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

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

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

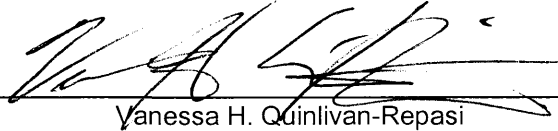
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APPROVAL PAGE

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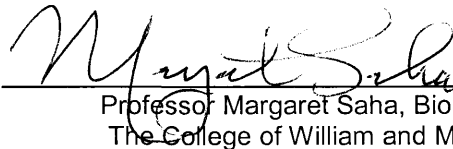
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COMPLIANCE PAGE

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ABSTRACT PAGE

The bacterial pathogen *Helicobacter pylori* exclusively colonizes the human gastric epithelium, causing chronic inflammation which leads to gastritis in most of those who are infected. The long-term presence of *H. pylori* also promotes the development of more severe conditions such as ulcers, and is a significant risk factor for gastric cancers such as adenocarcinoma. In addition to being an important human pathogen, *H. pylori* is also an ideal system for basic study of bacterial gene regulation. This species controls expression of a wide array of target genes in response to environmental signals using only three two-component signal transduction (TCST) systems: ArsRS, FlgRS, and CrdRS. Evidence of co-regulation of some genes by more than one of these pathways suggests that *H. pylori* compensates for this apparent deficiency in regulatory ability through a complex network of cross-regulation interactions. The acetone carboxylase operon, *acxABC*, may be regulated by all three of these signal transduction pathways. This extensive regulation of acetone metabolism suggests that this capability is important for *H. pylori*'s ability to infect and survive in the gastric mucosa, and may reveal promising targets for drug development. In order to characterize the activity of transcription factors that regulate this operon, I have cloned, expressed, and purified the response regulator component of each of *H. pylori*'s TCST systems along with the orphan response regulator HP1021. Electrophoretic mobility shift assays (EMSAs) were performed with these response regulators and DNA probes amplified from the putative upstream regulatory region of *acxABC*. Results confirm the direct binding of ArsR and HP1021 to the *acxA* promoter observed in previous studies, and suggest that as many as four ArsR binding sites may be involved in regulation of *acxABC*. Results also suggest that regulation of *acxA* by CrdR is direct and involves multiple binding sites or conformations. FlgR bound to the *acxA* promoter region in a nonspecific manner, suggesting that FlgR must interact with another DNA-binding protein to activate *acxA* transcription, or that regulation of *acxA* by FlgR is indirect. These results establish *acxA* as a model target gene for future study of how *H. pylori* can finely modulate transcription of a single operon via multiple direct regulatory systems and mechanisms. Furthermore, the optimization of methods and protocols in the course of this study has made additional tools for investigation of TCST networking available to our research group.

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Introduction

Pathogenicity of *Helicobacter pylori*

Helicobacter pylori was first identified in 1982 as a gram negative microaerophilic helical bacterium endemic to the human gastric mucosa, and belongs to the class ϵ -proteobacteria. Soon after this discovery, a strong correlation was established between infection with this pathogen and the presence of gastric and duodenal ulcers, which were previously thought to be caused by stress or diet (1). These ulcers may now be treated with a high rate of success using antibiotics to eradicate the infection along with additional medications to reduce pain and inflammation (2). However, *H. pylori* is still highly prevalent: approximately 50% of the world population and 30% of people in the United States are currently *H. pylori*-positive. Infection rates vary with geographical location, race, gender and socioeconomic status (3). Most of those infected will develop gastritis at some point in their lives, but the chronic inflammatory response to infection can also lead to more severe symptoms and conditions such as peptic ulcer disease (including gastric and duodenal ulcers, which occur in ~10% of those infected), non-Hodgkins lymphoma, and gastric cancers such as adenocarcinoma (4). In 2000, gastric adenocarcinoma was the fourteenth most common cancer worldwide, with *H. pylori* infection as its second-strongest risk factor after family history (5).

Total genome sequences have been published for multiple strains of *H. pylori*, including the pathogenic strains J99 and 26695 (6). Some strains are more virulent in humans than others. One genetic factor that increases virulence is the cytotoxin associated antigen pathogenicity island (*cag* PAI). The *cag* PAI is a 40kB region of DNA including genes homologous to a known type IV secretion system that moves bacterial protein and DNA into host cells, leading to tumorigenesis in the host (7). *H. pylori* strains isolated from patients

without severe gastric disease frequently lack the *cag* PAI, while infection with *cag* PAI-positive strains has been shown to increase risk of gastric adenocarcinoma relative to risk faced by uninfected individuals and those infected with a strain lacking this pathogenicity island (4). Additionally, there is significant genetic variation between *H. pylori* strains that goes beyond the presence or absence of the *cag* PAI. For example, while the average genome size is ~1.7 Mb, this can vary between strains by more than 24 kb in addition to the 40 kb pathogenicity island (6). The *H. pylori* strains used in the research described in this thesis were 26695 and J99, both of which possess the *cag* PAI and were isolated from patients with *H. pylori*-associated gastric disease. Since genome content is variable among strains, raising the possibility that some genetic regulatory mechanisms may not be conserved, experiments with known pathogenic strains for which the full-genome sequence is already available should yield the most medically relevant information.

