activated by the His residue, allowing it to act as a nucleophile and attack the phosphate atom in the phosphoaspartate. Further diverging from the phosphorylation reaction, a sulfate ion between the Asp of the RR and the His of the HK facilitates the dephosphorylation of the RR.

Although the classic paradigm for TCS signaling is relatively simple, recent research suggests that many TCSs are much more complex than originally thought (Marcus E. A., Sachs, Wen, & Scott, 2016). TCSs have been shown to require accessory proteins, multiple interactions between sensory proteins and RRs, and/or interaction with other TCSs in order to transmit their given signals and induce a cellular response. This study investigates a complex TCS in *Helicobacter pylori* by drawing from findings of non-paradigmatic TCSs in other species.

**Response regulators may respond to multiple stimuli and show a gradient of activity**

There is evidence that in some cases there may be more than one step necessary to activate a RR (Hortsmann, Sahasrabhojane, Hui, Su, & Shelburne, 2017). RRs are generally activated by phosphorylation of conserved aspartic acid residues by their cognate HK, however evidence has shown that phosphorylation is also common in bacterial TRs on serine and threonine residues by Hanks-type serine/threonine kinases (STKs) (Kalantari, Derouiche, Shi, & Mijakovic, 2015). Tyrosine phosphorylation by bacterial tyrosine kinase (BY kinase) is also possible, though there is no evidence that this type of phosphorylation is common in TCS RRs.

STK phosphorylation is more stable than HK phosphorylation as many HKs also possess phosphatase activity and phospho-histidine itself has a short half-life (Kalantari, Derouiche, Shi, & Mijakovic, 2015). Phosphorylation of RRs by STKs can serve to antagonize or enhance
phosphorylation by HKs. In the CovRS TCS of *Streptococcus pyogenes*, an OmpR/PhoB type TCS, Stk1 phosphorylates T65 in CovR, antagonizing D53 phosphorylation by CovS (Hortsmann, Sahasrabhojane, Hui, Su, & Shelburne, 2017). This phosphorylation antagonism works in a reciprocal manner as well, i.e., phosphorylation of D53 by CovS inhibits phosphorylation of T65 by Stk1. Phosphorylation of CovR at D53 activates the RR so that it may bind to the promoters of virulence genes and repress them. Phosphorylation of CovR T65 prevents phosphorylation of D53, thus keeping CovR in its inactive state, allowing the expression of virulence factors.

To further complicate the interaction of these two activation systems, in some cases, cross-phosphorylation may occur between STKs and HKs (Kalantari, Derouiche, Shi, & Mijakovic, 2015). In *Bacillus subtilis* it was found that two STKs are capable of phosphorylating a serine residue in the signal-sensing domain of DegS, a cytosolic HK in the DegS/DegU TCS. Phosphorylation at this residue stimulates the protein's kinase activity, serving as an activating input for the TCS.

There is also evidence that in some cases response proteins perform regulatory functions in their inactivated form, and that activation allows these proteins to regulate additional genes. In a study investigating the role of CovR phosphorylation in the regulation of gene expression in *S. pyogenes*, investigators found that a mutant CovR protein that could not be phosphorylated was still able to bind DNA but was unable to dimerize in the same manner as the phosphorylated form of wild type CovR (Hortsmann, Sahasrabhojane, Hui, Su, & Shelburne, 2017). Furthermore, transcriptome analysis showed that, although the non-phosphorylated CovR was still able to bind gene promoters in the CovRS regulon, it was only able to regulate a subset of genes from the regulon. Analysis of genes in the CovRS transcriptome revealed structural
differences in the promoters of genes that were regulated by monomeric CovR versus those that could only be regulated by the dimeric protein. These findings imply that response regulators may exhibit some regulatory activity in their inactive form, and that activation by an HK induces further regulatory activity.

Collectively, these studies paint a much more complex picture of gene regulation in bacteria than the traditional single-phosphorylation model. It appears that RR activation is not a simple on/off switch, but that RRs may show varying levels of activation in response to multiple stimuli.

**Cross Talk Between TCSs**

A mounting body of evidence suggests that TCS signal transduction may be much more complex than originally thought, potentially involving cross talk between multiple TCS pathways, such that signals are integrated to produce a coordinated intracellular response (Yamamoto, et al., 2005; Leday, et al., 2008). In some cases, cross talk between TCSs may be the result of direct communication between TCSs in which TCS component proteins regulate non-cognate proteins from other TCSs. In *E coli*, the sensory protein UhpB is able to phosphorylate nine different response regulators (Yamamoto, et al., 2005). This mode of cross talk is unlikely to be prevalent, however, due to the high degree of specificity characteristic to sensory HKs and their cognate RRs.

Cross talk can also occur through less direct routes either through interaction with intermediate molecules or through cross-regulation of the same genes. In group A *Streptococcus* (GAS), the CovRS and TrxRS TCSs interact through multiple mechanisms to control the expression of the Mga virulence regulon (Leday, et al., 2008). TrxR activates the Mga regulon to activate Mga-regulated virulence factors by binding the *mga* promoter and inducing Mga.
expression. Both *trxS* and *trxR* are, however, in the same operon and are repressed by CovR. CovR is also known to repress the expression of both *rivR* and *rivX*. RivR is a stand-alone regulator that promotes Mga expression, while rivX is a small RNA that enhances Mga activity in inducing translation of the Mga regulon. The CovRS and TrxRS TCSs show many levels of indirect cross-regulation, including interaction with multiple intermediate molecules. These findings indicate that intermediate molecules are a promising candidate for communication between TCSs.

The use of intermediate molecules in coordinating signals between TCSs may explain the role of TCS accessory proteins. Accessory proteins have been found to allow TCSs to respond to multiple stimuli and to alter the way in which they respond. In GAS, the CovRS TCS has been shown to interact with an accessory protein called the Regulator of Cov (RocA) protein (Jain, et al., 2017). Increased RocA concentration impairs the activation and inactivation of the CovRS TCS by the host factors Mg$^{2+}$ and antimicrobial peptide LL-37 respectively. Investigators thus propose a model in which RocA responds to an unidentified stimulus from the host and interacts with CovS through membrane-spanning domains in both proteins. Interaction between RocA and CovS enhances the kinase activity in CovS. This interaction results in increased levels of phosphorylated CovR and therefore altered expression of virulence factors. The investigators explored several explanations for the evolution of the interaction between CovS and RocA, but did not consider the potential role of RocA in integrating TCS responses to multiple signals.

STKs offer another potential class of molecule that could serve as an intermediate to coordinate signaling between TCSs. Although STK phosphorylation is more stable than HK phosphorylation, the interactions between STKs and their target proteins are much less specific than the interactions between sensory HKs and their cognate RRs (Kalantari, Derouiche, Shi, &
Mijakovic, 2015). This lack of specificity could allow STKs to interact with multiple RRs and/or sensory HKs and coordinate the activation and inactivation of multiple systems.

**Phosphomimetic amino acids**

Phosphomimetic amino acids are frequently implemented in the research of phosphoproteins (Hortsmann, Sahasrabhojane, Hui, Su, & Shelburne, 2017; Delaune, et al., 2012; Marcus E. A., Sachs, Wen, & Scott, 2016). Both asparagine and glutamate have properties that affect protein structure similarly to phosphorylation of an aspartic acid residue (Delaune, et al., 2012). Asparagine is a large amino acid and glutamic acid is capable of carrying a negative charge. In the study of bacterial phosphoproteins, the conserved aspartate residue of a RR can be altered to asparagine or glutamate to generate a RR that acts as if it is always phosphorylated and is thus partially or fully constitutively active.

A phosphomimetic amino acid mutation was employed in a study on *Staphylococcus aureus* (Delaune, et al., 2012). The RR WalR from the TCS WalKR was mutated to WalR D55E. This mutant strain constitutively showed a significantly altered phenotype and transcriptome with increased biofilm formation and apha-hemolytic activity as well as increased transcription of virulence factors. This study shows that phosphomimetic amino acids at the site of phosphorylation in RRs can produce a constitutively active form of the phosphoprotein.

A study using phosphomimetic amino acid substitutions in *S pyogenes*, however, yielded different results (Hortsmann, Sahasrabhojane, Hui, Su, & Shelburne, 2017). Instead of showing constitutive activity, a phosphor-site mutation in CovR to CovR D53E generated a RR possessing only partial activity. This mutant was able to regulate some genes in the CovRS regulon but not others. Further research determined that the CovR D53E was able to bind to promoters in the CovRS regulon, but was unable to form a dimer. The investigators thus
hypothesized that the monomeric form of CovR is able to regulate a subset of genes in the CovRS regulon but unable to regulate others and that this distinction was dependent upon the promoter architecture of the genes.

