Transcriptional Regulation of the Acetone Carboxylase Operon via Two-Component Signal Transduction in Helicobacter pylori

Samuel Emerson Harvey

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Transcriptional Regulation of the Acetone Carboxylase Operon via Two-Component Signal Transduction in Helicobacter pylori

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

By

Samuel Emerson Harvey

Accepted for __________________________

(Honors)

_______________________________________
Dr. Mark Forsyth, Chair

_______________________________________
Dr. Oliver Kerscher

_______________________________________
Dr. Kurt Williamson

_______________________________________
Dr. Randolph Coleman

Williamsburg, VA

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Abstract

*Helicobacter pylori* is a gram negative gastric pathogen that infects the mucosal lining of the human stomach and is present in nearly half of the human population. *H. pylori* is the etiologic agent of peptic ulcer disease, and infection is highly associated with the development of gastric cancer. The *H. pylori* genome encodes three complete two-component signal transduction systems (TCSTs): ArsRS, CrdRS, and FlgRS. Each system regulates many genes in response to environmental stimuli. The genome also encodes an essential orphan response regulator, HP1021. Previous transcriptional profiling experiments indicate that each of these TCSTs regulates the expression of virulence genes. The acetone carboxylase operon, *acxABC*, is associated with virulence and is regulated by all *H. pylori* TCSTs and HP1021. We characterized the TCST-mediated transcriptional regulation of *acxABC* expression by examining the physical interaction of response regulators ArsR and HP1021, a repressor and activator of *acxABC* transcription, respectively, with the promoter region of *acxA*. Electrophoretic mobility shift assays suggest that both ArsR and HP1021 bind upstream and downstream of the -35 hexamer promoter element, with ArsR binding two distinct sites and HP1021 binding as many as six sites. All of the ArsR binding sites overlap with HP1021 binding sites, suggesting possible binding competition. Also, *acxA* expression was assayed via quantitative real-time PCR in *H. pylori* strains 26695 and J99 under both neutral and acidic conditions. Under neutral conditions, abrogation of *arsS* in *H. pylori* strain J99 resulted in a 4.4-fold increase in *acxA* transcription, however no significant change in transcription was observed in strain 26695. Grown under acidic conditions, the J99 *arsS* null mutant exhibited approximately a 2.6-fold increase in *acxA* transcription with no
significant differential regulation occurring in the 26695 arsS null mutant. Collectively, this study suggests that *H. pylori* uses multiple TCSTs to regulate the expression of the acxABC operon, forming an overlapping regulatory network that allows finely tuned control over transcriptional regulation. This multi-layered mechanism of regulation may apply to other *H. pylori* virulence genes and represents a unique way for *H. pylori*, a bacterium with a relative paucity of TCSTs, to maintain tight control over gene expression. Inter-strain variation in the ArsRS-mediated regulation of acxA occurs between *H. pylori* strains J99 and 26695, offering the acxABC operon as a model for understanding strain-specific variation in TCST-mediated regulation of virulence factors.
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1. Introduction

1.1 - *Helicobacter pylori*

*Helicobacter pylori* is a gram negative bacterium that infects the human gastric mucosa. It has a characteristic helical shape as well as lophotrichous flagella at one pole. *H. pylori* was first isolated in 1982 by Drs. Barry Marshall and Robin Warren at the Royal Perth Hospital (Marshall & Warren, 1984). After its discovery, the association between *H. pylori* infection and the presence of gastric disease became increasingly clear (Dunn *et al.*, 1997). Today, *H. pylori* is accepted as the primary causative agent of peptic ulcer disease and is also strongly associated with the development of gastric cancer. More than 90% of patients with duodenal ulcers and nearly 80% of gastric ulcer patients are colonized by *Helicobacter pylori*. Gastric cancer is the fourth most common cancer in the world, and rates of gastric cancer are twice as high in infected persons compared to those uninfected (Parkin, 2001; Vogiatizi *et al.*, 2007).

Epidemiologically, *H. pylori* is one of the most widespread human pathogens, infecting approximately 50% of the human population around the world. In developing countries, incidence of infection approaches 70-90%, and in the developed world 20-30% infection rates are common (Dunn *et al.*, 1997). However, only a fraction of those infected ever present overt symptoms, and symptoms usually appear during middle-age. A definitive route of transmission from individual to individual is not known, however most individuals are infected during childhood, and clustering of *H. pylori* strains occurs within families. This supports the hypothesis that *H. pylori* is transmitted between mother and child via an oral-oral or fecal-oral route through close contact (Brown, 2000; Vogiatzi *et al.*, 2007). After infection, *H. pylori* is able to maintain a life-long, persistent
colonization of an individual. Much work has been done on treating patients infected with *H. pylori*, and many FDA-approved antibiotic regimens are employed today. The most common therapy involves a triple drug regimen with a proton pump inhibitor to lower the acidity of the stomach and facilitate clearance of the infection (Vogiatzi *et al.*, 2007).

Due to its widespread infection rates, potential for intrafamilial transmission, and high mutation rate, *H. pylori* is highly genetically diverse. *H. pylori* also has a long history of infecting human beings. Genetic variation in *H. pylori* has been used as a marker to track human evolutionary history, a testament to the close association of the bacterium and mankind over the course of human evolution (McNulty *et al.*, 2004; Wirth *et al.*, 2004). Different strains of *H. pylori* cluster in various geographic areas, and some strains are more virulent than others. This has led to the observation of an interesting disconnect between infection rates and gastric disease incidence, first noted in Africa where infection with *H. pylori* is nearly universal but incidence of gastric disease is low (Holcombe, 1992). This contrasts with infected populations of East Asia, particularly in Japan and Korea, where infection rates are lower than Africa but rates of gastric adenocarcinoma are much higher (Miwa *et al.*, 2002). This discordance is attributable to the highly variable pathogenicity of *H. pylori* strains as well as cultural, dietary, and lifestyle factors.

**1.2 - Pathogenicity in* Helicobacter pylori***

*H. pylori* has a relatively small genome of ~1.7 million base pairs, and as such was the first bacterium to have two genetically distinct strains, 26695 and J99, fully sequenced (Tomb *et al.*, 1997; Alm *et al.*, 1999). After the discovery of *H. pylori*, many new strains were collected from patients suffering from gastric disease all over the world.
Large differences in the pathogenicity of these strains were noted, and genetic elements correlated to this difference in pathogenicity were identified and sequenced. One genetic element associated with more severe disease outcomes is the cytotoxin-associated genes pathogenicity island (cag-PAI), a 40-kb genomic insert containing 27-30 genes that codes for a Type IV Secretion System (T4SS), a needle-like appendage used by bacteria to translocate products into an adjacent cell (Wang *et al.*, 2012). The most well-studied virulence factor encoded within the cag-PAI is CagA, a high molecular weight protein that is translocated via the T4SS into epithelial cells of the stomach. CagA+ strains induce more severe gastric inflammation via increased production of pro-inflammatory cytokines compared to cagA- strains (Blaser *et al.*, 1995). Once inside the cell, CagA can be phosphorylated by human Abl and Src kinases and subsequently activates SHP-2, a eukaryotic tyrosine phosphatase. Sustained activation of SHP-2 disregulates multiple signaling pathways within human cells, leading to cellular instability and damage (Liu *et al.*, 2012). In mice, direct transgenic expression of CagA induces hyperplasia in the gastric epithelium, ultimately resulting in cancer (Ohnishi *et al.*, 2008). Both *H. pylori* strains used in this study, 26695 and J99, contain the cag-PAI and were isolated from patients suffering from gastric disease. Therefore these strains are associated with severe gastric disease and serve as excellent genetically tractable models for studying medically important molecular systems of *H. pylori*.

Other important virulence factors encoded by *H. pylori* include VacA, a vacuolating cytotoxin; urease, an enzyme responsible for catalyzing the hydrolysis of urea into carbon dioxide and ammonia; and SabA, an adhesin that allows the bacterium to adhere to the sialyl-Lewis\(^x\) antigen on gastric epithelial cells (Cover &
Blaser, 1992; Scott et al., 2002; Yamaoka et al., 2006). Virulence is not solely
determined by bacterial virulence factors, but is influenced by host responses as well.
Polymorphisms in epithelial cells that increase secretions of proinflammatory cytokines
and decrease stomach acid secretion in response to *H. pylori* infection are also associated
with more severe disease outcomes (El-Omar, 2001; Wroblewski et al., 2010).

**1.3 - Two-Component Signal Transduction**

*H. pylori* resides in close association with the gastric mucosa and the gastric
epithelium, a relatively restricted microbial niche adjacent to the highly variable
environment of the stomach. Within this environment, *H. pylori* is exposed to a range of
environmental stressors including fluctuating pH and metal ion concentrations. Some of
the molecular mechanisms *H. pylori* uses to sense and respond to environmental changes
are two-component signal transduction systems (TCSTs) (Beier & Frank, 2000). TCSTs
are widespread conserved signal transduction systems present almost exclusively in
prokaryotes. TCSTs are activated by environmental ligands and control a diverse range of
cellular processes. TCSTs are generally composed of a transmembrane sensor histidine
kinase protein and a cytoplasmic response regulator. Upon the binding of an
environmental ligand to the histidine kinase, autophosphorylation occurs on a conserved
histidine residue present on the cytosolic domain of the histidine kinase. This phosphoryl
group is transferred to an aspartate residue in the N-terminal receiver domain of the
cognate response regulator. Once phosphorylated, the response regulator alters its
biological activity. Most TCSTs in bacteria function to alter gene expression in response
to an external stimulus, however TCSTs are also capable of directly modifying the
function of existing proteins (Bourret & Silversmith, 2010).
In the canonical model, once the response regulator has been phosphorylated, a conformational change occurs in the protein that activates its C-terminal domain, also known as the output domain, which usually facilitates DNA binding (Figure 1) (Beier & Frank, 2000). The response regulator then interacts directly with its DNA targets, and all of the DNA targets of a particular response regulator are known as a “regulon.” A histidine kinase may not exclusively phosphorylate one response regulator. Instead, regulatory networks combining signaling between multiple histidine kinases and response regulators are prevalent. Understanding the mechanisms that govern this intricate molecular crosstalk is a challenge for researchers in the field, especially when some organisms maintain dozens of TCSTs operating in parallel. Small molecule phosphate donors such as acetyl phosphate are also capable of activating response regulators independently of histidine kinases, and many response regulators display inherent phosphorylation-independent activity (Bourret & Silversmith, 2010). These regulatory networks should not overshadow the detailed mechanisms governing regulatory specificity that exist as well (Laub & Goulian, 2007). Although sequence homology readily identifies new TCSTs, it is very difficult to surmise the environmental signal, regulon, or networking of a particular TCST from amino acid sequence alone.