The purpose of the research described in this thesis is to broaden understanding of how *H. pylori* alters its global gene expression in response to environmental changes. Much work has been done regarding the mechanisms of virulence employed by *H. pylori* once an infection has been established, but little is known about the genetic mechanisms that regulate the process of infection, host colonization, and ability to survive in a constantly changing, often harsh environment. In addition to contributing to general understanding of bacterial gene regulation, knowledge of *H. pylori* survival mechanisms could facilitate the development of targeted drugs, which could address the growing problem of antibiotic resistance. The FDA-approved eradication protocols recommended for *H. pylori* as of 2006 all included at least two antibiotics, to decrease the possibility of treatment failure due to resistance. Simultaneous treatment with three antibiotics is becoming increasingly common (2). Treatment with broad-spectrum antibiotics such as amoxicillin, which is called for by

four of the FDA's eight approved eradication protocols, carries its own risks for patients whether or not they are infected with antibiotic-resistant *H. pylori*. In addition to eradicating pathogens, antibiotics affect the symbiotic and commensal flora of the gut and in doing so put the patient at risk for secondary infections. Antibacterial drugs designed to disable the metabolic pathways of pathogens in a species-specific or strain-specific manner represent a promising solution. A complete map of the regulatory pathways of *Helicobacter pylori* would reveal a wider range of options for drug targets.

Two-Component Signal Transduction

Two-component signal transduction (TCST), also called histidine-aspartate phosphotransfer, is the most prevalent means by which bacteria detect and respond to environmental signals by inducing or repressing gene expression. (Environmental signals may be ligands such as metal ions or nutrients, or they may be conditions such as osmolarity or temperature.) TCST systems typically consist of a transmembrane histidine kinase sensor protein and a cytoplasmic DNA-binding response regulator protein. When the sensor detects an environmental signal, it autophosphorylates at a histidine residue. This phosphoryl group is then transferred to an aspartate residue in the N-terminal receiver domain of the response regulator, altering its regulatory activity (Figure 1). The response regulator is usually activated through a change in conformation that makes its C-terminal DNA-binding domain more readily available to bind its target sequence. Two-component system response regulators cannot autophosphorylate by cleaving ATP, but they can accept phosphate groups from small-molecule phosphodonors such as acetyl phosphate. In some systems where small-molecule phosphodonors are present in the cytosol in significant amounts, this can lead to a low baseline level of response regulator phosphorylation (8).