**Two Component Systems in *Helicobacter pylori***

*Helicobacter pylori* is a gram negative human pathogen that infects approximately 50% of the global human population (Kabamba, Tuan, & Yamaoka, 2018). Patients infected with *H. pylori* can present with a number of symptoms ranging from mild gastritis to gastric cancer. Varying strains of *H pylori* may possess a number of virulence factors such as outer membrane adhesion proteins, *cag* pathogenicity island (*cag* PAI), vacuolating cytotoxin (VacA), γ-glutamyl transpeptidase (gGT), and (Duodenal Ulcer Promoting protein A (DupA) that contribute to chronic inflammation (Ansari & Yamaoka, 2017). This chronic inflammation can, over time lead to cancer. The exact mechanism by which *H pylori* infection leads to cancer, however, is still not fully understood.

The TCSs in *H. pylori* interact with host factors to regulate the expression of virulence factors and mediate colonization of the human stomach. Colonization and virulence factor expression are both vital for *H. pylori* pathogenesis, thus understanding how TCSs act to regulate gene expression is vital for understanding *H. pylori* pathogenesis and developing effective therapies. The mechanisms behind these TCSs are poorly characterized and are thus a major research opportunity. This study aims to characterize the mechanism of the Acid response system TCS (ArsRS) that is necessary for colonization of the human stomach. Although the focus of this study in on ArsRS, it is imperative to understand the role of all TCSs in *H pylori* and how they might interact with ArsRS due to the increasing evidence of cross-talk between TCSs.
The *H. pylori* genome encodes four sensory proteins and seven response regulators (Joseph & Beier, 2007). This is a relatively small number compared to most bacterial species, likely due to the adaption of *H. pylori* to its highly specific niche in the human stomach. Two of the RRs are orphan RRs with no cognate HK. They are referred to by their unique genome identifiers, HP1043 and HP1021. Both these response regulators have atypical REC domains. HP1043 is in the OmpR family of RRs, while HP1021 does not show sequence similarity with any other TCS RR in its output domain (Beier & Frank, 2000). The function of these response regulators remains unknown, but research has shown that HP1043 expression is necessary for *H. pylori* viability (Joseph & Beier, 2007).

The HK CheA can phosphorylate two types of response regulator: CheY and CheV (Joseph & Beier, 2007). CheY regulates chemotaxis and flagellar motion. Phosphorylated CheY interacts with the flagellar switch protein complex to change the flagellar rotation from counterclockwise to clockwise (Lowenthal, et al., 2009). The exact functions of CheV RRs are unknown, though they have been shown to play a role in chemotaxis as well (Joseph & Beier, 2007).

The CrdRS TCS is responsive to copper concentrations and helps mediates copper resistance through the regulation of copper resistance determinants A and M (CrdAM) and CzcAB (Waidner, Melchers, Stähler, & Bereswill, 2005; Waidner, et al., 2002). This TCS has an additional role in response to nitric oxide stress, which is important for adapting to the host immune response (Hung, et al., 2015). The CrdRS response to nitric oxide species also involves the differential expression of *crdA* (Hung, et al., 2015). Finally, CrdRS has also been implicated in the regulation of acid resistance genes (Loh & Cover, 2006; Joseph & Beier, 2007). The CrdS is an orthodox HK of 397 amino acids belonging to the IIA class of sensor proteins (Beier &
Frank, 2000). This class of sensor proteins is membrane bound with the sensory domain in the periplasm and the signaling domain in the cytoplasm. CrdR is in the OmpR family of response regulators.

Both the ArsRS and the FlgRS TCSs are acid responsive (Marcus E. A., Sachs, Wen, & Scott, 2016; Scott, et al., 2010). Like CrdS, the ArsS HK is in the IIIA class of sensory proteins, indicating that it is localized to the inner membrane and senses periplasmic pH (Beier & Frank, 2000). ArsR is also an OmpR type RR. The FlgS HK, in contrast, belongs to the HK subclass IIIB. This classifies FlgS a cytoplasmic HK, and the protein thus responds to changes in cytoplasmic pH. It has been suggested that FlgS functions independently of FlgR in acid acclimation, and plays a key role in urease assembly and activation as well as in the maintenance of cytoplasmic and periplasmic pH. In addition to the regulation of acid-acclimation genes, the FlgRS TCS is also responsible for the regulation of intermediate flagellar genes (Niehus, Ye, Suerbaum, & Josenhans, 2002).

The redundancy between TCSs for regulation of acid acclimation indicates the importance of this process in H. pylori gastric colonization. It also raises questions of whether cross-talk between TCSs may be involved in mediating the acid response.

**H. pylori TCSs in Acid acclimation and colonization**

*H. pylori* infects gastric epithelial tissue and the mucus layer covering these cells, and thus must be able to respond to the shifts in acidity of the human stomach, which can range from a pH of about 1-5 (Ansari & Yamaoka, 2017). *H. pylori* is bioenergetically a neutralophile, and thus cannot persist in such intensely acidic conditions without the aid of acid response mechanisms, which are largely regulated by TCSs. (Wen, Feng, Scott, Marcus, & Sachs, 2006)
Upon infection of the host, *H pylori* enters the stomach lumen where the pH is approximately 2.0 (Ansari & Yamaoka, 2017). In order to survive the acidity of the gastric lumen before establishing itself within the mucus covering the gastric epithelium, *H pylori* relies on the intracellular production of the multisubunit enzyme urease (Ansari & Yamaoka, 2017). Urease is expressed in a pH-dependent manner through the acid-sensing TCSs ArsRS and FlgRS. Urease is a metalloenzyme and requires association with 24 nickel ions to become fully active, thus urease expression is also dependent on the nickel-responsive regulatory protein NikR. Although urease is produced intracellularly, the enzyme has also been found on the bacterial surface due to the lysis of some bacterial cells in the infecting population. Surface urease differs in function from cytoplasmic urease. The extracellular form of the enzyme appears to interact with host components to influence the persistence of colonization.

The cytoplasmic urease enzyme works by breaking down urea to produce products that act as a buffering system in the periplasmic space (Ansari & Yamaoka, 2017). Urea is broken down into ammonia (NH$_3$) and carbamate (NH$_2$COOH) (Figure 3). Carbamate further decomposes to ammonia and carbonic acid (H$_2$CO$_3$), and the carbonic acid breaks down to CO$_2$ and H$_2$O. The CO$_2$ and ammonia are then excreted into the periplasmic space through the UreI channel. In the periplasmic space, ammonia is reduced to ammonium (NH$_4^+$) and CO$_2$ to bicarbonate (HCO$_3^-$), raising the pH. This buffering system generates a nearly neutral microenvironment at the inner membrane with a pH of 6.1.

Urea diffuses across the outer membrane of *H. pylori* through porins and is then actively imported from the periplasmic space into the cytoplasm by urea-specific influx protein, permease (UreI). UreI is a proton gated channel at the inner-membrane, thus the rate of urea import increases as the pH in the periplasmic space drops. At an acidic pH, cytoplasmic urease
associates with the UreI channel, giving the enzyme direct access to urea (Marcus E. A., Sachs, Wen, & Scott, 2016). The gastric urea concentration of 1 mM at physiological conditions is not, however, sufficient to sustain persistent *H pylori* infection in the face of high gastric acidity as seen during fasting when the luminal pH can reach 1 (Ansari & Yamaoka, 2017). The bacteria must rely on additional means to survive gastric acidity such as the endogenous production of urea through Arginase or RocF, which hydrolyze arginine yielding ornithine and urea.

*H. pylori* does not remain in the gastric lumen for long. Instead, the bacteria utilize chemotaxis to direct their movement toward the higher pH region of the gastric mucosa (Ansari & Yamaoka, 2017). The bacteria colonize the gastric mucosa where they can be protected from the acidity of the lumen, hide from host immune factors, and gain nutrients from the host. The capacity of urease to increase the pH around the bacterium also alters the properties of the mucus so that it is less viscous, while the *H pylori* enzyme thioredoxin reduces the disulfide bonds between the mucins of the mucus layer allowing for increased motility of the bacteria. The helical shape of the bacteria also aids its ability to move into the mucus layer to escape acidity using a corkscrew-like movement.

Once *H pylori* moves through the mucus layer, outer membrane proteins (OMPS) on the bacterial cell surface bind to receptors on the gastric epithelium to mediate the final stages of infection (Matsuo, Kido, & Yamaoka, 2017). The *H pylori* genome encodes approximately 32 OMPs including sialic acid-binding adhesin (SabA), blood-group-antigen-binding adhesin (BabA), adherence-associated lipoprotein A and B (AlpA/B), outer inflammatory protein A (OipA), and *Helicobacter* outer membrane protein Q (HopQ). The sheer quantity of OMPs indicates the importance of these proteins for colonization. The timing of OMP expression is critical as these proteins bind to host surface proteins, and the expression of these host surface
proteins changes throughout the course of *H pylori* infection (Marcos, et al., 2008). As this change in OMP expression is dependent on the host environment, TCS signaling has been implicated in the regulation of several OMPs (Forsyth, Cao, Garcia, Hall, & Cover, 2002; Goodwin, et al., 2008; Harvey, et al., 2014; Acio-Pizzarello, et al., 2017).