1.4 - Two-Component Signal Transduction in *Helicobacter pylori*

*H. pylori* encodes relatively few regulatory genes, with four histidine kinases and six response regulators identified through sequence analysis. Of these genes, three complete TCSTs involved in transcriptional regulation have been characterized: ArsRS (acid response system), CrdRS (copper resistance determinant), and FlgRS (flagellum-based motility) (Beier & Frank, 2000). All three of the histidine kinases in these systems
are essential for colonization of a mouse model and thus control *H. pylori* virulence factors necessary for the establishment and maintenance of infection (Panthal *et al.*, 2003). Another system has been shown to serve as the functional equivalent of the CheA/CheY system in *Escherichia coli* that serves to directly control the activity of flagella (Beier *et al.*, 1997). The additional two response regulators, HP1021 and HP1043, are identified as orphan response regulators because they do not have an apparent cognate histidine kinase (Mueller *et al.*, 2007). *H. pylori* exhibits a relative paucity of TCST systems compared to other gram negative organisms, however obligate pathogens are often characterized by a small repertoire of transcriptional regulatory pathways that respond to common changes in the environment (Kim & Forst, 2001). One hypothesis for this phenomenon is that obligate pathogens colonize a narrow range of environments and thus devote fewer resources than free-living bacteria to responding to diverse conditions. In the case of *H. pylori*, it is restricted to one tissue type solely within human beings. While it resides in the challenging environment of the stomach, little competition from other microorganisms and a consistent set of environmental stressors warrant fewer TCSTs. A recent study of transcriptional regulatory networks in *H. pylori* concluded that the few genetic regulatory systems in *H. pylori* are densely interconnected with regulatory overlap between separate TCSTs. In this way, *H. pylori* is an excellent model system for studying TCST regulatory networking (Danielli *et al.*, 2010).

TCSTs are also subject to the high degree of genetic variation existing between *H. pylori* strains. Each of the TCSTs in *H. pylori* involved in transcriptional regulation controls a discrete regulon of genes. Studies comparing the regulons of homologous TCSTs between various stains have noticed differences in regulon membership. An
example includes transcriptional profiling of the ArsRS regulon in strains 26695 and B128. By generating a null mutation in the \textit{arsS} histidine kinase gene in both strains, macroarrays were used to identify genes that were differentially regulated between wild-type and mutant strains. The regulons between 26695 and B128 matched entirely, but B128 showed upregulation of two additional genes, HP1408 and HP0427, that are found only in \textit{H. pylori} (Forsyth \textit{et al.}, 2002). This and additional evidence of TCST inter-strain regulon variation from our lab make \textit{H. pylori} an ideal candidate for studying strain-specific gene regulation.
Figure 1. Conventional Paradigm for Two-Component Signal Transduction

**Systems.** This diagram displays a TCST with only one histidine kinase that interacts with only one cognate response regulator. Other systems are possible, and response regulators may be capable of phosphorylation-independent activity as well. (A) The periplasmic domain of the histidine kinase detects an environmental signal. (B) Binding of the signal causes an ATP driven autophosphorylation event on cytoplasmic domains of the histidine kinase at conserved histidine residues. (C) Phosphotransfer of the phosphoryl group occurs from the histidine kinase histidine residue to an aspartate residue on the cytoplasmic response regulator. (D) The phosphorylated response regulator undergoes an allosteric change that allows binding of target DNA sequences and alters gene expression. (E) The response regulator is dephosphorylated.

1.5 - The Acid Response System (ArsRS) Two-Component Signal Transduction System

ArsRS is currently the most well-studied TCST in *H. pylori*. The system is encoded by two adjacent genes, HP0166 and HP0165, and was first described in *H.*
*H. pylori* strain 26695. In this system, ArsS is the histidine kinase situated in the cytoplasmic membrane and undergoes autophosphorylation when environmental pH falls below 5.0. ArsR is the cytosolic response regulator with a C-terminal DNA-binding-domain and an N-terminal receiver domain where phosphotransfer occurs to the conserved aspartate residue. ArsR appears to exist as a monomer at biological concentrations (Gupta *et al.*, 2009). ArsR is capable of functioning both as a repressor and an activator of transcription, and it is an essential gene (Beier & Frank, 2000). Null mutants of *arsS* are tolerated while *arsR* null mutants are not viable. The ArsRS system stands for the Acid Response System and is primarily responsible for acid acclimation. Phosphorylated ArsR upregulates key acid adaptive genes such as those of the urease operon (Pflock *et al.*, 2005). *H. pylori* is a neutrophile, thriving best near pH 7, but over the course of infection and persistent colonization the bacterium may be exposed to very low pH. One mechanism the bacterium uses to survive in acidic conditions is the expression of urease, which catalyzes the hydrolysis of urea to ammonia. This creates a neutral microenvironment around the bacterium, buffering it from the harsh conditions of the stomach lumen (Pflock *et al.*, 2006). One study confirmed the binding of ArsR in a region 60 bp upstream of the Pribnow box within the promoter of *amiE*, a gene upregulated under acidic conditions, however no consensus binding sequence has been identified (Pflock *et al.*, 2006).

ArsRS does not only control the expression of acid adaptive genes. Unphosphorylated ArsR is capable of binding DNA, indicating the presence of another phosphorylation-independent regulon of genes essential for growth (Wen *et al.*, 2006). Other genes regulated by phosphorylated ArsR do not depend on the presence of ArsS, indicating that ArsR may be phosphorylated through another mechanism. A review of *H. pylori*
*pylori* TCSTs describes an overlap between the regulon of phosphorylated ArsR and another TCST histidine kinase, FlgS, leading to the hypothesis that FlgS may be capable of phosphorylating ArsR in an example of regulatory overlap between distinct TCSTs (Danielli *et al.*, 2010). In *H. pylori* strain J99, additional regulon overlap exists between ArsRS and CrdRS, the system responsible for regulating copper resistance genes (Loh *et al.*, 2006). This overlap is not observed between *H. pylori* strains 26695 and G27, indicating the significance of inter-strain regulon variation as well as the difficulty of comparing studies when variable strains are used (Pflock *et al.*, 2007a).

### 1.6 - The Copper Resistance Determinant (CrdRS) Two-Component Signal Transduction System

CrdRS is encoded by histidine kinase CrdS (HP1364) and response regulator CrdR (HP1365). Based on sequence homology to known response regulators, CrdR appears to have an N-terminal receiver domain with a conserved aspartate phosphorylation site and a C-terminal DNA-binding domain. CrdRS has been characterized based on its role in copper resistance. The system is essential for upregulating the expression of CrdA, CrdB, and CzcB, a metal export system that exports intracellular copper. It is unclear whether *H. pylori* frequently encounters copper in its environment, but copper is also important in generating reactive-oxygen species in aqueous environments through Fenton reactions (Waidner *et al.*, 2005). It is possible that the CrdRS system may be a more general regulator of the *H. pylori* response to oxidative stress in the stomach, but few studies exist that have explored the full CrdRS regulon.

### 1.7 - The Flagellar FlgRS Two-Component Signal Transduction System

Functioning flagellar-motility is essential for the establishment of persistent
colonization of animal models by *H. pylori* (Eaton *et al.*, 1996). FlgR (HP0703), the response regulator of the system, activates the transcription of five operons encoding the components of the flagella. These operons are controlled by a $\sigma^{54}$ promoter, and FlgR only activates these genes in the presence of *H. pylori* or *E. coli* $\sigma^{54}$ RNA polymerase. The C-terminus of FlgR is not predicted to be a DNA-binding domain, but instead shows homology with other $\sigma^{54}$ RNA Polymerase activators. This makes FlgR unique among the response regulators encoded by *H. pylori* (Brahmachary *et al.*, 2004). FlgS (HP0244), a histidine kinase, is predicted to localize to the cytoplasm, making it the only cytoplasmic histidine kinase in *H. pylori*. FlgS is also involved in the acid response as well as upregulating cagA, the important *H. pylori* virulence gene (Wen *et al.*, 2009). The ligand that FlgS detects is poorly defined, but it is possible that FlgS acts as a sensor of cytosolic pH, much as ArsS senses extracellular pH (Wen *et al.*, 2008). Mutant strains lacking FlgR do not form flagella, but they are capable of surviving at a lower pH than strains lacking FlgS. This supports the idea that any acid acclimation processes FlgS contributes to are effected by another response regulator.