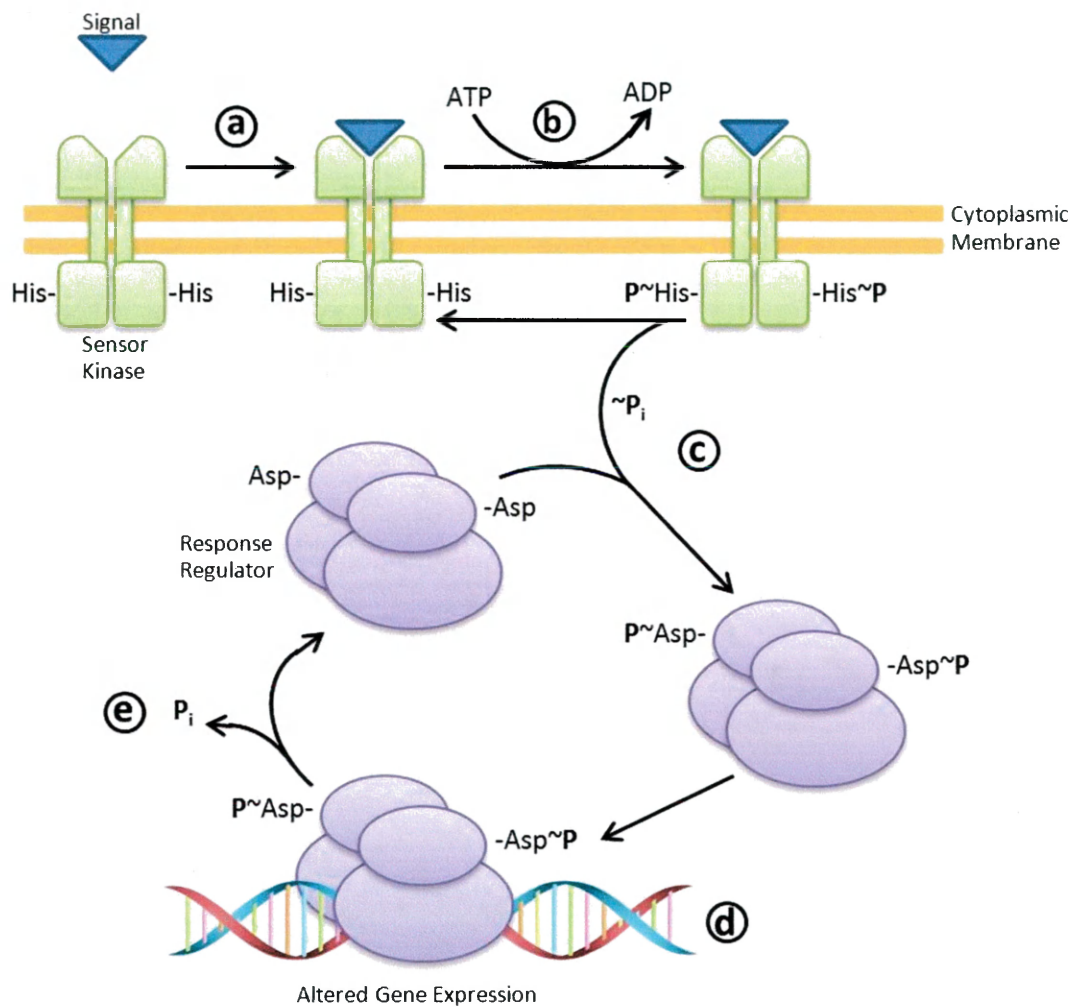


Figure 1. A Typical Two-Component Signal Transduction System. A TCST system in which the response regulator is only active in gene regulation when phosphorylated is shown for the sake of simplicity. Other mechanisms are possible, including systems in which phosphorylation shifts the response regulator between two distinct functions. (a) The periplasmic domain of the sensor kinase senses an environmental signal. (b) The cytoplasmic domain of the sensor kinase autophosphorylates at a histidine residue. (c) The phosphate group is transferred from the sensor kinase to an aspartate residue in the receiver domain of the response regulator. (d) The response regulator binds DNA and activates or represses transcription of its target gene(s). (e) The response regulator is dephosphorylated. DNA clipart © DragonArt Designs, 2010.

Response regulators usually act directly on their target genes as transcription factors, but some multistep phosphorelay systems and TCST response regulators that interact with other transcription factors have been identified as well (8). While it is possible that some TCST systems are isolated and thus regulate one gene or set of genes each in response to one type of environmental signal, there is some evidence of interaction between systems (9). Overall, little is known about most bacteria TCST systems aside from the general model described above. According to a recent review, novel TCST systems can be reliably discovered by searching for amino acid sequence homology, but amino acid sequences cannot yet be used to make predictions about regulatory targets, environmental signals, or regulatory networks (8).

Two-component Signal Transduction and Pathogenicity

Complex Regulation of Virulence Factor Expression *via* TCST in the *Bordetellae*

Though regulation of virulence factors other than motility *via* two-component signal transduction has not yet been described in *Helicobacter pylori*, this type of regulatory mechanism takes a central role in the pathogenicity of several other bacterial species. *Bordetella pertussis*, an exclusive human pathogen and the causative agent of whooping cough, and *Bordetella bronchiseptica*, which can colonize and cause respiratory infections in most mammals, regulate expression of virulence factors in response to environmental conditions *via* the BvgAS two-component signal transduction system. BvgAS controls expression of *Bordetallae* virulence factors including adhesins and secreted toxins by transitioning between at least three modes of global gene expression: Bvg⁺, Bvgⁱ, and Bvg⁻. The Bvg⁺ phase can be induced by growth in the laboratory at 37°C, and is thought to be a response to the host environment. BvgS, a transmembrane sensor kinase, responds to this