The expression of the BabA OMP is lost during the first 2-12 weeks of infection in animal models, and this loss of BabA expression has also been seen in human clinical isolates (Kable, et al., 2017). BabA binds the Lewis B (Le\(^b\)) blood group antigen, although the specificity of this adhesin molecule to particular blood group antigens varies between strains of *H pylori* (Matsuo, Kido, & Yamaoka, 2017). BabA enhances translocation of the pathogenic factor CagA into host gastric epithelial cells via a Type IV secretion system encoded by the *cag* PAI, inducing severe inflammation. It has been suggested that the loss of BabA expression may be key to persistence of infection in the face of changing levels of inflammation and glycosylation patterns in the host epithelium (Kable, et al., 2017). Although the loss of BabA expression is partly due to slip-strand mispairing within the *babA* coding sequence, it appears that host factors may regulate BabA expression through other routes as well. A recent study found that gender is the most important host determinant for BabA expression (Kable, et al., 2017). As gastric pH differs significantly between males and females (AC, AW, Choudhary, Piong, & Merchant, 2011), it is possible that acid-responsive TCS signaling may regulate BabA expression. Recently generated preliminary data from our lab further supports the hypothesis that *babA* is acid induced (Ilanchezhian and Forsyth – unpublished results).

The timing of SabA expression is important as the host antigen bound by SabA, sialyl LewisX/a (sLe\(^x\) and SLe\(^a\)) is not expressed in healthy gastric mucosa (Matsuo, Kido, & Yamaoka, 2017). As mentioned previously, it is hypothesized that *H pylori* induces sLe\(^x\)
expression through products of the *cag* PAI after *H pylori* has adhered to the gastric epithelium through other OMPs. Thus, it is only advantageous for *H pylori* to express *sabA* in the later stages of gastric infection, and *sabA* is regulated by acid-responsive signaling through the ArsRS TCS (Acio-Pizzarello, et al., 2017; Forsyth, Cao, Garcia, Hall, & Cover, 2002; Goodwin, et al., 2008). This study uses *sabA* acid repression as a measure of the ArsRS regulated acid response in *H pylori*.

![Diagram](image)

**Figure 3. The acid regulation of urease in H. pylori**
Both the ArsRS TCS and the FlgS TCS sensory protein are involved in the induction *ureA* and *ureB* expression under acidic conditions. UreA and UreB are both subunits of the urease enzyme, which breaks down urea to carbon dioxide and ammonia. The products are then released into the periplasmic space where they act as a buffering system. (Adapted from Krulwich *et al* 2011)

The complicated nature of the signaling paradigm in the *H. pylori* ArsRS TCS demonstrated using phosphomimetic amino acids

The *H. pylori* ArsRS TCS belongs to the OmpR/PhoB family of response regulators. The cognate TCS proteins are encoded in the same operon in the *H pylori* genome (Tomb, et al., 1997; Alm, et al., 1999). In the traditional model for this family of TCS, the membrane-bound ArsS HK detects changes in periplasmic pH via protonation of H94 in the periplasmic sensory
domain (Müller, Götz, & Beier, 2009). This signal is then transduced via the phosphorylation of H214 in the DHp domain (Figure 4). It is not known whether the activation of ArsS occurs through cis- or trans-phosphorylation. The activated form of ArsS transfers its phosphate group to ArsR at D52 in the REC domain, activating the response protein. The activated form of ArsR dimerizes, increasing ArsR affinity for DNA and enabling the protein complex to bind to promoters in the ArsRS regulon and alter gene transcription. Genes in the ArsRS regulon include amiE, amiF, omp11, carbonic anhydrase, hypA, ureAB, ureI, and sabA (Forsyth, Cao, Garcia, Hall, & Cover, 2002) (Joseph & Beier, 2007) (Wen, Feng, Scott, Marcus, & Sachs, 2006) (Loh, Gupta, Friedman, Krezel, & Cover, 2010). Changes in the transcription of these genes are vital for acid adaptation. The ArsS protein has also been shown to be vital for acid-induced trafficking of urease and its accessory proteins to the inner membrane (Marcus E. A., Sachs, Wen, & Scott, 2016).

There have been multiple studies, however, that challenge this traditional model of acid adaptation. A number of research groups have successfully generated mutants with single amino acid substitutions at the phosphor-accepting site in ArsR (Shär, Sickmann, & Beier, 2005; Marcus E. A., Sachs, Wen, & Scott, 2016; Wen, Feng, Scott, Marcus, & Sachs, 2006). These studies further confirm that this substitution abolishes phosphorylation of ArsR by ArsS. ArsR deletion mutants are non-recoverable, indicating that ArsR performs a vital function for H pylori under normal conditions (McDaniel, DeWalt, Salama, & Falkow, 2001). The viability of ArsR phospho-site mutants has thus led some investigators to suggest that ArsR is able to perform some vital function(s) independently of phosphorylation by ArsS (Shär, Sickmann, & Beier, 2005). This hypothesis is further supported by the fact that ArsS deletion mutants are readily recoverable.
Analysis of ArsR D52N *H pylori* under acidic versus neutral conditions revealed that the ArsR D52N mutation not only produced viable *H pylori*, but also allowed for survival under acidic conditions (Marcus E. A., Sachs, Wen, & Scott, 2016). These results have led some investigators to suggest that either phosphorylation of ArsR is not necessary for ArsR activation, or that non-phosphorylated ArsR may be capable of regulating one set of genes, while another set of genes requires a phosphorylated form of ArsR in order to be regulated. The viability of the ArsR D52N mutant has thus been interpreted to indicate that ArsR phosphorylation may not be necessary for ArsR activation (Shär, Sickmann, & Beier, 2005).

As discussed previously, however, phosphomimetic mutations are known to confer phosphorylation-like properties to proteins. ArsR activation could follow a model similar to CovR activation as seen in *S. pyogenes* such that the phosphomimetic mutation in ArsR is sufficient to allow ArsR to bind to promoters in the ArsRS regulon, but is not sufficient to allow ArsR to dimerize. Then, in a manner similar to the phosphomimetic CovR, ArsR D52N may be able to regulate some genes in the ArsRS regulon but not others according to the architecture of the promoters of affected genes. Additionally, there is increasing evidence that many bacteria implement TCSs in more complex signaling systems that exert multiple levels of control over individual genes. Thus the variation in gene expression seen between the phosphomimetic ArsR *H pylori* mutants and the wild type does not necessarily indicate that the phosphorylation of ArsR is unnecessary for acid regulation through the ArsRS TCS.

We thus developed three alternate hypotheses that could explain the viability of phosphomimetic ArsR *H. pylori* mutants. We first hypothesized that the phosphomimetic substitution sufficiently mimics phosphorylation of ArsR by ArsS to allow ArsR to perform an unknown vital function, but that ArsR requires an additional signal through another post
translational modification from ArsS in order to become fully active as a regulator of the transcriptional response to acidity. Alternatively, we hypothesized that ArsS may interact with an intermediate molecule that then regulates ArsR activity. Finally, we hypothesized that genes in the ArsRS regulon could be cross-regulated such that they are also under the control of other TCSs. Thus, in the ArsR D52N mutant, it could be that the phosphomimetic ArsR is unable to regulate acid acclimation genes, and instead other TCS proteins take over. This project seeks to examine the mechanism behind ArsRS acid acclimation through the lens of these three hypotheses.

![Diagram of the acid response (ArsRS) TCS in *H. pylori*](image)

**Figure 4. The acid response (ArsRS) TCS in *H. pylori***
The sensory protein (ArsS) autophosphorylates H214 in response to low pH. ArsS then transfers the phosphate to ArsR at D52, activating ArsR. The active form of ArsR then regulates genes in the ArsRS regulon, including *sabA* and *ureA*.

**Objectives**

TCSs are critical for pathogenesis in many microbes (Hortsmann, Sahasrabhojane, Hui, Su, & Shelburne, 2017; Delaune, et al., 2012; Casino, Rubio, & Marina, 2010; Goodwin, et al., 2008). In *H. pylori*, the ArsRS TCS is critical for the colonization of the human stomach and regulation of factors involved in pathogenesis. Given the specificity of TCSs to a particular bacterial species and the absence of this signal transduction system among vertebrates, TCS proteins are a promising target for therapeutics. It is especially important to explore the potential for targeting TCSs in light of the growing antibiotic resistance seen in bacteria. The mechanism
behind the ArsRS TCS has not been explored in depth. Recent studies have found that the regulation of genes in the ArsRS regulon may not always rely on phosphorylation of ArsR, suggesting a more complex mode of regulation than in the traditional TCS paradigm (Shär, Sickmann, & Beier, 2005; Marcus E. A., Sachs, Wen, & Scott, 2016; Wen, Feng, Scott, Marcus, & Sachs, 2006). The principal objective of this study is to examine how ArsS and ArsR interact under acidic conditions to alter the transcriptome of *H. pylori*.