1.8 - The Orphan Response Regulator HP1021

HP1021, an orphan response regulator lacking a cognate histidine kinase, is an essential protein for normal growth in *H. pylori*. Mutations in *hp1021* are tolerated, resulting in a highly growth-impaired phenotype (Schar *et al.*, 2005). One of the reasons HP1021 is an essential protein is its role in upregulating the transcription of *nifS* and *nifU*, two housekeeping genes encoding nitrogenases. HP1021 functions as both a repressor and activator of transcription, and its regulon has been characterized through transcriptional profiling experiments (Pflock *et al.*, 2007a). HP1021 contains a C-
terminal DNA-binding domain but does not show homology to other DNA-binding domain families. The N-terminal region of the protein lacks the conserved aspartate residue required for activation of other response regulators, and mutations induced in this region have no effect on the growth of \textit{H. pylori} (Beier & Frank, 2000). The transcription of HP1021 is upregulated under acidic conditions, however it does not belong to the regulons of \textit{H. pylori}'s other acid responsive systems, ArsRS and FlgRS (Merrell \textit{et al.}, 2003; Wen \textit{et al.}, 2003; Loh \textit{et al.}, 2010) One study investigating the importance of transcriptional control of HP1021 found that significantly increasing its expression had no detrimental impacts on cell growth (Mueller \textit{et al.}, 2006).

1.9 - The Role of Two-Component Signal Transduction in Acetone Metabolism

Acetone, as well as acetoacetate and \(\beta\)-hydroxybutyrate, is one of the ketone bodies produced in liver tissue and secreted into the bloodstream during fatty acid metabolism. The process of ketogenesis occurs when glycogen stores in the liver are low, and high blood ketone levels are present during periods of starvation (Garber \textit{et al.}, 1974). Acetone is particularly enriched in gastric tissue due to the spontaneous decomposition of acetoacetate under acidic conditions, and some pathogens, including \textit{H. pylori}, can utilize this excess acetone as a carbon source (Brahmachary \textit{et al.}, 2008). \textit{H. pylori} is able to convert acetone to acetyl-CoA, the key biochemical necessary for carrying out the TCA cycle and generating energy. This biochemical pathway begins with the conversion of acetone to acetoacetate and is carried out by the enzyme acetone decarboxylase. \textit{H. pylori} encodes a three gene operon, \textit{acxABC} (HP0695-HP0697), that encodes acetone carboxylase (Corthésy-Theulaz \textit{et al.}, 1997). This operon is not essential for \textit{H. pylori} survival, but deletion of the operon reduces the ability of \textit{H. pylori} to colonize a mouse
model (Brahmachary et al., 2008). In this way, expression of acetone carboxylase may contribute to virulence.

The acxAABC operon is transcribed using RNA polymerase in combination with the housekeeping factor, $\sigma_{80}$ (Sharma et al., 2010). The operon contains an AT-rich region between the $-35$ and $-10$ sites, and possesses a Pribnow box at the $-10$ site (TATACT), which is characteristic of promoters in H. pylori (Forsyth & Cover, 1999). The acxAABC operon is of particular interest because evidence exists that all three of H. pylori’s TCSTs as well as HP1021 regulate its expression (Pflock et al., 2007a; Wen et al., 2009; Loh et al., 2010; Markowsky et al., 2004 - unpublished). ArsR and the cloned DNA-binding domain of ArsR bind to the upstream regulatory region of acxA in vitro, and higher levels of acetone carboxylase are present when a null mutation in arsS is introduced in H. pylori strain J99 (Loh et al., 2010). In H. pylori strains G27 and 26695, acxAABC transcription is repressed in pH conditions below 5.0. This suggests ArsR may be a repressor of acxAABC under acidic conditions. Multiple ArsR DNA binding sites have been described within the promoter region of acxA, both upstream and downstream of the $-35$ hexamer (Quinlivan-Repasi et al., 2011 - unpublished). Unpublished macroarray data from our lab indicates that the CrdRS system also regulates acxAABC. Null mutations in both crdR and crdS cause an increase in expression, indicating that CrdR may serve as a repressor (Markowsky et al., 2004 – unpublished). CrdR has been shown to interact directly with the upstream regulatory region using in vitro DNA binding studies (Quinlivan-Repasi et al., 2011 – unpublished). A microarray study identified acxAABC as a member of the pH-independent regulon of FlgRS, where expression of all three genes of the operon was down-regulated in an flgS null mutant strain of 26695 (Wen et al.,
FlgR typically regulates genes under the control of a $\sigma^{54}$ promoter (Brahmachary et al., 2004), therefore the $\sigma^{80}$ promoter of the $acxABC$ operon may preclude direct interaction between FlgR and the promoter of $acxABC$. The mechanism by which FlgS mediates regulation of $acxABC$ is unknown, but FlgS may be able to phosphorylate other response regulators (Danielli et al., 2010). HP1021, the orphan response regulator, induces $acxABC$ transcription and interacts physically with the upstream regulatory region of the operon. DNA-binding experiments produced four discrete complexes between HP1021 and $acxA$ upstream DNA, indicating that HP1021 may bind multiple sites of the promoter (Pflock et al., 2007a).

Characterizing the multi-system regulation of $acxABC$ has the potential to inform and improve therapeutic interventions in $H. pylori$ infections. TCSTs have long been recognized as potential antibiotic targets in pathogenic bacteria due to their essential control over bacterial growth and virulence, however early attempts at developing drugs to interfere with signaling stalled due to poor selectivity (Gotoh et al., 2010). If $acxABC$ is regulated by all $H. pylori$ TCSTs, then it represents a nexus of regulation that is influenced by responses to multiple environmental stressors. The role of $acxABC$ in $H. pylori$ pathogenicity has not been fully explored, but the investment of multiple regulatory systems suggests that acetone metabolism may significantly affect the fitness of $H. pylori$ in the dynamic environment of a human host. Understanding how TCSTs in $H. pylori$ interact with each other, $acxABC$, and other critical regulon members will inform drug design by identifying key components of regulatory networks optimal for pharmacological intervention.
1.10 - Research Goals and Experimental Approach

The acetone carboxylase operon is an excellent model for studying TCST and regulatory overlap in *H. pylori*. Due to the fact that multiple regulatory systems impinge on the expression of the acetone carboxylase operon and the operon contributes to *H. pylori*’s ability to colonize an animal model, understanding the TCST-mediated mechanism of its regulation will provide insight into how *H. pylori* fine-tunes the regulation of genes important to survival. The research in this thesis focuses on further characterizing the physical interactions of ArsR and HP1021, a repressor and activator of *acxABC*, respectively, with the upstream regulatory region of this operon. The primary experimental techniques used to investigate the physical binding of response regulators to specific DNA sequences are electrophoretic mobility shift assays (EMSA). By localizing the binding sites of each response regulator relative to the promoter of *acxABC*, a better understanding of how each response regulator increases or decreases transcription as well as identification of the binding sequences recognized by each protein can be determined. Also, the networking or competition between DNA binding proteins can be assessed. Additionally, gene expression studies were conducted to ascertain inter-strain differences in the regulation of *acxABC* between *H. pylori* strains J99 and 26695. Comparing wild-type *H. pylori* with strains containing null mutations in *arsS*, the expression of *acxA* in cells grown under neutral or acidic conditions was assayed via quantitative real-time PCR (qPCR). Understanding heterogeneity in TCST regulation of *acxABC* between strains of *H. pylori* will enhance our understanding of how strains differ in their ability to cause disease.
2 - Methods

2.1 - Expression of ArsR and HP1021

Freezer stocks of M15 *E. coli* strains known to express 26695 ArsR and 26695 HP1021 were obtained from Daniel Hallinger and Vanessa Quinlivan-Repasi, respectively (Quinlivan-Repasi et al., 2011 - unpublished). These strains are M15 *E. coli*/*pQE30*-26695arsR + pREP4 and M15 *E. coli*/*pQE30*-26695hp1021 + pREP4. These strains contained the open reading frame of each protein inserted within a pQE30 expression vector (Qiagen). This expression vector system appends a 6X Histidine tag to the N terminus of each protein as an aid in purification. Each protein was expressed using a modified version of the native batch purification protocol from the QIAexpressionist manual. A freezer stock of each M15 *E. coli* strain containing pQE30 with the desired response regulator was inoculated into 10 mL of Luria-Bertani (LB) broth supplemented with 100 µg/mL ampicillin (Amp) and 50 µg/mL kanamycin (Kan). After overnight incubation at 37°C, the entire culture was used to inoculate 250 mL of prewarmed LB Amp/Kan in a sterile baffled culture flask. The culture was then grown at 37°C with shaking at 250 rpm until reaching an OD₆₀₀ of 0.5-0.7. Prior to inducing expression, a 1 mL sample, serving as an uninduced control, was taken from the culture, harvested via centrifugation, and resuspended in 25 µL of Laemmli Sample Buffer (BioRad). IPTG was added to the remaining culture to a final concentration of 1 mM to induce protein expression, and the culture was returned to the incubator. When expressing ArsR or HP1021, the culture was allowed to incubate for 4 hours or 2 hours, respectively. After incubation, another 1 mL sample was taken from the culture, pelleted, and resuspended in 25 µL Laemmli Sample Buffer to serve as an induced control. The rest of the culture was
split into 50 mL aliquots and centrifuged at 4000 g for 20 minutes at 4°C. Next, the 50 mL cell pellets were resuspended in 4 mL NPI-10 lysis buffer (50mM NaH$_2$PO$_4$, 300mM NaCl, 10mM imidazole). Lysozyme (Fisher Scientific) was added to a final concentration of 1 mg/mL, and the samples were incubated for 30 minutes on ice with occasional mixing by inversion. The cells were lysed via sonication at maximum power on ice with six 10 second bursts with a 10 second pause in between each burst. Twelve units of Benzonase® nuclease (Novagen) was added per mL of lysate and incubated an additional 15 minutes on ice. Each lysate was then centrifuged for 30 mins at 10,000 g at 4°C to pellet cellular debris. The supernatant, containing the soluble fraction, was removed and stored at 4°C while the pelleted insoluble fraction was resuspended to the original volume before centrifugation in NPI-10 lysis buffer and also stored at 4°C. A 25 µL aliquot of both the soluble and insoluble fractions was mixed with 25 µL Laemmli Sample Buffer. In order to assess the solubility of the expressed protein, the uninduced control, induced control, soluble fraction, and insoluble fraction suspended in Laemmli Sample Buffer were boiled at 100°C for 5 minutes and then run on SDS-PAGE gels stained with Coomassie Brilliant Blue. SDS-PAGE was conducted using 12% acrylamide 0.75 mm thick gels using the Mini-PROTEAN® III system (BioRad). After electrophoresis and staining, the expression level and solubility of the desired protein could be assessed.