temperature (and possibly other host signals) by autophosphorylating and then transferring its phosphate groups to the response regulator BvgA. BvgA~P then activates transcription of the *bvg*-activated regulon, which includes virulence factors and the *bvgAS* operon itself. This positive feedback loop results in increased expression of the third member of this operon, *bvgR*. BvgR is a transcription factor that represses expression of a distinct regulon referred to as the *bvg*-repressed genes (10). The *bvg*-repressed genes of *B. pertussis* are mostly of unknown function, but in *B. bronchiseptica* this regulon is known to include genes required for motility and survival of nutrient deprivation. Exposure of the sensor domain of BvgS to modulating signals, which include temperatures below 26°C and millimolar concentrations of MgSO₄ or nicotinic acid, causes the cells to move into the Bvg⁻ phase. BvgS does not autophosphorylate in this phase, so transcription of the *bvg*-activated regulon is no longer up-regulated and transcription of the *bvg*-repressed regulon is free to occur. Bvgⁱ, an intermediate phase, is characterized by high expression of adhesins, low expression of toxins, and the expression of a set of highly immunogenic membrane proteins with homology to *E. coli* intimins (11). The Bvg⁺ phase appears to be optimized for infection and the Bvg⁻ phase appears to be important for survival of *B. bronchiseptica* outside the host. (*B. pertussis* cannot survive for long outside the host, so the Bvg⁻ phase may be vestigial in this species.) It has been suggested that the expression of putative intimins and genes that facilitate aggregation of *B. pertussis* cells during the Bvgⁱ phase makes this phase important for transmission of *B. pertussis* (12).

The transition of global gene expression between the Bvg⁺ and Bvg⁻ states has been described as analogous to a rheostat as opposed to a simple two-pole switch (11). BvgA~P has a higher affinity for the promoters of some members of its regulon than others, resulting in increased sensitivity of the expression levels of these genes to phosphorylation of BvgS.

These are termed "early" promoters, and loss of activation by BvgA at these promoters requires higher environmental levels of modulating signals. Transcriptional activation of members of the BvgAS regulon with "late" promoters is less responsive to BvgS phosphorylation, but is more sensitive to modulation by environmental signals (10). This model of graded transcriptional control and multiple modes of global gene expression controlled by a single TCST system exemplifies the complexity of function that may characterize the TCST systems of *H. pylori*.

TCST Networking and Virulence in the *Salmonellae*

Two-component signal transduction is also important for pathogenesis in the *Salmonellae*, which cause enteric disease including typhoid fever. To establish a successful infection, *Salmonella enterica* serovar Typhimurium must be able to survive phagocytosis by a macrophage or neutrophil. *S. enterica* Typhimurium senses the phagosomal environment via the PhoQ two-component signal transduction sensor kinase, which leads to phosphorylation of the PhoP response regulator (13). PhoP~P activates transcription of the *pag* (PhoP-activated genes) regulon and represses expression of the *prg* (PhoP-repressed genes) regulon. The *pag* regulon includes a variety of outer membrane proteins and lipopolysaccharide-modifying enzymes. Because the host innate immune response recognizes the lipid A portion of lipopolysaccharide (LPS), constant structural modification of this virulence factor by the bacterium is necessary for evasion of the immune response. *Salmonellae* also resist the immune system in part through the PhoP-activated expression of genes that promote resistance to antimicrobial peptides produced by host cells. As in the case of BvgA in *Bordetellae* described above, variations in the response by different members of the *pag* regulon to phosphorylation of PhoP is thought to be caused by differences in regulatory sequence of these target genes (14). When *S. enterica* Typhimurium is in the

extracellular host environment, concentrations of Mg^{2+} and Ca^{2+} are higher than in the phagosome. PhoQ does not autophosphorylate in the presence of these cations, which leads to a decrease in the amount of PhoP~P present in the cell. These conditions promote transcription of the *prg* regulon, which consists of genes required for epithelial cell invasion that are repressed by Pho~P (15). The PhoPQ TCST contributes to regulation of approximately 3% of the *S. typhimurium* genome (14).

Like the BvgAS TCST of the *Bordetellae*, the PhoPQ TCST is more complex than a switch between two gene expression patterns. Recent research has revealed extensive networking between PhoPQ and other TCST systems of the *Salmonellae*. Transcription of the *pmrAB* operon, which encodes a TCST system required for expression of genes involved in resistance to antimicrobial peptides, is activated by PhoP~P in response to phagocytosis. PmrA also activates transcription of the *pmrAB* operon in a positive feedback loop (16). Additionally, there is evidence of protein-protein interaction between the RstB TCST sensor kinase and PhoQ that results in increased expression of the *pag* regulon (17). The existence of at least two different types of interaction between TCST systems in a single species suggests that precise control of genes required for survival and virulence in response to changing environmental conditions may be achieved through extensive TCST networking. Like *Salmonella*, *H. pylori* encounters a wide range of environmental conditions during the infection and colonization process. The importance of TCST networking in control of the transition of *S. enterica* serovar Typhimurium from colonization to intracellular infection suggests that similar networking may be required during the infection process for *H. pylori*.