In order to evaluate the role of ArsR phosphorylation in ArsRS acid regulation, we used site-directed mutagenesis and natural transformation to compare the success rates of phosphomimetic versus non-phosphomimetic substitutions at the site of phosphorylation (D52) in ArsR. We then used qRT-PCR to compare the expression of acid regulated genes between wild type *H pylori* and the ArsR D52E mutant cultured under neutral and acidic conditions. The results from these experiments led to the development of three alternate hypotheses: (1) acid regulation in *H pylori* involves interaction between ArsS and other TCSs in which ArsS phosphorylates non-cognate TCS proteins, (2) in addition to phosphorylating ArsR D52, ArsS also phosphorylates an accessory protein that then also regulates ArsR activity, or (3) ArsR requires an additional post translational modification from ArsS in addition to phosphorylation at D52 in order to become fully active.

The first hypothesis was evaluated by quantifying changes in the expression of acid-regulated genes in *H. pylori* strains with null mutants of *crdR, flgR, crdR/flgR*, or *crdS/crdR*. In order to explore both the first and second hypotheses, an *arsS* deletion mutant and an *arsS* H214A mutant with a no kinase capability were used to examine the role of phosphorylation by ArsS. To address the third hypothesis, we are attempting to insert a 6 histidine (6H) tag into the ArsR in order to purify the protein from *H pylori* cultured under acidic conditions for mass
spectrometry analysis. Through this multifaceted approach we hope to elucidate the regulatory factors involved in the ArsRS mediated acid response in *H pylori* (Krulwich, Sachs, & Etana, 2011).

**Materials and Methods**

*H. pylori* Culture

*H pylori* strain 26695 was cultured on tryptic soy agar II with 5% sheep’s blood (BBL) at 37°C in an ambient air/5% CO₂ atmosphere for 24-48 hours. If a strain was grown for longer than 48 hours, it was passed onto a new plate. Cell lines were never passed more than three times. After being passed three times, strains were started fresh from freezer stock. Liquid cultures of *H pylori* were grown in 6 well plates with sulfite-free Brucella broth (SFBB) supplemented with 1X cholesterol (Gibco® by Life Technologies™), and 20µg/mL vancomycin shaking at 150 rpm.

**Producing pArsR D52G and pArsR D52S mutant plasmids**

Cultures of single colony isolates of *E coli* previously transformed with an *arsR* plasmid containing an upstream CAT chloramphenicol resistance gene and stored at -80°C were started from freezer stock in 5mL LB/1X ampicillin. Cultures were incubated at 37°C Shaking at 225 rpm for 24 hours. The cells were then pelleted at 4,360 rpm for 5 minutes and the plasmid was isolated and purified.

Site directed mutagenesis was conducted on the purified *arsR* plasmid using the QuikChange Lightning Multi Site-Directed Mutagenesis Kit by Agilent Technologies according to the manufacturer’s instructions. 100ng of the purified plasmid was used in each reaction.
Mutagenic primers were designed according to the QuickChange protocol and purchased through IDT. 100ng of the mutagenic primers ArsR D52S and ArsR D52G were used to generate plasmids with non-phosphomimetic mutations at the site of phosphorylation in ArsR (Table 1). *DpnI* enzyme was used to digest the original template, leaving only the mutated plasmid. The products of the mutagenesis reactions were used to transform XL10-Gold Ultracompetent *E. coli* cells. The transformed cells were grown up 16-24 hours in 5mLs LB broth at 37°C shaking at 225rpm and then plated at volumes of 1µL, 10µL and 100µL on LB/amp agar plates. The plates were grown overnight, and isolated amp^R^ colonies were selected and grown up in 5mL LB/1Xamp broth at 37°C, 225 rpm shaking overnight. The plasmids were then purified and isolated. These purified plasmids were transformed into *H. pylori* alongside a simultaneous transformation with the previously purified ArsR D52E phosphomutant and ArsR D52D* plasmids as a control.

### Plasmid purification and isolation

5mL of liquid LB/ampicillin were inoculated with a single isolated colony from the antibiotic plates. Cultures were grown overnight at 37° for 12-24 hours shaking at 225rpm. Cells were spun down at 4360 rpm for 5 minutes. A Midsci Mini Hi-Speed Plasmid Kit by IBI was used to lyse the cells and isolate the plasmids. The DNA yield was quantified using an Implen nanophotometer with a lid factor of 10.

### Natural transformation of *H. pylori*

The strain of *H. pylori* 26695 to be transformed was started from freezer stock by spotting onto a blood agar plate. After 3-5 days, the cells were collected and passed onto 4 new blood agar plates to grow 24-36 hours. Cells were harvested in 2mL SFBB/cholesterol/vancomycin and pelleted at 4360 rpm for 5 minutes. The cell pellets were then resuspended in 100µL.
SFBB/cholesterol/vancomycin via pipetting. The transforming plasmid was heat-sanitized at 80ºC for 20 minutes to avoid contamination. 7-10μg of plasmid were added to the cells, and solution was gently mixed.

The plasmid-cell mixture was spotted in 30µL spots over a blood agar plate and incubated at 37ºC in 5% CO₂. The plate was incubated agar-side down for 4 hours, and then flipped to avoid the accumulation of condensation and incubated in the same environment agar-side up for 24 hours. After this time the spots were spread onto separate SFBB agar plates containing 10% newborn calf serum along with the selection antibiotic. Antibiotic plates were incubated at 37ºC, 5% CO₂ for 4-7 days. Isolated colonies were selected from antibiotic plates and patched onto blood agar plates. Patches were incubated 1-2 days at 37ºC, 5% CO₂ and then spread onto two new blood agar plates for freezer stock and sequencing.

**Analysis of transformation rate of ArsR D52S and ArsR D52G H. pylori transformants**

The plasmid products purified from the ArsR D52G and ArsR D52S mutagenesis reactions contained a CAT gene conferring Cm⁺, thus the *H pylori* cells transformed with these plasmids were plated on chloramphenicol plates as a selection mechanism for successfully transformed colonies. 10 isolated colonies were selected from each transformation and grown up on blood agar plates for sequencing analysis to confirm successful transformation at the D52 site. Cells for sequencing were harvested from the plates and pelleted at 6,000 rpm for 5 minutes. The Mini gBAC genomic DNA kit by IBI was used to extract genomic DNA following the manufacturer’s instructions for Gram-negative bacteria. Sequencing analysis was used to confirm successful transformation. Sequencing reactions were conducted using primer HP166-12 (Table 1). The rate of transformation at the D52 site was then compared between the ArsR D52S and D52G mutants. ArsR D52D* (in which the GAT codon encoding the 52nd aspartate residue
was substituted with an alternate aspartic acid codon, GAC) and ArsR D52E were used as controls.

**Sequencing analysis to confirm successful transformation**

Sequencing analysis was conducted to confirm successful transformation of the plasmid into the *H pylori* genome at the site of interest. The region of interest was amplified via PCR. Agarose gel electrophoresis with a 1kb ladder was used to confirm the size of the PCR amplicons were within the expected range. PCR products were then purified using the Midsci Gel/PCR DNA-Fragment Extraction Kit by IBI following the manufacturer’s protocol. A sequencing reaction was then conducted using BigDye stock for sequencing by Invitrogen with the purified PCR products and the appropriate primers.

**Broth culture at acidic versus neutral pH**

The pH of SFBB/cholesterol/vancomycin was measured and the broth was then divided in half. HCl was added to one aliquot of the broth to reach a pH of 5. NaOH was used to adjust the remaining aliquot to a pH of 7. Both aliquots were then filtered through a 2µM filter to sterilize the broth. *H pylori* strains of interest were started from freezer stock on TSA II plates with 5% sheep’s blood (Becton-Dickinson) and incubated at 37°C in ambient air/5% CO₂. Cells were harvested in 2 mL pH7 SFBB/cholesterol/vancomycin. The OD₆₀₀ was measured, and cultures were started at an OD₆₀₀ of 0.2 in 6 mL of pH 7 SFBB/cholesterol/vancomycin in a 6 well plate. Cultures were incubated overnight at 37°C in an ambient air/5% CO₂ atmosphere shaking at 150rpm. The OD₆₀₀ of each culture was then taken to ensure that all cultures showed growth, and cultures were passed to a new 6 well plate at a starting OD₆₀₀ of 0.20 in 6mL. These cultures were incubated another 5-7 hours, and then each culture was divided in two and spun down at 1800 x g for 5 minutes. One aliquot of each culture was resuspended in 3 mL pH 7
broth, and the other in 3 mL pH 5 broth. These cultures were incubated for another hour under the same conditions as the previous cultures. The cells were then spun down at 1800 x g and suspended in 1 mL Trizol reagent for mRNA isolation via MagMax or 1mL RNAzol for RNAzol mRNA extraction. Cells were stored at -80ºC until RNA extraction.