2.2 - Response Regulator Purification

Each protein was purified using the Ni-NTA gravity filtration system from Qiagen. Up to 12 mL of the soluble fraction of each protein was added to a 5 mL bed volume drip column containing 3 mL 50% Ni-NTA agarose slurry. Eluted fractions were run on SDS-PAGE gels to determine the fraction containing the purest response regulator.
protein, and the protein concentration was determined on a Nanodrop®
spectrophotometer (Thermo). If the concentration was less than 2 mg/mL total protein,
the protein was concentrated using 10kD Amicon Ultra-15 Centrifugal Units (Millipore).
The protein was aliquoted in 80 µg increments and 10% glycerol was added by volume.
The protein aliquots were stored at −20˚C for future experiments.

2.3 - Electrophoretic Mobility Shift Assays

2.3.1 - Probes

The DNA Probes for EMSAs detectable using the LightShift® Chemiluminescent
EMSA Kit (Thermo) were biotin labeled. These probes (Table 2, Figure 2) were
amplified via PCR from a plasmid template containing the entire upstream regulatory
region of acxA (pCR-26695acxABC) obtained from Vanessa Quinlivan-Repasi
(Quinlivan-Repasi et al., 2011 – unpublished). Later, some probes were amplified from
existing probe stocks. All PCR reactions were conducted with Bullseye Taq® (Midsci)
and biotinylated primers (Table 1). Unlabeled probes (Table 2) used in specific
competition assays were also amplified via PCR using unbiotinylated primers (Table 1).
The unrelated DNA used in nonspecific competition reactions was Epstein-Barr Nuclear
Antigen DNA (EBNA) provided in the Lightshift® EMSA Optimization Kit (Thermo)
(Table 1).

EMSA probes required further purification after PCR amplification. Biotinylated
probes were run on native 6% acrylamide gels containing 4 µg/mL ethidium bromide.
After electrophoresis, the probes were excised from the gel and purified using the “crush
and soak method” (Sambrook & Russell, 2001). Gel samples were crushed using a small
mortar within a microcentrifuge tube, mixed with 300 µL of acrylamide gel elution buffer
(300 mM NaOAc, 1mM EDTA pH 8.0), and shaken 24-72 hours at room temperature. Then the samples were centrifuged 10 minutes at 10,000 g to pellet the acrylamide gel fragments, and the supernatant containing the purified probes was collected and frozen at −20°C. Unlabeled probes were purified using the Gel/PCR DNA Fragment Extraction Kit (IBI Scientific).

<table>
<thead>
<tr>
<th>Name</th>
<th>5’ to 3’ sequence</th>
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</thead>
<tbody>
<tr>
<td>HP0694 Fwd.2</td>
<td>TCCCAGCCTAAGCACCATC</td>
</tr>
<tr>
<td>HP0694 Fwd.2.btn</td>
<td>Biotin-TCCCAGCCTAAGCACCATC</td>
</tr>
<tr>
<td>AcxA-35Rev</td>
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<tr>
<td>Up.1 Rev</td>
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<td>Up.2 Rev</td>
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<td>Up.7 Fwd</td>
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<td>Up.8 Fwd</td>
<td>ACGCCAAATTTCCCTTATT</td>
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<td>Probe Name</td>
<td>Description</td>
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<td>Down.6 Fwd</td>
<td>CCTATTAGAAGTATAAGGCT</td>
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**Table 2. EMSA Probes**

<table>
<thead>
<tr>
<th>Probe Name</th>
<th>Description</th>
<th>Probe Size</th>
<th>Primers Used to Amplify Probe</th>
</tr>
</thead>
</table>
| Up         | Spans end of HP0694 translation to the −35 hexamer of H. pylori acxABC | 209 bp | HP0694 Fwd.2  
|            |            |            | AcxA-35Rev                   |
| Up*        | Biotinylated Up |            | HP0694 Fwd.2.btn  
|            |            |            | AcxA-35Rev                   |
| Up.1       | Up probe truncated 39 bp from 3’ end | 170 bp | HP0694 Fwd.2  
|            |            |            | Up.1 Rev                    |
| Up.1*      | Biotinylated Up.1 |            | HP0694 Fwd.2.btn  
|            |            |            | Up.1 Rev                    |
| Up.2       | Up probe truncated 79 bp from 3’ end | 130 bp | HP0694 Fwd.2  
|            |            |            | Up.2 Rev                    |
| Up.2*      | Biotinylated Up.2 |            | HP0694 Fwd.2.btn  
|            |            |            | Up.2 Rev                    |
| Up.3       | Up probe truncated 119 bp from 3’ end | 90 bp | HP0694 Fwd.2  
<p>|            |            |            | Up.3 Rev                    |
| Up.3* | Biotinylated Up.3 | HP0694 Fwd.2.btn Up.3 Rev |
| Up.4 | Up probe truncated 159 bp from 3’ end | 50 bp | HP0694 Fwd.2 Up.4 Rev |
| Up.4* | Biotinylated Up.4 | HP0694 Fwd.2.btn Up.4 Rev |
| Up.5 | Up probe truncated 50 bp from 5’ end | 159 bp | Up.5 Fwd AcxA-35Rev |
| Up.5* | Biotinylated Up.5 | Up.5 Fwd AcxA-35Rev.btn |
| Up.6 | Up probe truncated 90 bp from 5’ end | 119 bp | Up.6 Fwd AcxA-35Rev |
| Up.6* | Biotinylated Up.6 | Up.6 Fwd AcxA-35Rev.btn |
| Up.7 | Up probe truncated 130 bp from 5’ end | 79 bp | Up.7 Fwd AcxA-35Rev |
| Up.7* | Biotinylated Up.7 | Up.7 Fwd AcxA-35Rev.btn |
| Up.8 | Up probe truncated 170 bp from 5’ end | 39 bp | Up.8 Fwd AcxA-35Rev |
| Up.8* | Biotinylated Up.8 | Up.8 Fwd AcxA-35Rev.btn |
| Down | Starts at –35 hexamer of <em>H. pylori</em> acxABC and ends downstream of the start of translation | 175 bp | AcxA-35Fwd AcxARev2 |</p>
<table>
<thead>
<tr>
<th>$Down^*$</th>
<th>Biotinylated $Down$</th>
<th>AcxA-35Fwd.btn AcxARrev2</th>
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<td>$Down$ probe truncated 40 bp from 5’ end</td>
<td>135 bp Down.1 Fwd AcxARrev2</td>
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<tr>
<td>$Down.1^*$</td>
<td>Biotinylated $Down.1$</td>
<td>Down.1 Fwd AcxARrev2.btn</td>
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<tr>
<td>$Down.2$</td>
<td>$Down$ probe truncated 80 bp from 5’ end</td>
<td>95 bp Down.2 Fwd AcxARrev2</td>
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<tr>
<td>$Down.2^*$</td>
<td>Biotinylated $Down.2$</td>
<td>Down.2 Fwd AcxARrev2.btn</td>
</tr>
<tr>
<td>$Down.3$</td>
<td>$Down$ probe truncated 120 bp from 5’ end</td>
<td>55 bp Down.3 Fwd AcxARrev2</td>
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<td>$Down.3^*$</td>
<td>Biotinylated $Down.3$</td>
<td>Down.3 Fwd AcxARrev2.btn</td>
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<tr>
<td>$Down.4$</td>
<td>$Down$ probe truncated 55 bp from 3’ end</td>
<td>120 bp AcxA-35Fwd Down.4 Rev</td>
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<td>$Down.5$</td>
<td>$Down$ probe truncated 95 bp from 3’ end</td>
<td>80 bp AcxA-35Fwd Down.5 Rev</td>
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<td>$Down.5^*$</td>
<td>Biotinylated $Down.5$</td>
<td>AcxA-35Fwd.btn Down.5 Rev</td>
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<tr>
<td>$Down.6$</td>
<td>$Down$ probe truncated 135 bp from 3’ end</td>
<td>40 bp AcxA-35Fwd Down.6 Rev</td>
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<td>Probe</td>
<td>Description</td>
<td>AcxA-35Fwd.btn Down.6 Rev</td>
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<tr>
<td>-------------</td>
<td>------------------------------------------------------------------------------</td>
<td>---------------------------</td>
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<tr>
<td>Down.6*</td>
<td>Biotinylated Down.6</td>
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</tr>
<tr>
<td>EBNA</td>
<td>Unlabeled Epstein-Barr nuclear antigen DNA with the binding site 5’- …TAGCATATGCTA…-3’)</td>
<td>25 bp</td>
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</table>

**Figure 2. Sizes and locations of acxA EMSA probes.**

(A) This schematic shows the location of the \textit{Up} probe and all of its truncations relative to the −38 nucleotide in the upstream regulatory region of \textit{acxA}. (B) This schematic shows the location of the \textit{Down} probe and all of its truncations relative to the −37 nucleotide of the \textit{acxA} promoter. The blue arrow indicates the transcriptional start site of \textit{acxA} while the coding region of \textit{acxA} is within the labeled rectangle.