Two-component Signal Transduction in *Helicobacter pylori*

Helicobacter pylori is known to possess three two-component signal transduction systems: CrdRS (copper resistance determinant), ArsRS (acid response system), and FlgRS

(flagellum-based motility). This is a small number of TCST pathways compared with most bacteria, though not surprising as obligate pathogens like *H. pylori* tend to have relatively few genetic regulatory pathways dedicated to transduction of environmental signals (18). A possible explanation for this trend is that bacteria that are limited to colonization of a narrow range of environments (and in the case of *H. pylori*, one type of tissue in one host species) will experience less selective pressure to devote resources to environmental response compared with free-living organisms which may exist in much more variable conditions. Because of its relatively small number of TCST systems, *H. pylori* is an ideal model for the basic study of cross-regulation or networking among TCST networks (19). Through a series of experiments designed to detect protein/DNA interactions, I have begun to characterize the mechanisms for transcriptional regulation of the acetone carboxylase operon, which is a common target gene of multiple TCST response regulators in *H. pylori*.

The ArsRS Two-Component Signal Transduction System

ArsRS (HP0166 and HP0165 in *Helicobacter pylori* strain 26695) is currently the best-characterized two-component signal transduction system of *H. pylori*. The histidine kinase ArsS resides within the cytoplasmic membrane with domains in both the periplasm and cytoplasm and autophosphorylates in response to pH values <5.0. ArsR (25.9 kDa, predicted pI = 5.27) is a cytoplasmic response regulator consisting of a C-terminal DNA-binding effector domain containing a helix-turn-helix motif, and an N-terminal receiver domain with homology to known ATPases (20). This domain architecture is typical of response regulators. Though a small part of the receiver domain of ArsR is homologous to a common dimerization interface, ArsR is only known to exist as a monomer (21). Depending on the position of the ArsR-binding site relative to the promoter of its target gene, ArsR or ArsR~P can repress or activate transcription. Currently, no consensus sequence has been

identified for ArsR binding sites. The major function of this TCST system is regulation of the acid acclimation response, including the activation of urease genes in response to acidification of the periplasm. Although *H. pylori* is a neutrophile, it must tolerate the highly acidic gastric environment during infection and colonization. One survival mechanism employed by *H. pylori* in acidic conditions is the creation of a pH-neutral microenvironment through the expression of urease and amidase enzymes, which catalyze ammonia production. ArsR, when phosphorylated in response to ArsS phosphorylation, directly activates transcription of these acid acclimation genes (22).

The regulatory function of ArsRS beyond its role in acid acclimation is not as thoroughly understood. Though both ArsR and ArsS are necessary for survival of *H. pylori* in both neutral and acidic environments, strains in which ArsR cannot be phosphorylated by ArsS can grow at neutral pH (23, 24). Additionally, unphosphorylated ArsR is still capable of binding DNA *in vitro* (24). These results suggest the existence of an essential phosphorylation-independent regulon for ArsR. There is also a subset of the ArsR regulon that requires phosphorylation of ArsR and is not deregulated in *arsS*- mutants; that is, expression levels of these genes change in response to acid exposure by the same amounts in *arsS*- mutants as they do in wild-type *H. pylori* (19). This suggests at least one additional mechanism of ArsR phosphorylation beyond phosphotransfer from ArsS. A recent review of *H. pylori* regulatory networks contends that overlap between the FlgS regulon and the genes regulated by ArsR~P but not ArsS suggests that FlgS may also be able to mediate phosphorylation of ArsR (19). There is also overlap between the regulons of CrdRS (the TCST system that regulates transcription of copper resistance genes) and ArsRS in some *H. pylori* strains, suggesting cross-regulation between the metal homeostasis and acid acclimation regulatory networks (25). The transcription of the ArsRS operon itself is activated

directly by the transcription factor Fur, which regulates intercellular iron homeostasis and contributes to the oxidative stress response, in response to increases in cytoplasmic iron concentrations (26).

The CrdRS Two-Component Signal Transduction System

The CrdRS operon includes the inner membrane-associated histidine kinase CrdS (HP1364) and the cytoplasmic response regulator CrdR (HP1365, 24.9 kDa, predicted pI = 5.67). Based on its amino acid sequence, CrdR is composed of an N-terminal receiver domain with an aspartic acid phosphorylation site and a C-terminal DNA-binding effector domain. CrdR and ArsR, though similar in domain architecture, have only 53% amino acid sequence homology (20).