MagMax RNA isolation

Cells suspended in 1mL Trizol were lysed in a bead-ruptor. BCP Phase Separation Reagent was used to separate out cellular debris. Each sample was cycled through the MagMax instrument, and magnetic beads were used to isolate the RNA. Eluted RNA was quantified using an Implen nanophotometer and was stored at -80ºC.

RNAzol RNA extraction

Cells were suspended in 1mL RNAzol and 2µL polyacryl carrier were added to each sample to assist in RNA precipitation. Cells were lysed in a beadrupter for 45 seconds at a speed of 5.0. Water was added and the tubes were spun at 12,000 x g for 8 minutes to isolate the RNA. A series of ethanol washes were used to purify the RNA. RNA pellets were then resuspended in 30µL PCR grade water. Yield was measured using a nanodrop and the isolated RNA was stored at -80ºC for qRT-PCR analysis.

Real-time quantitative PCR (qRT-PCR)

qRT-PCR was used to compare relative levels of sabA and ureA under acidic versus neutral conditions. Expression levels of both genes were standardized to the housekeeping gene fisZ, which encodes the cell division protein FtsZ. The experiment was performed using a TaqMan Gene Expression assay (Life Technologies) in the 96-well Applied Biosystems StepOne™. The assay was conducted according to the manufacturer’s protocol using the custom TaqMan Gene Expression probes sabA.Taq, ureA.Taq, and fisZ.Taq. Each assay was run in
technical triplicate with three biological replicates for each experiment. Relative gene expression was calculated following the $2^{\Delta \Delta Ct}$ method described by Livak and Schmittgen (Livak & Schmittgen, 2001).

**Generating a genetic knockout of arsS in H pylori**

*H pylori ΔarsS* was generated by Mark Forsyth in the lab via chloramphenicol/metronidazole counter-selection method developed by Mark McClain at Vanderbilt University Medical Center (Loh, et al., 2011). A previously synthesized *arsS* plasmid was grown up in *E coli* for 16-24 hours and then purified and isolated. A CAT-*rdxA* gene cassette was inserted into the plasmid via site-directed mutagenesis, rendering the *arsS* gene nonfunctional (*parsS/CAT/rdxA*). The CAT gene confers chloramphenicol resistance while the *rdxA* gene confers metronidazole sensitivity when placed into an *rdxA*− genetic background. This plasmid was cloned in *E coli*, purified, and then transformed into *H. pylori ΔrdxA*. This strain is Mtz-R due to the loss of RdxA, a gene encoding a nitroreductase enzyme that reduces metronidazole, thus activating its bactericidal properties. Transformed colonies of *H pylori* were selected for on chloramphenicol plates. Ten isolated colonies were grown up for gDNA extraction, and the *arsS* gene was PCR amplified and run on an agarose gel to confirm the insertion of the CAT-*rdxA* cassette.

Another site directed mutagenesis was performed on the original *arsS* plasmid to delete 390 bp of the gene, rendering it nonfunctional (*pΔarsS*). Previously, *H pylori* 26695 had been successfully transformed with the *parsS/CAT/rdxA* to produce *H pylori arsS::CAT-rdxA*. The *pΔarsS* plasmid was then cloned, purified, and transformed into *H pylori arsS::CAT-rdxA*. Transformed colonies were selected for on metronidazole plates, as transformation with *pΔarsS* would have excised the CAT-*rdxA* gene cassette. The successful allelic replacement would now
be metronidazole resistant. Successful transformation was confirmed via sequencing, and 5 transformed colonies were grown up and stored at -80°C.

**Generating the ArsS H214A H pylori kinase mutant**

The ArsS H214A mutant *H pylori* strain was generated by Clara Sartor. Site-directed mutagenesis was conducted on the *parsS* plasmid as described above to mutate the H 214 codon to an alanine codon, generating *parsS*H214A. This mutant plasmid was then used to naturally transform HP 26695/∆rdxA/arsS::cat-rdxA. Transformed colonies were selected on SFBB/metronidazole plates. The H214A mutation was confirmed through PCR amplification and sequencing of the *arsS* locus.

**Generating genetic knockouts of flgR and crdR in H pylori**

*crdR* and *flgR* deletion strains were generated by Mark Forsyth prior to the start of this study (Forsyth & Cover, unpublished data). An *H pylori* mutant with genetic knockouts in both *flgR* and *crdR* was generated by transformation of the *H. pylori* strain 26695/∆rdxA/∆crdR strain with p*flgR::CAT-rdxA*, generating 26695/∆rdxA/∆crdR/∆flgR-CAT-rdxA. Successfully transformed colonies were selected for on chloramphenicol plates. Insertion of the CAT-rdxA cassette was confirmed by gDNA extraction, PCR amplification of the *flgR* gene, and agarose gel electrophoresis. Insertion of the cassette renders *flgR* non-functional, and no further mutations were desired in this strain, thus the 26/∆crdR/∆flgR-CAT-rdxA strain was used for broth culture pH 7 vs pH 5 experiments.

**Electrophoretic mobility shift assays (EMSAs)**

PCR using gDNA from *H. pylori* J99 was used to generate *hopZ*, *labA*, and *sabB* biotin end-labelled probes. Primers *hopZ*-Bio and *hopZ*-R3 were used to amplify the *hopZ* promoter region (-141 to +113) for use as a probe in EMSAs. Primers *labA*-Bio and *labA*-R3 were used to
amplify the labA promoter region (-204 to +125), also for use as an EMSA probe. For the final probe, primers sabB-Bio and sabB-R were used as primers to amplify the sabB promoter region. Non-biotin labelled versions of hopZ-Bio, labA-Bio and sabB-Bio were amplified for use in the EMSA as unlabeled, specific DNA competitors. rArsR protein was expressed in E. coli and purified following a method previously described by Mark Forsyth (Harvey, et al., 2014). EMSAs were conducted with the LightShift Chemiluminescent EMSA Kit (Thermo) following manufacturer’s instructions. 0.2nmol rArsR was incubated with or without nonspecific competitors in 10X Binding Buffer, 50% glycerol, 100 μM MgCl₂, 1% NP40, and Poly dI/dC at room temperature for 10 minutes. Incubations without ArsR or with varying concentrations of unlabeled, nonspecific competitors were used as controls (175-fold molar excess for labA, 500-fold molar excess for hopZ, and 300-fold molar excess for sabB. 2 µL Biotin labeled probe were added to the appropriate wells for final concentrations between 9 and 20fmol, and the reactions were incubated for an additional 20 minutes at room temperature. The resulting DNA and DNA-protein complexes were run on 5% TBE native polyacrylamide gels (Bio-Rad) at 100V, Western blotted to Zeta Probe membranes (Bio-Rad) and UV cross-linked. Biotin DNA and DNA-rArsR complexes were then detected using streptavidin-horseradish peroxidase and chemiluminescent imaging (Harvey, et al., 2014; Loh, Gupta, Friedman, Krezel, & Cover, 2010).

Results

Only phosphomimetic D52 mutations are viable

Previous studies have found H pylori 26695 mutant strains with asparagine (N) in the place of aspartic acid (D) at the 52<sup>nd</sup> site are viable (Marcus E. A., Sachs, Wen, & Scott, 2016; Shär, Sickmann, & Beier, 2005). As functional ArsR is essential for H pylori viability, this has
led some investigators to claim that at least part of the acid response regulated by ArsRS may be regulated independent of ArsR phosphorylation. Asparagine, however, is a phosphomimetic amino acid. This means that asparagine has physical properties similar to those of a phosphorylated Asp residue. We thus proposed that the ArsR D52N mutant was viable due to its phosphomimetic ability, and that phosphorylation of ArsR was necessary for a functional acid response.

Figure 5. Transformation of HP 26695 with phosphomimetic and non-phosphomimetic ArsR D52 mutations
A) Construct of plasmid used in site-directed mutagenesis reactions. (B) Crossover at the D52 codon was only successful with the D52D* control plasmid and the plasmids containing the D52N and D52E phosphomimetic mutations. This indicates that phosphoactivity at this site is necessary for a vital ArsR function.