2.3.2 - Binding Reactions

The EMSA protocol used in this study was adapted from the LightShift® EMSA Optimization Kit Protocol (Thermo) and ArsR EMSAs performed by Loh et al. (Loh et al., 2010). Generally, four binding reactions were conducted for each biotinylated probe:

1. Biotinylated Probe
2. Biotinylated Probe + Response Regulator
3. Biotinylated Probe + Response Regulator + 500x Unlabeled Probe
4. Biotinylated Probe + Response Regulator + 500x Unlabeled nonspecific DNA

The amount of biotinylated probe, unlabeled probe, and protein was varied between experiments in an effort to empirically determine binding conditions and specificity, but within the binding reactions of an individual experiment the amounts were kept constant.

The total volume of each binding reaction was 30 µL. Reagents were combined in the following order: 6 µL 5x binding buffer, 2 µL poly dI-dC (1 mg/mL), unlabeled probe (if necessary), 3-6 µg protein (if necessary), and ddH₂O to 29 µL. The binding reaction was then incubated for 10 minutes at room temperature. Lastly, 1 µL (0.5-2 ng) labeled probe was added to each binding reaction and incubated for an additional 20 minutes at room temperature.

The binding buffers used in the EMSAs were obtained from previous studies. The ArsR 5x binding buffer contained 50 mM Tris-HCl, 250 mM KCl, 5 mM DTT, 12.5% glycerol, 25 mM MgCl₂, and 0.25% NP-40 (Loh et al., 2010). The HP1021 5x binding buffer contained 50 mM Tris-HCL, 50 mM KCl, 5 mM DTT, 5% glycerol, and 25 mM EDTA (Pflock et al., 2007a).
2.3.3 - Electrophoresis

Binding reactions were electrophoresed immediately after incubation with the biotinylated probe. First, 7.5 µL loading buffer (Thermo) was added to each binding reaction, then 20 µL of each reaction was loaded onto a 6% acrylamide native PAGE gel. Gels were electrophoresed for at least 30 minutes, 100V prior to loading samples. The binding reactions along with 3 µL of 2-log biotinylated DNA ladder (New England Biolabs) were electrophoresed in 0.5x TBE at 100 V until the unbound biotinylated probes were approximately 1 cm from the bottom of the gel. Total run time depended on the specific biotinylated probe being used.

2.3.4 - Membrane Blotting and Detection

Native PAGE gels were blotted to Zeta-Probe GT® nylon membranes (BioRad) immediately after electrophoresis. Blotting was conducted according to the manufacturer’s protocol using the BioRad Mini Trans-Blot® cell. Fiber pads, filter paper, and nylon membranes were soaked in cold 0.5x TBE for 10 minutes before blotting. Blotting transfer was conducted in cold 0.5x TBE for 30 minutes at 380 mA. After blotting, the nylon membranes were crosslinked in a GS Gene Linker® (BioRad) at 125 µJoules for 3 minutes and stored dry at 4˚C until the detection protocol could be completed.

The detection procedure was carried out using the LightShift® EMSA kit protocol. After detection, the blots were exposed to CL-Xposure® X-ray film (Thermo) for 30s and processed in a Konica film processor. Additional exposures of shorter or longer intervals were conducted if necessary including “burnout” exposures where the X-ray film was exposed until luminescence ceased.
2.4 - *Helicobacter pylori* Liquid Culture and Acid Shock

Freezer stocks of *Helicobacter pylori* strains (Table 3) were inoculated on TSA plates supplemented with 5% Sheep’s Blood and incubated 24-48 hours at 37°C in a 5% CO₂ atmosphere. Each strain was then passed to a fresh agar plate 24 hours before liquid inoculation. One full plate of cells was swabbed into 1 mL of sterile Sulfite Free Brucella Broth (SFBB) with 10% fetal calf serum and 20 µg/mL vancomycin at pH 7. The OD₆₀₀ of this suspension was recorded and used to inoculate 25 mL of sterile SFBB in a baffled vented 125 mL culture flask to an OD₆₀₀ of 0.1. Culture flasks were then incubated at 37°C in a 5% CO₂ atmosphere in a rotating incubator. The cultures were allowed to grow until the cells reached an OD₆₀₀ of 0.8 – 1.0. At this point, the cultures were split into two 12.5 mL aliquots and centrifuged at 4000 rpm for 5 minutes. The supernatant was discarded, and one pellet serving as a control was resuspended in 12.5 mL sterile SFBB pH 7 while the other pellet undergoing the acid shock was resuspended in 12.5 mL sterile SFBB pH 5. Each culture was then incubated at 37°C in a 5% CO₂ atmosphere in a rotating incubator for 1 hour. After the incubation, the cultures were removed to room temperature and the OD₆₀₀ of each was determined. Using a standard conversion factor of 1 OD₆₀₀ unit = 5x10⁸ cells/mL, an aliquot containing 1x10⁹ cells was transferred from each culture flask to a microcentrifuge tube. These aliquots were pelleted at 4000 g for 5 minutes, the supernatant was discarded, and the pellets were frozen at −80°C.

2.5 - RNA Extraction

Total RNA was extracted from each cell pellet according to the manufacturer’s protocol using the MagMAX™-96 for Microarrays Total RNA Isolation Kit (Life Technologies) and the AM1839 Spin Program on a MagMAX™ Express Magnetic
Particle Processor (Life Technologies). Purified RNA concentrations were analyzed on a Nanodrop® spectrophotometer (Thermo) and frozen at −80°C.

| Table 3. *Helicobacter pylori* Strains |
|-------------------------------|---------------------------------|
| Name             | Purpose                                | Source                |
| 26695 WT         | Fully-sequenced *cag* PAI+ laboratory strain | Mark Forsyth          |
| 26695/*arsS::cat*| 26695 with *arsS* null mutation        | College of William & Mary |
| J99 WT           | Fully-sequenced *cag* PAI+ laboratory strain |                         |
| J99/*arsS::km*   | J99 with *arsS* null mutation         |                         |

2.6 - Quantitative Real Time PCR

Quantitative real time PCR (qPCR) using the $2^{-\Delta\Delta Ct}$ method was carried out to determine the expression levels of *acxA* relative to housekeeping gene *glk* encoding glucokinase (HP1103) in all *H. pylori* strains grown at pH 7 or pH 5. The primers used for *acxA* were *acxA.2 Fwd* (5’-TGCAATAAGGGTTTTTGAGCA-3’) and *acxA.2 Rev* (5’-GATAACCACTTGCCCCTTGA-3’) amplifying a product of 177-bp. The primers used for *glk* were *glk Fwd* (5’-GGCTTGGGGGAAGCAGTCTT-3’) and *glk Rev* (5’-ACCTTTCCGCGCTACAT-3’) amplifying a product of 156-bp. The qPCR was performed using the Power SYBR® Green RNA-to-Ct™ 1-Step Kit (Life Technologies) on a StepOne™ Real Time PCR System (Applied Biosystems) according to manufacturer’s protocols. PCR conditions were run according to manufacturer’s protocols with an annealing temperature of 58°C. Reactions were 20 μL with 1-2 ng of total RNA serving as a template and were performed in triplicate. Data was processed using DataAssist™ software (Applied Biosystems).
3 - Results and Discussion