The best-characterized function of CrsRS is its role in copper homeostasis. Though copper is a necessary cofactor for many essential metabolic processes in *H. pylori*, an excess of intracellular copper can cause DNA damage through oxidative stress. CrsRS activates expression of the copper resistance determinant CrdA in response to increases in environmental copper concentrations. Whether CrdS directly senses an increase in copper ions or another environmental signal indicative of this change has not been proven. When copper ions are introduced into an aqueous environment they cause the formation of reactive oxygen species via Fenton-like reactions (27). It is possible that CrdS, rather than being dedicated solely to copper homeostasis, is a global sensor of oxidative stress. CrsRS is also involved in the acid acclimation response in *H. pylori* strain J99, but not in strains 26695 and G27 (25, 28). This inter-strain difference in regulatory processes highlights the need to confirm experimental results in multiple strains of *H. pylori*.

The FlgRS Two-Component Signal Transduction System

Flagellar-based motility is necessary for persistent colonization of a host by *H. pylori* (29). The transcription of at least five operons encoding structural components of the flagella is under the control of a σ^{54} promoter and is activated by the response regulator FlgR (HP0703, 43.4 kDa, predicted pI = 6.31). Unlike some of its orthologs in similar species, *H. pylori* FlgR does not appear to possess a DNA-binding domain. In a study performed with FlgR from *H. pylori* strain 26695, FlgR was able to activate transcription of flagellar genes by interacting with either *E. coli* or *H. pylori* σ^{54} -RNA polymerase in the presence of minimal upstream regulatory sequence, suggesting that FlgR does not require an enhancer. Based on its amino acid sequence, *H. pylori* FlgR possesses an N-terminal receiver domain containing a potential dimerization interface, and a C-terminal ATPase domain of the AAA+ superfamily similar to those of other σ^{54} -holoenzyme activators (30). (There is no experimental evidence that FlgR is able to autophosphorylate by cleaving ATP.)

FlgS (HP0244), the cognate histidine kinase of FlgR, is thought to localize to the cytoplasm based on its amino acid sequence. If so, FlgS would be the only cytoplasmic TCST sensor in *H. pylori*. FlgS is required by *H. pylori* strain 26695 for survival at pH values <2.5 but not at pH values between 2.5 and 4.5, and a subset of the known FlgS regulon including genes involved in the acid acclimation response and the *cag* PAI gene *cagA* (which encodes a toxin secreted into host cells) is activated at low pH (31). It is possible that FlgS acts as a sensor of cytoplasmic acidification and regulates the acid acclimation response in concert with ArsRS (32). Though *flgR*- *H. pylori* mutants are non-motile, they are able to survive acidic conditions that *flgS*- mutants are not. This suggests that the contribution of

FlgS to acid acclimation is unrelated to motility, and that FlgS may transmit its pH-responsive signal through another response regulator, potentially ArsR. The pH-independent subset of the FlgS regulon includes the flagellar regulon of FlgR and 11 other genes (31). Transcription of some of these target genes is regulated in response to acid, but not by FlgS (6, 33). The signal or signals detected and transduced by FlgRS to activate transcription of this regulon have not yet been identified.

The Orphan Response Regulator HP1021

The orphan response regulator HP1021 (35.2 kDa, predicted pI = 8.09) is essential for normal growth of *Helicobacter pylori*: deletion of ORF *hp1021* from the *H. pylori* strain 26695 genome is either lethal or causes severely impeded growth (34). (The term “orphan” is used because although HP1021 is structurally similar to other TCST response regulators, no cognate sensor kinase for HP1021 has been identified.) HP1021 activates transcription of the essential housekeeping genes *nifS* and *nifU*, which encode nitrogenases necessary for the assembly of iron-sulfur clusters and may account for the impaired growth of the *hp1021*-mutant (35). Whole-genome transcriptional profiling identified increased expression levels of 28 genes and decreased expression levels of 51 genes in a *hp1021*-*H. pylori* mutant, suggesting that HP1021 may be both a repressor and activator of transcription (35).

HP1021 has been classified as a response regulator based on domain architecture. The C-terminal DNA-binding domain of HP1021 contains a helix-turn-helix motif but shows no significant homology to any DNA-binding domain or domain superfamily in the NCBI Conserved Domains database (20). The N-terminal receiver domain of HP1021 has some homology with the receiver domains of the other *Helicobacter pylori* response regulators, but the aspartic acid at the consensus phosphorylation site is replaced in HP1021 by serine. This atypical serine residue does not appear to be necessary for the essential function of HP1021,

as *H. pylori* with amino acid substitutions at and on either side of this residue display a wild-type growth phenotype (36). There is no evidence that HP1021 can be phosphorylated by either a histidine kinase or acetyl phosphate, and an alternate post-translation mechanism by which the activity of HP1021 could be modulated has not yet been identified. The transcription of HP1021 is up-regulated in response to an acidic pH, though HP1021 is not known to be included in either the ArsRS or FlgRS regulons (6, 37, 31). It is possible that HP1021 itself is only regulated at the level of transcription. However, stringent regulation of HP1021 may not be necessary for normal growth of *H. pylori*, as overexpression of HP1021 in *H. pylori* strain G27 had no detrimental effects on growth *in vitro* (38).