In order to investigate this hypothesis, we evaluated the viability of an additional phosphomimetic ArsR mutant (D52E) and a series of non-phosphomimetic mutants (D52A, D52G, and D52S). We generated mutant plasmids through site-directed mutagenesis reactions on a plasmid containing a CAT gene cassette upstream of arsR in HP0167 (Figure 5A). ArsR D52D* was used as a control. Then we naturally transformed these plasmids into H. pylori 26695. Antibiotic selection for chloramphenicol resistance was used to isolate transformed colonies. We then selected ten transformed colonies from each reaction for sequencing to confirm complete crossover of the mutant plasmid. Results showed that in both the ArsR D52N and D52E phosphomimetic mutants, the mutations at the 52nd site went in at a high rate (90% and 80% respectively). Conversely, in the non-phosphomimetic mutants, D52A, D52G and
D52S, none of the colonies selected showed successful transformation at the 52nd site (Figure 5B), suggesting that these non-phosphomimetic mutants are nonviable, and that the ArsR D52N and D52E mutants are viable due to their phosphomimetic activity.

The ArsR D52E mutant shows an intact acid response but not constitutive acid regulation

If phosphomimetic activity at the 52nd amino acid was sufficient to produce a functional acid response in *H. pylori*, then we would expect to see constitutive acid regulation of genes in the ArsRS regulon under neutral conditions. We cultured *H. pylori* 26695 *arsR* D52E along with wild type *H. pylori* 26695 under acidic and neutral conditions and then extracted mRNA for cDNA synthesis. qRT-PCR using TaqMan (Invitrogen/Thermo-Fisher) probes was used to evaluate the mRNA levels of both an acid induced and an acid repressed gene in the ArsRS regulon (ureA and sabA respectively). Results showed significantly reduced expression of *sabA* (Figure 6) but not *ureA* (Figure 7) in the HP/26695/arsR D52E mutant relative to wild type *H. pylori* at pH 7. The phosphomimetic mutant still showed further acid repression of *sabA* at pH 5 (Figure 6) and intact acid induction of *ureA* (Figure 7), indicating that the mutant did not show full constitutive acid regulation of either *sabA* or *ureA*, contrary to our prediction at the outset of these experiments, suggesting that the phosphomimetic ArsR D52E may be only partially active and thus able partially regulat *sabA* but not *ureA*. The ability of the phosphomimetic mutant to induce *ureA* expression and further repress *sabA* at pH 5 suggests that genes in the ArsRS regulon may be regulated by other proteins in addition to ArsR, or that ArsR may require an additional post translational modification besides phosphorylation at D52 in order to become fully active.
Figure 7. Relative expression of *ureA* in phosphomimic HP/26695/arsR D52E
qRT-PCR was used to quantify relative mRNA levels to compare acid induction of *ureA* in the ArsR D52E strain of *H pylori* to those in wild type *H. pylori*. mRNA levels in the wild type at pH 7 were set to one as a standard. There was no significant change in *ureA* expression at pH 7 or acid induction of *ureA* at pH 5 in the ArsR D52E mutant relative to the wild type control (p=0.46 and p=0.0935 respectively). The ArsR D52E mutant still showed a very significant acid induction of *ureA* at pH 5 relative to at pH 7 (p=0.0045). p-values were determined via Welch’s unpaired *t* test of unequal variance.

Figure 6. Relative expression of *sabA* in phosphomimic HP/26695/arsR D52E
qRT-PCR was used to quantify relative mRNA levels to compare acid induction of *sabA* in the ArsR D52E strain of *H pylori* to those in wild type *H. pylori*. mRNA levels in the wild type at pH 7 were set to one as a standard. Results showed a significant decrease in *sabA* expression at pH 7 in the ArsR D52E mutant relative to the wild type control (p=0.0124). There was no significant change in *sabA* repression at pH 5 in the ArsR D52E mutant relative to the wild type control (p=0.324). The ArsR D52E mutant still showed a very significant acid repression of *sabA* at pH 5 relative to at pH 7 (p=0.0066). p-values were determined via Welch’s unpaired *t* test of unequal variance.
Phosphorylation by ArsS required for acid response in *H. pylori*

In order to investigate the role of phosphorylation by ArsS in the acid response of *H. pylori*, we generated a series of ArsS mutants using site-directed mutagenesis and evaluated their ability to both acid induce *ureA* ([Figure 8A](#)) and acid repress *sabA* ([Figure 8B](#)). Relative gene transcription levels were measured using qRT-PCR. We found that an *arsS* deletion mutant failed to both acid induce *ureA* and acid repress *sabA*, indicating that the ArsS protein is necessary for acid regulation of these two genes. Next, we produced an *arsS* H214A mutant in which the ArsS histidine residue involved in phosphorylation was changed to alanine, and thus cannot be phosphorylated. This mutant also failed to both acid induce *ureA* ([Figure 11A](#)) and acid repress of *sabA* ([Figure 8B](#)).

Given that the phosphomimetic ArsR mutant *arsR* D52E produced a functional acid response in spite of being incapable of phosphorylation by ArsS, we introduced the *arsR* D52E mutation into the *arsS* deletion mutant in order to examine whether the phosphomimetic form of ArsR was sufficient to mediate the acid response in the absence of ArsS. We found that this 26695/Δ*arsS*/*arsRD52E double mutant was also incapable of acid induction of *ureA* ([Figure 8A](#)) or acid repression of *sabA* ([Figure 8B](#)), indicating that ArsS still plays a role in acid regulation in the *arsR* D52E mutant. There are at least two alternative hypotheses regarding the role of ArsS in this mutant: either ArsS is involved in an additional posttranslational modification of ArsR beyond phosphorylation at D52, or ArsS mediates the acid regulation of *ureA* and *sabA* through a means independent of ArsR. ArsS possesses some structural similarities to an acetyl transferase, indicating that it may be able to acetylate ArsR in addition to its HK capabilities. A growing body of evidence of cross-talk between TCSs, however, supports the latter hypothesis that ArsS might be able to mediate the acid regulation of *ureA* and *sabA* independently of ArsR.
Phosphorylation by ArsS still required in the ArsR D52E mutant

As phosphorylation by ArsS is required for acid induction of ureA and the phosphomimetic arsR D52E mutation is viable, we generated a mutant H. pylori strain with a loss in ArsS kinase ability coupled with the D52E phospho-mutation in ArsR (HP/26695/arsS H214A/arsR D52E). If this mutant were able to acid regulate ureA, then that would suggest that ArsR requires multiple posttranslational modifications (including phosphorylation at D52) from ArsS in order to become fully active. qRT-PCR analysis of ureA expression in this mutant at pH 7 and pH 5, however, showed no significant acid induction of ureA at pH5 relative to pH7 (Figure 12). Furthermore, this strain showed an expression pattern of ureA similar to the H. pylori strain with a loss in ArsS kinase ability but without phosphomimetic ArsR. These results

Figure 8. Relative expression of ureA and sabA in H. pylori 26695 with mutations in arsS
(A) qRT-PCR results show a significantly decreased acid induction of ureA in both the arsS null mutant (∆arsS) and the non-phosphorylatable arsS mutant (arsSH214A). Introduction of the phosphomimetic arsR D52E mutation into the arsS knockout mutant failed to restore the acid response. (B) qRT-PCR analysis of sabA expression showed that these mutants also exhibited a significant decrease in acid repression of sabA.
indicate that ArsS can regulate the expression of *ureA* independently of ArsR, potentially through the phosphorylation of a noncognate RR.

![Graph showing relative expression of *ureA* in different conditions](image)

**Figure 9.** Relative expression of *ureA* in a phosphomimetic ArsR mutant lacking ArsS kinase ability (HP/26695/arsS H214A/arsR D52E)

qRT PCR showed that the HP/26695/arsS H214A/arsR D52E phosphomutant showed no significant acid induction of *ureA* relative to wild type *H. pylori* at pH 5 and showed an expression pattern of *ureA* similar to the HP/26695/arsS H214A strain lacking phosphomimetic ArsR. mRNA levels in the wild type at pH 7 were set to one as a standard. Statistical analysis was conducted via Welch’s unpaired *t* test of unequal variance.

No evidence of cross-talk between ArsRS and either FlgR or CrdRS

As studies on TCSs in other bacteria have shown cross-regulation between non-cognate TCS proteins in the same cell (Leday, et al., 2008), we investigated the potential for cross-talk between ArsRS and two other TCSs in *H. pylori*: CrdRS and FlgRS. We generated a deletion mutant of *crdR* (Δ*crdR*) and a deletion mutant of *flgR* (Δ*flgR*). We cultured these mutants at pH 7 and pH 5 and then compared transcript levels of *ureA* and *sabA* in neutral versus acidic
conditions in order to evaluate the acid response in these mutants. Wild type *H. pylori* was used as a control.

**Figure 10. Relative expression of ureA and sabA in crdR and flgR mutants**

(A) qRT-PCR results show significant change in the acid induction pattern of *ureA* in HP/26695/*ΔcrdR*. There was a significant increase in acid induction of *ureA* in HP/26695/*ΔflgR*, but this was only a 1.4-fold increase and there was no significant increase in *ureA* expression at pH 5 in the HP/26695/*ΔcrdR*/*ΔflgR* mutant, thus the involvement of FlgR in the regulation of *ureA* does not appear to have a significant effect. (B) There was no significant change in the acid repression of *sabA* in the *crdR* and *flgR* mutants. Statistical tests were conducted via Welch’s unpaired t test of unequal variance.