3.1 - Localizing ArsR Binding Sites via Electrophoretic Mobility Shift Assays

All DNA probes used for electrophoretic mobility shift assays (EMSAs) were amplified from the upstream regulatory region of acxA from H. pylori strain 26695 (Table 2, Figure 2). The cloned and expressed ArsR used in this study was also from strain 26695. The strategy used to localize the ArsR binding sites involved interrogating ArsR with successively truncated DNA probes from both the 5’ and 3’ ends of the DNA. In this way, one can infer the presence of a binding site by looking for the loss of a shift between successive truncations (Figures 3 through 6). The binding sites were localized to approximately 40 base pair regions of the acxA promoter, although the actual sequence ArsR binds to may be smaller (Table 4). The data from Figures 3 through 6 suggests that ArsR binds upstream of the acxA −35 hexamer as well as 139 nucleotides downstream of the acxA transcriptional start site. Examination of protein expression in H. pylori suggests ArsR is a repressor of acxA transcription, therefore a binding site within the coding sequence of acxA may function to interfere with successful transcription of the gene (Loh et al., 2010). Quantitative real time PCR data shown in the current study corroborates the classification of ArsR functioning as a repressor (see below).
Figure 3. ArsR EMSA with Up.1*-Up.4* probes. This figure illustrates ArsR interrogated with DNA probes amplified from the upstream regulatory region of acxA. In this case, the probes are amplified from the region upstream of the −38 nucleotide before the start of acxA transcription but downstream of the neighboring gene, hp0694. The schematic shows the length of each biotinylated probe. Each EMSA is shown below the particular biotinylated probe used in the experiment. Lanes labeled L contain a 2-log biotinylated DNA ladder (New England Biolabs). Reactions A1-D1 contain 1 ng Up.1*. Reactions A2-D2 contain 1 ng Up.2*. Reactions A3-D3 contain 1 ng Up.3*. Reactions A4-D4 contain 1 ng Up.4*. All A reactions contain only the biotinylated probe. All B reactions contain biotinylated probe and 5 µg ArsR. All C reactions contain biotinylated probe, 5 µg ArsR, and 100x concentration of unbiotinylated probe as a specific competitor. Reaction D1 contains biotinylated probe, 5 µg ArsR, and 52x EBNA as a nonspecific competitor. Reaction D2 contains biotinylated probe, 5 µg ArsR, and 40x EBNA. Reaction D3 contains biotinylated probe, 5 µg ArsR, and 28x EBNA. Reaction D4 contains biotinylated probe, 5 µg ArsR, and 15x EBNA. The yellow arrows indicate the loss of an ArsR-probe complex between Up.3* and Up.4*, and a yellow rectangle labeled “ArsR BS 1” indicates the suspected binding site of ArsR between nucleotides −196 and −156.
Figure 4. ArsR EMSA with Up.5* - Up.8* probes. This figure illustrates ArsR interrogated with DNA probes amplified from the upstream regulatory region of acxA. In this case, the probes are amplified from the region upstream of the −38 nucleotide before the start of acxA transcription but downstream of the neighboring gene, hp0694. The schematic shows the length of each biotinylated probe. Each EMSA is shown below the particular biotinylated probe used in the experiment. Lanes labeled L contain a 2-log biotinylated DNA ladder (New England Biolabs). Reactions A1-D1 contain 0.5 ng Up.5*. Reactions A2-D2 contain 0.5 ng Up.6*. Reactions A3-D3 contain 0.5 ng Up.7*. Reactions A4-D4 contain 0.5 ng Up.8*. All A reactions contain only the biotinylated probe. All B reactions contain biotinylated probe and 5 µg ArsR. All C reactions contain biotinylated probe, 5 µg ArsR, and 500x concentration of unbiotinylated probe as a specific competitor. All D reactions contain biotinylated probe, 5 µg ArsR, and 500x EBNA as a nonspecific competitor. The yellow arrows indicate the loss of an ArsR-probe complex between Up.5* and Up.6*, and a yellow rectangle labeled “ArsR BS 1” indicates the suspected binding site of ArsR between nucleotides −196 and −156.
Figure 5. ArsR EMSA with Down.1*-Down.3* probes. This figure illustrates ArsR interrogated with DNA probes amplified from the upstream regulatory region of acxA. In this case, the probes are amplified from the region downstream of the −37 nucleotide into the open reading frame of acxA. The schematic shows the length of each biotinylated probe. Each EMSA is shown below the particular biotinylated probe used in the experiment. Lanes labeled L contain a 2-log biotinylated DNA ladder (New England Biolabs). Reactions A1-D1 contain 1 ng Down.1*. Reactions A2-D2 contain 1 ng Down.2*. Reactions A3-D3 contain 1 ng Down.3*. All A reactions contain only the biotinylated probe. All B reactions contain biotinylated probe and 5 µg ArsR. All C reactions contain biotinylated probe, 5 µg ArsR, and 100x concentration of unbiotinylated probe as a specific competitor. Reaction D1 contains biotinylated probe, 5 µg ArsR, and 42x EBNA as a nonspecific competitor. Reaction D2 contains biotinylated probe, 5 µg ArsR, and 29x EBNA. Reaction D3 contains biotinylated probe, 5 µg ArsR, and 17x EBNA. The purple arrow indicates the presence of an ArsR-probe complex persisting to the smallest probe, and a purple rectangle labeled “ArsR BS 2” indicates the suspected binding site of ArsR between nucleotides +84 and +139.
### A

**Down.5* - 120 bp**

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<thead>
<tr>
<th>A1</th>
<th>B1</th>
<th>C1</th>
<th>D1</th>
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**Down.5* - 80 bp**

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**Down.6* - 40 bp**

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<tr>
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### B

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<td>ArsR (5 µg)</td>
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<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>Down</em></td>
<td></td>
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<td>500x</td>
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<td>EBNA</td>
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---

**Down* + ArsR Complex**

**Down* Probe**
Figure 6. ArsR EMSA with Down.4* - Down.6* probes. This figure illustrates ArsR interrogated with DNA probes amplified from the upstream regulatory region of acxA. In this case, the probes are amplified from the region downstream of the −37 nucleotide into the open reading frame of acxA. (A) The schematic shows the length of each biotinylated probe. Each EMSA is shown below the particular biotinylated probe used in the experiment. Lanes labeled L contain a 2-log biotinylated DNA ladder (New England Biolabs). Reactions A1-D1 contain 0.5 ng Down.4*. Reactions A2-D2 contain 0.5 ng Down.5*. Reactions A3-D3 contain 0.5 ng Down.6*. All A reactions contain only the biotinylated probe. All B reactions contain biotinylated probe and 5 µg ArsR. All C reactions contain biotinylated probe, 5 µg ArsR, and 500x concentration of unbiotinylated probe as a specific competitor. All D reactions contain biotinylated probe, 5 µg ArsR, and 500x EBNA as a nonspecific competitor. None of the probes bound ArsR in these EMSAs. A purple rectangle labeled “ArsR BS 2” indicates the suspected binding site of ArsR between nucleotides +84 and +139. (B) This EMSA shows ArsR binding to the Down* probe. All lanes contain 1 ng of Down*.

3.2 - Localizing HP1021 Binding Sites via Electrophoretic Mobility Shift Assays

The same strategy used to localize ArsR binding sites was employed to localize binding of HP1021 to EMSA probes (Figures 7 through 10). The data displayed in Figures 7 through 10 suggest the presence of three HP1021 binding sites upstream of the −35 hexamer of the acxA promoter and three binding sites downstream of this hexamer (Table 4). The EMSAs performed with HP1021 were different than those performed with ArsR in that multiple HP1021-probe complexes were visible within a single gel. Multiple shifted complexes of differing molecular weight indicate the potential presence of multiple HP1021 binding sites on the same probe. We hypothesize that the lower molecular weight complexes occur when HP1021 is bound to fewer binding sites and higher molecular weight complexes occur when more sites on the probe are bound to HP1021. In Figure 7, the first HP1021 binding site is predicted to lie between nucleotides −196 and −156 relative to the +1 transcriptional start site of acxA whereas Figure 8 indicates that the first binding site lies between nucleotides −156 and −116. The reason
for this discrepancy is unclear and could be resolved by interrogating HP1021 with distinct 40 bp probes from nucleotides $-196$ to $-156$ or $-156$ to $-116$. HP1021 binding sites 2/3 are located within the same 40 bp sequence identified using this strategy. The same is true of HP1021 binding sites 5/6. A previous transcriptional profiling study of *H. pylori* strain 26695 identified HP1021 as an activator of *acxA* transcription and also confirmed the binding of HP1021 to a 210 bp DNA probe encompassing the promoter of *acxA* (Pflock *et al.*, 2007a). That study also demonstrated the presence of four distinct HP1021-probe complexes in gel retardation experiments with this probe, similar to the multiple complexes detected in the current study (Figures 7 through 9). The identification of HP1021 binding sites upstream of the $-35$ hexamer in the *acxA* promoter are consistent with the hypothesis that HP1021 enhances transcription of the *acxABC* operon, however the presence of binding sites within the open reading frame of *acxA* would be more typical of transcriptional repressor sites. EMSAs such as these provide information on the binding ability of transcription factors to specific DNA probes but do not elucidate the complex binding dynamics of response regulators within a living cell.
**Figure 7. HP1021 EMSA with Up.1* - Up.4* probes.** This figure illustrates HP1021 interrogated with DNA probes amplified from the upstream regulatory region of *acxA*. In this case, the probes are amplified from the region upstream of the −38 nucleotide before the start of *acxA* transcription but downstream of the neighboring gene, *hp0694*. The schematic shows the length of each biotinylated probe. Each EMSA is shown below the particular biotinylated probe used in the experiment. Lanes labeled L contain a 2-log biotinylated DNA ladder (New England Biolabs). Reactions A1-D1 contain 0.5 ng Up.1*. Reactions A2-D2 contain 0.5 ng Up.2*. Reactions A3-D3 contain 0.5 ng Up.3*. Reactions A4-D4 contain 0.5 ng Up.4*. All A reactions contain only the biotinylated probe. All B reactions contain biotinylated probe and 5 µg HP1021. All C reactions contain biotinylated probe, 5 µg HP1021, and 500x concentration of unbiotinylated probe as a specific competitor. All D reactions contain biotinylated probe, 5 µg HP1021, and 500x EBNA as a nonspecific competitor. The black arrows indicate the loss of the two highest molecular weight HP1021-probe complexes between Up.1* and Up.2*, and the red arrow indicates the loss of the lowest molecular weight HP1021-probe complex between Up.3* and Up.4*. The red rectangle labeled “HP1021 BS 1” indicates a binding site of HP1021 between nucleotides −196 and −156. The black rectangle labeled “HP1021 BS 2/3” indicates the two suspected binding sites of HP1021 between nucleotides −116 and −76.
Figure 8. HP1021 EMSA with Up.5* - Up.8* probes. This figure illustrates HP1021 interrogated with DNA probes amplified from the upstream regulatory region of acxA. In this case, the probes are amplified from the region upstream of the −38 nucleotide before the start of acxA transcription but downstream of the neighboring gene, hp0694. The schematic shows the length of each biotinylated probe. Each EMSA is shown below the particular biotinylated probe used in the experiment. Lanes labeled L contain a 2-log biotinylated DNA ladder (New England Biolabs). Reactions A1-D1 contain 0.5 ng Up.5*. Reactions A2-D2 contain 0.5 ng Up.6*. Reactions A3-D3 contain 0.5 ng Up.7*. Reactions A4-D4 contain 0.5 ng Up.8*. All A reactions contain only the biotinylated probe. All B reactions contain biotinylated probe and 5 µg HP1021. All C reactions contain biotinylated probe, 5 µg HP1021, and 500x concentration of unbiotinylated probe as a specific competitor. All D reactions contain biotinylated probe, 5 µg HP1021, and 500x EBNA as a nonspecific competitor. The red arrow indicates the loss of the highest molecular weight HP1021-probe complex between Up.6* and Up.7*. The black arrows indicate the loss of two HP1021-probe complexes between Up.7 and Up.8*. The red rectangle labeled “HP1021 BS 1” indicates a suspected binding site between nucleotides −156 and −116. The black rectangle labeled “HP1021 BS 2/3” indicates two suspected binding sites of HP1021 between nucleotides −116 and −76.
Figure 9. HP1021 EMSA with Down.1* - Down.3* probes. This figure illustrates HP1021 interrogated with DNA probes amplified from the upstream regulatory region of acxA. In this case, the probes are amplified from the region downstream of the −37 nucleotide into the open reading frame of acxA. The schematic shows the length of each biotinylated probe. Each EMSA is shown below the particular biotinylated probe used in the experiment. Lanes labeled L contain a 2-log biotinylated DNA ladder (New England Biolabs). Reactions A1-D1 contain 0.5 ng Down.1*. Reactions A2-D2 contain 0.5 ng Down.2*. Reactions A3-D3 contain 0.5 ng Down.3*. All A reactions contain only the biotinylated probe. All B reactions contain biotinylated probe and 5 µg HP1021. All C reactions contain biotinylated probe, 5 µg HP1021, and 500x concentration of unbiotinylated probe as a specific competitor. All D reactions contain biotinylated probe, 5 µg HP1021, and 500x EBNA as a nonspecific competitor. The green arrows indicate the loss of an HP1021-probe complex between Down.1* and Down.2*. The orange arrows indicate the persistence of two HP1021-probe complexes until the smallest probe. A green rectangle labeled “HP1021 BS 4” indicates the suspected binding site of HP1021 between nucleotides +4 and +44. The orange rectangle labeled “HP1021 BS 5/6” indicates the presence of two binding sites of HP1021 between nucleotides +84 and +139.
**Figure 10: HP1021 EMSA with Down.4*-Down.6* probes.** This figure illustrates HP1021 interrogated with DNA probes amplified from the upstream regulatory region of acxA. In this case, the probes are amplified from the region downstream of the −37 nucleotide into the open reading frame of acxA. (A) The schematic shows the length of each biotinylated probe. Each EMSA is shown below the particular biotinylated probe used in the experiment. Lanes labeled L contain a 2-log biotinylated DNA ladder (New England Biolabs). Reactions A1-D1 contain 0.5 ng Down.4*. Reactions A2-D2 contain 0.5 ng Down.5*. Reactions A3-D3 contain 0.5 ng Down.6*. All A reactions contain only the biotinylated probe. All B reactions contain biotinylated probe and 5 µg HP1021. All C reactions contain biotinylated probe, 5 µg HP1021, and 500x concentration of unbiotinylated probe as a specific competitor. All D reactions contain biotinylated probe, 5 µg HP1021, and 500x EBNA as a nonspecific competitor. The green arrows indicate the loss of an HP1021-probe complex between Down.5* and Down.6*. A green rectangle labeled “HP1021 BS 4” indicates the suspected binding site of HP1021 between nucleotides +4 and +44. The orange rectangle labeled “HP1021 BS 5/6” indicates the presence of two binding sites of HP1021 between nucleotides +84 and +139. (B) This EMSA shows HP1021 binding to the Down* probe. All lanes contain 1 ng of Down*.