Two-Component Signal Transduction and Acetone Metabolism

Acetone, along with acetoacetate and 3- β -hydroxybutyrate, is produced in the mammalian liver during fatty acid metabolism and enters the bloodstream (39). Because the spontaneous decomposition of acetoacetate to acetone is accelerated at low pH, gastric tissues may be enriched in acetone in comparison to the rest of the body, conferring a survival advantage to gastric pathogens that can use acetone as a carbon source. *Helicobacter pylori* absorbs acetone from its host tissue and converts it to acetyl-CoA, which is essential for initiation of the Krebs cycle. The first step in this process is the conversion of acetone to acetoacetate, carried out by the enzyme acetone carboxylase. Though deletion of the acetone carboxylase operon (*acxABC*; HP0695-7 in *H. pylori* 26695) is not lethal for *H. pylori*, the presence of functional acetone carboxylase enhances the ability of *H. pylori* to colonize and infect mice (40). Because mice do not experience *H. pylori*-induced gastric illness, any connection of *acxABC* to virulence beyond colonization remains unknown. (*H. pylori* infection is usually exclusive to humans, but animal-acclimated strains can be created in the laboratory.)

There is approximately 190 bp of nontranscribed sequence upstream of the *acxA* transcription start site that will be referred to as the *acxA* upstream regulatory region. The *acxABC* operon is under the control of a housekeeping σ^{80} promoter (41). The AT-rich region between the -35 and -10 sites of the *acxA* promoter along with the Pribnow box at the -10 site (TATACT) is typical of *H. pylori* promoters (42). An inverted repeat (with one mismatch) directly upstream of the -35 site (TTATTaCAAA . . . TTTGaAATAA) is a potential binding site for transcription factors (Figure 2).

There is evidence that transcription of the acetone carboxylase operon, *acxABC*, is regulated directly or indirectly by all three TCST systems of *H. pylori* as well as the orphan response regulator HP1021. HP1021 has been shown to induce *acxABC* transcription and to bind directly to the upstream regulatory region of *acxA*, but the mechanism of regulation has not been characterized in detail (35). Unpublished macroarray data obtained recently in our lab indicates that *acxABC* is regulated by the CrdRS TCST system. Transcription of *acxA* is increased in *H. pylori* strains with null mutations in either *crdR* or *crdS* (43). It is interesting to note that another gene involved in acetone metabolism, *fadA*, exhibits similar de-repression in the absence of a functional CrdRS TCST system (43). Direct interaction of CrdR and the *acxA* promoter has not yet been demonstrated, and a signal sensed by CrdS that affects *acxABC* transcription has not been identified. A CrdR-binding site containing a mirrored repeat (AACACC . . . CCACAA) has been identified directly upstream of the -35 site of the *crdA* (a membrane-associated protein required for copper homeostasis) promoter, but the promoter region of *acxABC* does not contain this sequence or any other mirrored repeats (27). As CrdR is an activator of *crdA* and a potential repressor of *acxABC*, it is possible that CrdR binds to a different DNA sequence when it acts as a repressor. Recent microarray data suggest that the genes of the *acxABC* operon are members of the pH-

independent FlgRS regulon as well. Expression of all three genes in the acetone carboxylase operon was down-regulated in an *flgS-H. pylori* strain 26695 mutant in a manner irrespective of the pH of the growth media (31). The impact of deletion of *flgR* on *acxABC* transcription has not yet been studied. Because *acxABC* is not under the control of a σ^{54} promoter, the activation of *acxABC* transcription in response to autophosphorylation of FlgS may occur by an alternate type of FlgR activity or may be mediated by another response regulator. Both full-length ArsR and the ArsR DNA-binding domain bind the upstream regulatory region of *acxA in vitro*, and *acxABC* transcription is upregulated in an *arsS- H.pylori* strain J99 mutant (37). Transcription of *acxABC* is also repressed at pH values <5.0 in *H. pylori* strains G27 and 26695, but not in strain J99.