**Figure 11. ureA expression in the absence of CrdRS and in the absence of CrdR with phosphomimetic ArsR**

qRT-PCR analysis of HP/26695/*ΔcrdR*/*ΔcrdS* showed no significant change in the pattern of acid induction of *ureA* at pH 7 versus pH 5. qRT-PCR analysis of HP/26695/*crdR*/*arsR D52E* also showed no significant change in acid induction of *ureA*. Statistical analysis was conducted via Welch’s unpaired t test of unequal variance.
Our results showed no change in acid induction of *ureA* in either the Δ*crdR* mutant or the Δ*flgR* mutant (**Figure 10A**). Additionally, there was no significant change in acid repression of *sabA* in these mutants (**Figure 10B**). In order to investigate whether cross talk occurs between ArsRS and the CrdRS TCS at levels beyond the RR, a mutant with deletions in both *crdR* and *crdS* (HP/26695/Δ*crdR*/Δ*crdS*) was generated. This mutant also showed no significant change in acid induction of *ureA* (**Figure 11**). Finally, in order to determine whether cross talk between ArsS and CrdR has an effect on *ureA* expression that is only visible in the absence of phospho-accepting ArsR, we generated a mutant with phosphomimetic ArsR but lacking CrdR (HP/26695/Δ*crdR*/arsR D52E). This mutant strain also showed no significant differences in acid induction of *ureA* at pH 5 relative to wild type *H. pylori* (**Figure 11**). These results suggest that cross talk between the ArsRS TCS and ΔCrdR or ΔFlgR is not involved in the acid regulation of *ureA* or *sabA*, but does not rule out the possibility of cross talk between the ArsRS TCS and other response regulators.

**ArsR binds promoter regions of adhesin encoding genes labA, hopZ, and sabB**

Recent research has indicated that the ArsRS TCS may play a role in biofilm formation (Servetas, Carpenter, Haley, Gilbreath, & Gaddy, 2016). We thus investigated the role of ArsR and ArsS in biofilm formation. Mark Forsyth and Catherine Acio-Pizzarello designed a study in which qRT-PCR was used to examine the expression of adhesin genes *sabB*, *hopZ*, and *labA* in the absence of functional ArsS (Acio-Pizzarello, et al., 2017). We also performed EMSAs to test the ability of rArsR to bind to the promoter regions of these genes. qRT-PCR analysis showed significantly increased transcript levels of all three genes, indicating that ArsS plays a role in repressing the expression of these adhesin proteins (data not shown). Furthermore, EMSA analysis showed that rArsR bound to the promoter region of all three adhesin genes in a specific
manner (Figure 12). These findings suggest that ArsS represses the expression of the adhesin genes sabB, hopZ, and labA via ArsR.

Discussion

TCSs are classically thought of as relatively simple signal transduction systems with two proteins interacting in isolation in order to regulate the expression of genes in the TCS’s regulon (Casino, Rubio, & Marina, 2010). Recent research in multiple bacterial species, however, have indicated that TCS signal transduction may be much more complex than originally thought (Delaune, et al., 2012; Hortsmann, Sahasrabhojane, Hui, Su, & Shelburne, 2017; Jain, et al., 2017; Kalantari, Derouiche, Shi, & Mijakovic, 2015; Leday, et al., 2008). One such study indicated that in H. pylori, acid regulation by the ArsRS TCS may be operate independently of phosphorylation (Shär, Sickmann, & Beier, 2005). The findings were further supported by additional research by Marcus et al (Marcus E. A., Sachs, Wen, & Scott, 2016). The ArsR mutants used in these studies, however, had phosphomimetic amino acid substitutions at the phosphor-accepting, conserved aspartic acid at the 52nd position. The arsR gene is vital for H. pylori survival, as demonstrated by the fact that colonies with deletions of arsR are non-
recoverable (McDaniel, DeWalt, Salama, & Falkow, 2001). As the phosphomimetic ArsR colonies ArsR D52E and ArsR D52N are recoverable while mutants with deletions of *arsR* are not, we hypothesized that these mutants were only viable due to the phosphomimetic substitution at this site, and that wild type *H pylori* still relies on phosphorylation at this site for a functional acid response. We found that the non-phosphomimetic substitutions ArsR D52G, ArsR D52S, and ArsR D52A did not yield viable colonies. These findings support the hypothesis that acid regulation by ArsRS requires phosphorylation of ArsR, and that the ArsR D52E and ArsR D52N mutants are viable due to their phosphomimetic activity.

If these mutants were viable due to the phosphomimetic activity of ArsR, we would expect to see constitutive regulation of genes in the ArsRS regulon such that these genes would be constantly expressed as if the bacteria were under low pH conditions, even in neutral conditions. qRT-PCR analysis of these phosphomimetic mutants shows that *sabA*, a gene known to be repressed by ArsRS (Forsyth, Cao, Garcia, Hall, & Cover, 2002), does not show constitutive acid repression at pH 7 in either HP 26695 ArsR D52E or HP 26695 ArsR D52N. Additionally, *ureA*, a gene induced by ArsRS under acidic conditions (Forsyth, Cao, Garcia, Hall, & Cover, 2002), does not show constitutive up-regulation at pH 7 in either phosphomimetic mutant. These findings combined with previous findings that only phosphomimetic substitutions may be made at the 52nd position in ArsR suggest that phospho-activity at the 52nd position in ArsR is necessary for ArsR to perform a vital function in *H. pylori*, but that phosphomimetic mutations at this site are not sufficient to fully activate ArsR for acid regulation of genes in the ArsRS regulon.

These results are similar to findings describing regulation of virulence factors by the CovRS TCS in *Streptococcus pyogenes* (Hortsmann, Sahasrabhojane, Hui, Su, & Shelburne,
The phosphomimetic form of CovR (CovR D53E) is able to regulate some genes in the CovRS but not others. The phosphomimetic CovR D53E is able to bind DNA, but is unable to dimerize as the phosphorylated wild type CovR does. Analysis of the promoter regions of genes in the CovRS regulon indicates that genes regulated by the monomeric form of CovR D53E show different promoter architecture than those that do not show altered transcription in response to monomeric CovR D53E binding.

Both ArsR and CovR are OmpR type response regulators, and thus show similar properties (Casino, Rubio, & Marina, 2010; Joseph & Beier, 2007). It could be that ArsR D52E, similarly to CovR D53E, is able to bind genes in the ArsRS regulon but unable to dimerize, and that the monomeric form of ArsR D52E is able to regulate some genes in the ArsRS regulon according to promoter architecture. As ArsR D52E cannot be phosphorylated, however, this hypothesis would not explain the change in transcriptional activity seen in both sabA and ureA. There must be another means of inducing changes in the transcription of these genes in the absence of phosphorylatable ArsR. We hypothesized that the acid regulation seen in the ArsR D52E phosphomutant could be through regulation by another TCS, interaction between the ArsS sensory protein and a non-cognate response regulator, or through additional post-translational modifications to ArsR, either by ArsS or another regulatory molecule.

In order to investigate whether ArsS kinase activity is necessary for acid regulation, we generated a mutant strain lacking arsS (HP26695/ΔarsS) and a mutant strain in which ArsS lacked kinase activity (HP26695/arsS H214A). qRT-PCR analysis of gene expression in both ArsS mutant strains grown at pH 7 and pH 5 showed neither acid repression of sabA nor acid induction of ureA. These results suggest that the kinase activity of ArsS is necessary for acid regulation in wild type H. pylori. In order to determine whether these results were consistent in
the ArsR D52E *H. pylori* phospho-mutant strain, we introduced the *arsR* D52E mutation into the HP26695/∆*arsS* strain generating HP26695/∆*arsS/arsR* D52E. qRT-PCR analysis of this mutant also showed no acid repression of *sabA* or acid induction of *ureA*, indicating that ArsS is still vital for acid regulation of the ArsRS regulon even in the presence of phosphomimetic ArsR.

As only phosphomimetic substitutions are viable at the phospho-accepting site in ArsR, ArsS kinase activity is necessary for both acid induction of *ureA* and acid repression of *sabA*, and ArsS is still necessary for acid regulation of these genes in the presence of the ArsR D52E phosphomimetic, we hypothesized that ArsS interacts with a non-cognate response regulator in the acid regulation of *sabA* and *ureA*. We propose that the phosphomimetic activity of ArsR D52E is able to activate ArsR sufficiently to perform some function vital to *H. pylori* survival, but not sufficiently to allow ArsR to regulate genes in the ArsR regulon, allowing ArsR D52E and ArsR D52N to produce viable colonies, but not resulting in constitutive acid regulation of genes in the ArsRS regulon.