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<thead>
<tr>
<th>Table 4. Response Regulator Binding Sites</th>
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<tr>
<td><strong>Binding Site Designation</strong></td>
<td><strong>Sequence</strong></td>
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<tr>
<td>ArsR Binding Site 1</td>
<td>TTGATTTAATATCAGTTTAAATTTTT CTTCTATATGAT</td>
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<tr>
<td>ArsR Binding Site 2</td>
<td>ACCATGACGGACACATTTTTTTGTGAA AGAAAAATGGCAGTTTCTAGTTGGTA AAG</td>
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<tr>
<td>HP1021 Binding Site 1 (predicted based on results shown in Figure 7)</td>
<td>TTGATTTAATATCAGTTTAAATTTTT CTTCTATATGAT</td>
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<tr>
<td>HP 1021 Binding Site 1 (predicted based on results shown in Figure 8)</td>
<td>ATTTATATGATATTTTTGGGTAATTT AAGATGAATATCGG</td>
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<tr>
<td>HP 1021 Binding Sites 2 and 3</td>
<td>TAGCGTTTTGAATAAATTTGTTACTA CTTTTCACTTTATT</td>
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<tr>
<td>HP1021 Binding Site 4</td>
<td>TTGCCCTTGAGCAACACTTTAAATACAA GGAGTCTAAATGAA</td>
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<tr>
<td>HP 1021 Binding Sites 5 and 6</td>
<td>ACCATGACGGACACATTTTTTTGTGAA AGAAAAATGGCAGTTTCTAGTTGGTA AAG</td>
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3.3 - Binding Specificity and Competition Assays

In EMSA protocols, competition reactions are performed in order to examine binding specificity between the response regulator and the target sequence of the probe. Unlabeled probes identical in sequence to the labeled probe are added to test for specific competition. Another reaction containing unrelated DNA serves as a nonspecific competitor. The amount of nonspecific competitor should at least equal the fold concentration of the specific competitor, however for several EMSAs (Figures 3, 5, 6B, 10B), review of experimental records indicated an error where significantly less nonspecific competitor was added than previously thought. For these EMSAs, the fold concentration of nonspecific competitor Epstein-Barr Nuclear Antigen DNA (EBNA) is far below the fold concentration of the specific competitor, therefore the nonspecific reactions need to be repeated with an appropriate concentration of EBNA to ensure it does not interfere with specific binding between protein and probe. Specific competition is essential for confirming that the interaction between response regulator and probe is sequence specific, however in many of the EMSAs in this study, specific competition was difficult to demonstrate. In Figure 11, the ability of increasing concentrations of unlabeled Up probe to compete away ArsR binding to Up* was assayed. After 600x Up was added, an appreciable loss of shifting was observed, although even after the addition of 800x Up probe some shifting remained. The inability to completely compete away binding even with a large excess of specific competitor demonstrates a high avidity between ArsR and its binding sequences. The addition of up to 1032x of the nonspecific competitor EBNA did not compete away binding. In all other EMSAs, approximately 100x – 500x specific competitor was added, therefore there may not have been enough
unlabeled probe to demonstrate competition. In the case of Figure 9 where multiple HP1021-probe complexes were observed, specific competition was only able to compete away the formation of the lowest molecular weight protein-probe complex. This information may provide some insight into the binding affinity of HP1021 to its various binding sites, with sites of lowest affinity being more easily competed away than others.

It is uncertain why specific competition was so difficult to demonstrate compared to similar experiments performed in other studies. In a study by Loh et al., specific competition was demonstrated between the DNA binding domain of ArsR and labeled probes from the promoter regions of acxA with a 20x excess of unlabeled probe (Loh et al., 2010). Pflock et al. were able to significantly compete away binding between HP1021 and the promoter of acxA with a 50x excess of unlabeled probe (Pflock et al., 2007a). When binding reactions for specific competition are prepared, the protein of interest and the unlabeled probe serving as the specific competitor are co-incubated before the labeled probe is added. The protein should have the same affinity for the labeled probe and the unlabeled probe, therefore specific competition should occur most readily when the molar concentration of protein is lower than the molar concentration of specific competitor. However, in Loh’s study, competition was observed even when 1000 times more ArsR was present than total probe. If all of the ArsR was capable of binding DNA, then all of the probe, both labeled and unlabeled, should have shifted. One possible explanation for this discrepancy could be that not all of the ArsR used in this study, nor all of the ArsR DNA binding domain used in Loh’s study, was in a state capable of binding its target sequence. In the case of the EMSAs presented in this study, only a portion of the protein in each reaction may have been able to bind DNA. This behavior
could be an artifact of the experimental binding conditions present in EMSAs or due to
the presence of inactive/unphosphorylated ArsR.

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<td><strong>EBNA</strong></td>
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**Figure 11. ArsR EMSA with Up* and Up Competition Assays.** This figure illustrates
ArsR binding to the Up* probe and the effect of increasing concentrations of specific
competitor Up probe on shifting. Lanes marked L contain a 2-log biotinylated DNA
ladder (New England Biolabs). All EMSA reactions contained 1 ng of Up* labeled
probes. Successive loss of shifting was observed with increasing concentrations of Up
probe, however no effect on shifting was observed upon addition of the nonspecific
competitor EBNA.

3.4 - Modeling Regulation of acxA with ArsR and HP1021

Figures 12 and 13 present hypothetical models of the ArsR and HP1021 binding
landscapes near acxABC and the mechanism by which each protein alters acxABC
transcription. These models are based on EMSA data from this study and data from previous studies detailing the impact of each response regulator on \textit{acxA} transcription (Pflock \textit{et al.}, 2007a; Loh \textit{et al.}, 2010). It should be noted that the binding order predicted by these figures is speculative but could be confirmed experimentally by examining the binding affinity of each response regulator for a specific binding site. In Figure 12, at low concentrations of active ArsR only the high affinity binding site upstream of the $-35$ hexamer is bound, having little effect on \textit{acxA} transcription. As concentrations of active ArsR increase, perhaps by increased phosphorylation by the cognate histidine kinase ArsS, ArsR binds to the lower affinity binding site within the coding sequence of \textit{acxA}, repressing transcription. Other mechanisms such as interactions between ArsR facilitating DNA looping could stabilize repression of \textit{acxA}.