As a mutant strain of *H pylori* with phosphomimetic ArsR (HP/26695/arsRD52E) produces a functional acid regulation of *ureA* and *sabA* (but not constitutive acid-like regulation), this response is dependent on kinase activity of ArsS, and crosstalk via CrdR and/or FlgR is not involved, we hypothesized that phosphorylation of ArsR by ArsS at D52 is necessary but not sufficient for acid regulation of *sabA* and *ureA* and that a further post-translational modification of ArsR by ArsS is required for ArsR to become fully active in acid regulation. We proposed that the phosphomimetic activity of ArsR D52E is able to activate ArsR sufficiently to perform some function vital to *H. pylori* survival, but not sufficiently to allow ArsR to regulate genes in the ArsRS regulon, allowing ArsR D52E and ArsR D52N to produce viable colonies, but not resulting in constitutive acid regulation of *ureA* and *sabA*. 
In order to investigate this hypothesis, we first attempted to generate an *H. pylori* strain with a 6-His tagged ArsR protein for mass spectrometry analysis. Multiple attempts to generate this strain through a variety of methods failed. We initially attempted to generate a plasmid for 6-His tagged ArsR via site-directed mutagenesis on a plasmid for the ArsR locus. When this plasmid was transformed into *E. coli*, however, no colonies were recoverable. We then designed a synthetic plasmid with a 6-His tag at the end of the ArsR locus. This plasmid was naturally transformed into a strain of *H. pylori* with *rdxA* and CAT genes, conferring metronidazole sensitivity and chloramphenicol resistance respectively, inserted between the *arsR* and *arsS* loci as a marker of successfully transformation. Selection for transformed colonies on metronidazole blood agar plates, however, did not yield colonies. We hypothesized that the inability to recover colonies was due to the sequence of the synthetic plasmid failing to overlap sufficiently with the *rdxA* gene. We thus used PCR to ligate the synthetic plasmid to additional sequence downstream of the ArsR locus going into the ArsS locus. Attempts to naturally transform this product into the same *H. pylori* strain, however, also failed to produce viable colonies. As our lab has had trouble directly transforming PCR products into *H. pylori* in the past, we ligated this PCR product into a plasmid vector. Attempts to transform this plasmid into *E. coli*, however, have thus far been unsuccessful.

We thus decided to take another approach by evaluating whether an *H. pylori* strain with a loss in ArsS kinase ability but with ArsS otherwise intact could produce a functional acid response in the presence of phosphomimetic ArsR (HP/26695/arsRD52E/arsSH214A). Our previous findings suggested that ArsR D52E has some phosphomimetic activity as only phosphomimetic substitutions (such as glutamic acid) are viable at the 52nd site in ArsR. Thus, if ArsS activates the acid response in HP/26695/arsRD52E via an additional post translational
modification to ArsR other than phosphorylation at D52, then the HP/26695/arsRD52E/arsSH214A strain should produce a functional acid response at pH 5. qRT-PCR analysis HP/26695/arsRD52E/arsSH214 showed no significant acid induction of ureA under acidic conditions, suggesting that acid regulation in the ArsR D52E mutant may be independent of ArsR, and instead relies on phosphorylation of non-cognate proteins by ArsS. Cross talk between two component systems has been demonstrated in vitro (Fisher, Jiang, Wanner, & Walsh, 1995), but has never conclusively been found in vivo (Stock, Robinson, & Goudreau, 2000).

The FlgS sensory histidine kinase is required for acid regulation in *H. pylori* at a pH of 2.5 or less, and ureA is included in the acid-regulated regulon of FlgS (Wen, Feng, Scott, Marcus, & Sachs, 2006). Research suggests that acid regulation by FlgS does not require FlgR. As FlgS and FlgR are cognate RRs, however, the implication of FlgS in acid regulation makes FlgR a promising candidate for interaction with ArsS in acid regulation. qRT-PCR analysis of a mutant strain of *H. pylori* with a deletion in *flgR* cultured at pH 7 and pH 5 showed no significant changes in sabA or ureA expression at pH 5 relative to wild type *H pylori*. These results suggest that FlgR is not involved in the acid regulation of these genes. As FlgS is involved in the acid regulation of ureA, it would be worthwhile to investigate the expression of ureA in an ArsR D52E mutant with a deletion in *flgR* (HP/26695/arsRD52E/ΔflgR).

CrdR also provides a promising candidate for interaction with ArsS in the acid regulation of ureA and sabA. Both CrdR and ArsR are OmpR type RRs, indicating that the two RRs share significant structural homology (Joseph & Beier, 2007). The REC domain in OmpR type RRs is composed of a (βα)5 fold with 5 parallel β-strands surrounded by α-helices on both sides (Nguyen, Yoon, Cho, & Lee, 2015). The conserved phospho-accepting Asp residue is positioned
between β3 and α3. The REC domain of OmpR type RRs also show an additional conserved Asp residue in the Lβα1 loop involved in coordinating the Mg$^{2+}$ ion involved in phosphor-transfer, conserved catalytic Thr and Ser residues in the Lβα4 loop that interact with the oxygen atoms of the phosphoryl group, and a conserved Lys residue in the Lβα5 loop involved in phosphotransfer. This domain interacts with the sensory HK in the transfer of a phosphate group from a conserved His residue in the HK to the conserved Asp residue between β3 and α3 in the REC domain. The conservation of structure seen between REC domains in OmpR type RRs indicates that HKs may be able to interact with non-cognate RRs.

OmpR type RRs also show significant homology in their effector domains (Nguyen, Yoon, Cho, & Lee, 2015). At the C-terminus, OmpR type RRs show a winged helix turn helix (wHTH) motif ($α1-β1-α2-T-α3-β2-W-β3$) that binds the target gene. Although many types of DNA-binding proteins have a wHTH motif, the OmpR/PhoB is distinct in that it has a 4-strand antiparallel β-sheet before the wHTH motif. The sequence of the 4-strand antiparallel β-sheet is not well conserved, but it is conceivable that two OmpR-type RRs within the same bacteria could share significant sequence homology. Variation within this sequence allows for binding to specific genes, and there could be some redundancy in binding between OmpR-type RRs within a bacterium.

Mirroring results seen in HP/26695/ΔflgR qRT-PCR analysis of a mutant strain with a deletion in the crdR locus (HP/26695/ΔcrdR) showed no significant changes in acid induction of sabA or ureA pH 5. A mutant H pylori strain with deletions in both crdR and flgR (HP/26695/ΔcrdR/ΔflgR) also showed no significant change in sabA or ureA expression under acidic conditions. Furthermore, a mutant with deletions at both the crdR and crdS (HP/26695/ΔcrdS/ΔcrdR) These results suggest that the CrdRS TCS is not involved in acid
regulation of *ureA* or *sabA* and refute our hypothesis that the regulation of the ArsRS regulon relies on cross talk between ArsRS and the CrdR and FlgR noncognate RRs. These RRs may still be involved in the acid regulation of other genes in the ArsRS regulon, as the regulation of genes by RRs has been shown to vary depending on promoter architecture (Hortsmann, Sahasrabhojane, Hui, Su, & Shelburne, 2017). EMSA analysis showed that rArsR is capable of binding *sabB*, *hopZ*, and *labA* promoters, thus future research on cross-talk between ArsRS and non-cognate TCS proteins should expand to include the regulation of these genes. These findings further underscore the importance of the ArsRS TCS as these genes encode adhesin proteins that are tightly linked with *H. pylori* pathogenesis. Future research on gene expression regulated by the ArsRS TCS should thus expand to evaluate the expression of these adhesin genes.

Our findings led us to propose to new potential hypotheses to explain the acid regulation of *ureA* and *sabA* in the absence of phospho-accepting ArsR: (1) **ArsS phosphorylates a non-cognate response regulator besides CrdR or FlgR that also works to regulate *sabA* and *ureA***; or (2) **In addition to ArsR, ArsS also phosphorylates an accessory protein that then interacts with ArsR to further activate it** (Figure 13). Future research should focus on the potential for cross talk between the ArsS TCS and other non-cognate TCS proteins in the acid regulation of both *sabA* and *ureA*. A protein-pull down experiment of ArsS may reveal which proteins in *H. pylori* are capable of interacting with ArsS. Additionally, programs for the analysis of protein structure, such as RaptorX, can model the probability of protein-protein interactions and could give some insight as to which proteins are probable candidates for cross talk with ArsS. Further work to generate a 6-His tagged ArsR protein would allow for mass spectroscopy analysis of ArsR to evaluate what other post translational modifications might be occurring to activate ArsR in addition to phosphorylation at D52.
**Acknowledgements**

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**References**


Copper Resistance Determinants CrdA (HP1326), CrdB (HP1327), and CzcB (HP1328) in Helicobacter pylori. *Journal of Bacteriology, 184*(23), 6700-6708.


**Tables**

**Table 1: primers used in this study**

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