Figure 13 details a possible mechanism for HP1021-mediated induction of \textit{acxA} expression. At low concentrations of active HP1021, few high affinity sites would be bound, resulting in little effect on \textit{acxA} expression. As the concentration of active HP1021 increases, more of the binding sites upstream of the $-35$ hexamer would be bound, possibly increasing the strength of the \textit{acxA} promoter and increasing transcription. If the HP1021 binding sites located in the coding region of \textit{acxA} are only bound when HP1021 concentrations are very high, these sites could represent a negative regulatory mechanism to prevent overexpression of \textit{acxA} when HP1021 concentrations exceed a certain threshold. This could be an adaptive mechanism to prevent unnecessarily high expression levels of \textit{acxA}.
Figure 12. Hypothetical model for the transcriptional regulation of *acxA* by ArsR. ArsR binding sites are labeled in green. As the concentration of active, phosphorylated ArsR~P increases in the cell, high affinity sites would be bound first followed by low affinity sites, increasingly repressing *acxA* transcription. Possible protein-protein interactions between active ArsR~P could stabilize the repression of *acxA*.

Figure 13: Hypothetical model for the transcriptional regulation of *acxA* by HP1021. HP1021 binding sites are labeled in blue. When HP1021 concentration is low, few sites are bound and *acxA* concentration remains limited. As concentrations of HP1021 increase, high affinity sites in the promoter could enhance the transcription of *acxA*. Once HP1021 concentrations exceed a threshold, low affinity sites within the *acxA* coding region would be bound, serving as a negative regulatory mechanism to limit overexpression of *acxA*. 
3.5 - Overlapping Binding Sites of ArsR and HP1021

The complex binding patterns of ArsR and HP1021 to the upstream regulatory regions of $acxA$ as well as within the coding region of $acxA$ suggest an intricate mechanism of regulation. All ArsR binding sites identified in this study overlap with putative HP1021 binding sites. The DNA probe truncation technique used to localize binding sites was limited in resolution to 40 base pairs, so the actual binding sites of ArsR and HP1021 may not physically overlap, but they appear to be close together. Combined with evidence from the literature that ArsR functions as a repressor of transcription and HP1021 functions as an activator, these two proteins may compete for binding sites in order to effect differential regulation of $acxA$. ArsR has been shown to bind the promoter of $acxA$ most strongly when phosphorylated, but the factors that influence HP1021 binding are unknown (Pflock et al., 2007a; Loh et al., 2010).

3.6 - Inter-strain Variation in $acxA$ Expression

Quantitative real-time PCR (qPCR) was conducted on $H. pylori$ strains listed in Table 3 assaying for $acxA$ expression (Figures 14 and 15). Grown under either neutral or acidic conditions, relatively modest repression of $acxA$ was observed in the $H. pylori$ 26695 $arsS$ null mutant compared to wild-type. This is not consistent with observations that ArsR functions as a repressor of $acxA$, however the repression observed was less than two-fold that of the wild-type which may not be biologically significant. In the $H. pylori$ J99 strains, derepression of $acxA$ was observed in the $arsS$ null mutant at 4.4-fold and 2.6-fold when grown at pH 7 and pH 5, respectively. Thus derepression of $acxA$ in the J99 $arsS$ null mutant is consistent with a global analysis of protein expression (Loh et al., 2010). These qPCR data provide direct evidence that ArsRS-dependent regulation of
acxA occurs on the level of transcription, clarifying the Loh et al. study that only
analyzed protein levels of AcxA, AcxB, and AcxC. Loh et al. did not investigate H.
pylori strain 26695. Inter-strain variation in ArsRS regulation of acxA is exhibited by H.
pylori strains 26695 and J99, exemplifying the difficulty in comparing studies conducted
on different H. pylori strains. Differences in bacterial strains have confounded H. pylori
TCST research in the past. As an example, the CrdRS TCST system was determined to
be required for acid resistance in strain J99 but not in strains 26695 and G27 (Loh &
Cover, 2006; Pflock et al., 2007b). Variability in TCST regulation between H. pylori
strains has a profound impact on virulence, and understanding the mechanism by which
different strains modulate the expression of virulence genes can impact clinical strategies
in treating H. pylori infections.

3.7 – Future Considerations

One of the primary weaknesses of the EMSA data presented in this study is the
inconsistent ability to specifically compete away shifting. Concentrations of 100x – 500x
unlabeled DNA probes relative to labeled probes failed to demonstrate specific
competition in many cases. Only shifts observed with Down.2* and Down.3* binding to
ArsR (Figure 5) and the lowest molecular weight shift with Down.1* - Down.3* binding
to HP1021 (Figure 9) were clearly competed away in the specific competition reaction.
This data suggests that most of the binding sites observed are of such high affinity to each
response regulator that they cannot reasonably be competed away using the current
reaction conditions. Reducing the molarity of the protein in each reaction so that it is less
than the molarity of the unlabeled probe should theoretically ensure near complete
specific competition if all protein binds the unlabeled probe, however attempts to reduce
the amount of protein in each reaction to 3 µg or below failed to reproducibly produce shifts (data not shown). Another strategy to improve competition outcomes would be to obtain shorter chemically synthesized double stranded probes, both labeled and unlabeled, containing the suspected binding sequences of ArsR and HP1021 (Table 4). According to the manufacturer protocols for the Lightshift® Chemiluminescent EMSA Kit (Thermo), EMSA reactions containing probes with 20 – 60 base pairs are optimal. Perhaps performing the EMSAs with shorter probes that are professionally purified will resolve the challenge of demonstrating specific competition. Additional experiments titrating the amount of unlabeled probe required to compete away each shift would provide insight into the binding affinity of each suspected binding site with ArsR or HP1021. This information would greatly inform our hypothetical models of ArsR and HP1021-mediated regulation of acxA (Figures 12 and 13) as well as provide insight into the nature of competition between ArsR and HP1021 for specific binding sequences.

Additional experiments must be conducted to determine the mechanistic role of the three suspected HP1021 binding sites present near or within the coding region of acxA. Transcriptional profiling experiments have identified HP1021 as a transcriptional activator of acxA expression (Pflock et al., 2007a), and it is not typical of transcriptional activators to bind within the coding sequence of a gene. The hypothetical negative regulatory function these binding sites may serve to prevent overexpression of acxA in the case of high HP1021 concentrations is posited in Figure 13, but this hypothesis remains to be tested. A previous study by Mueller et al. sought to determine the impact of overexpression of HP1021 on the growth of H. pylori by creating strains merodiploid for hp1021, however no overt phenotype was observed (Mueller et al., 2007). Obtaining
hp1021 merodiploid strains and assaying for the expression of acxA and hp1021 using qPCR would allow for the correlation of acxA transcription with hp1021 transcription, providing an experimental means to test our existing hypothetical model. Generating hp1021 merodiploid mutants in multiple strains of H. pylori would also afford inter-strain comparisons similar to our study of ArsRS-mediated regulation of acxA in H. pylori strains 26695 and J99.

Figure 14. qPCR of H. pylori strains grown at pH 7. This figure shows the relative expression of acxA in 26695 and J99 arsS null mutants relative to their corresponding wild-type strains. The error bars represent the RQmin and RQmax calculated to a 95% confidence interval. These strains were grown under neutral pH conditions. The data represents results from triplicate samples from a single experiment. Similar results were obtained from three experiments.
Figure 15. qPCR of *H. pylori* strains grown at pH 5. This figure shows the relative expression of *acxA* in 26695 and J99 *arsS* null mutants relative to their corresponding wild-type strains. The error bars represent the RQmin and RQmax calculated to a 95% confidence interval. These strains were grown under neutral pH conditions. The data represents results from triplicate samples from a single experiment. Similar results were obtained from three experiments.

3.8 - Concluding Thoughts

Combined with previous studies of ArsR and HP1021, the data in this study supports direct regulation of *acxAB* transcription by the binding of ArsR and HP1021 to the *acxA* promoter as well as the coding region of the gene. Localization of the binding sites of ArsR and HP1021 indicates the presence of two binding sites for ArsR and six sites for HP1021. ArsR and HP1021 bind sites that are very close to one another and may overlap, suggesting that *H. pylori* utilizes multiple competing TCSTs to fine-tune the
expression of acxA. ArsRS-mediated regulation of acxA occurs at the transcriptional level, and variation in this regulation exists between H. pylori strains J99 and 26695.

Unpublished data from our lab also suggests that CrdR binds directly to the promoter of acxA, and FlgR may interact with the promoter region through an unknown cofactor (Quinlivan-Repasi et al., 2011 - unpublished). Along with this study, that data provides evidence for the physical interaction of all H. pylori TCSTs with the acxA promoter. While characterizing the precise mechanism of acxA regulation for these TCSTs will require further study, these results demonstrate the value of acxA as a model for understanding multiple TCST regulatory mechanisms converging on a single gene. The regulation of acxA is also an excellent example of how H. pylori, a bacterium with few regulatory systems, can precisely regulate gene expression. Other H. pylori virulence and survival genes that are subject to TCST regulation may be regulated in a similar manner. The presence of such a layered and complex mechanism of control over acxABC identifies the potential critical nature of acetone carboxylase expression in H. pylori physiology. Strain-specific differences in the regulation of acetone carboxylase add another layer of complexity in understanding the varied clinical outcomes associated with H. pylori infection. Combined with animal studies demonstrating the importance of acxABC in colonization and the establishment of infection (Brahmachary et al., 2008), these results highlight the acetone carboxylase operon as an important H. pylori virulence factor and nexus of TCST-mediated gene regulation that is worthy of future study.
4 - References


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