Constitutive expression of *acxABC* would be inefficient for *H. pylori*, as acetone levels in human tissue are highly variable (40). Though no *H. pylori* protein that can sense and respond to changes in acetone concentration has been identified, acetone is a potential environmental signal that could lead to activation or derepression of *acxABC*. *H. pylori* may also repress *acxABC* transcription to respond to various types of stress. Since acetone carboxylase is not necessary for survival, repression of *acxABC* transcription by ArsRS at low pH and by CrdRS at increased levels of reactive oxygen species would allow more resources to be devoted to the acid acclimation or oxidative stress response.

Experimental Approach

The research described in this thesis represents a contribution to the comprehensive characterization of transcriptional regulation of the acetone carboxylase operon *via* two-component signal transduction in *Helicobacter pylori*. The overall aim of the experiments described herein was to investigate whether the regulation of *acxABC* expression by the *H. pylori* transcription factors ArsR, CrdR, FlgR, and HP1021 is direct or indirect, and to begin

physical characterization of the *acxABC* regulation mechanism(s). Each of these response regulators was cloned, overexpressed in *E. coli*, and purified. Electrophoretic mobility shift assays (EMSAs) were then employed to test for direct interaction of each of the four *H. pylori* response regulators with DNA probes amplified from the upstream regulatory region of *acxA*.

Methods

Cloning the Putative Upstream Regulatory Region of *acxABC*

A 756-bp product containing the putative upstream regulatory region of *acxA* was amplified from *H. pylori* J99 and 26695 genomic DNA using the Expand High Fidelity PCR kit, which includes a proofreading polymerase (Roche Diagnostics). PCR was performed in 50 μ L reactions with primers HP0694 FWD.2 and *hyuA*-R-2 (Table 1) according to the manufacturer's instructions using a Gradient Multigene™ thermal cycler (Labnet). Amplicon purification by agarose gel extraction was performed with the IBI Gel/PCR DNA Fragment Extraction Kit according to the manufacturer's protocol (IBI). (This PCR protocol does not produce nonspecific amplification products, and gel purification may not be necessary in future work.) The purified PCR products were then ligated into the pCR2.1-TOPO® plasmid and transformed into OneShot® MAX Efficiency® DH5 α TM-T1^R chemically competent *E. coli* (Invitrogen) (Table 2). After ampicillin selection and X-gal screening, three single colonies per transformation were inoculated in Luria-Bertani broth containing 100 μ g/mL ampicillin (LB Amp) and incubated overnight at 37°C and 200rpm. Plasmid minipreps for pCR-J99-*acxA* and pCR-26695-*acxA* were performed with the IBI High-Speed Plasmid Mini kit (Table 3). Sequencing reactions were performed using M13 forward and reverse primers and Bigdye® Terminator v3.1 sequencing reagents (Applied Biosystems), and capillary sequencing was carried out on the Applied Biosystems 3130 Avant Genetic Analyzer. Samples of pCR-J99-*acxA* and pCR-26695-*acxA* showing 100% identity to the reference sequences in NCBI Genbank were kept to serve as templates for electrophoretic mobility shift assay (EMSA) probes. Freezer stocks of *E. coli* DH5 α /pCR-J99-*acxA* and *E. coli* DH5 α /pCR-26695-*acxA* were prepared with 15% glycerol and stored at -80°C.

Table 1) Primers: Lower-case letters indicate added restriction sites.		
	Name	5' to 3' sequence
1	HP0694 FWD.1 ¹	CAGGGTGGCTTTTACGATCACTG
2	hyuA-R-2 ¹	CGGTTCTTTCTGTAACGCCT
3	crdR Fwd.BamHI ¹	cccgatccATGCAAAAAAAGATTTTTTTACTAGAAAGACG
4	crdR Rev.PstI ¹	cccctgcagTCATAGTGGGTAAAGCGATAGCC
5	HP1021 Fwd.BamHI ¹	cccgatccATGAAAATCTTAATCATTGAAGACG
6	HP1021 Rev.PstI ¹	cccctgcagTTATTTGCGCGGTAAGTTATATTCCG
7	flgR Fwd.BamHI ²	cccgatccATGAAAATCGCCATTGTAGAAGATG
8	flgR Rev.PstI ²	cccctgcagCTACCTTTCCAAAAACAAATCTTTCT
9	pQE30 promoter ³	CCCGAAAAGTGCCACCTG
10	pQE30 reverse sequencing ³	GTTCTGAGGTCATTACTGG
11	HP0694 FWD.1.btn ²	Biotin-CAGGGTGGCTTTTACGATCACTG
12	acxARev1 ¹	GGCATCAATACCCATCACC
13	HP0694 FWD.2.btn ²	Biotin-TCCCAGCCTAAGCACCATC
14	acxARev2.btn ²	Biotin-CTTTACCAACTACGAAACT
15	acxA-35Fwd ²	ATGATTTCCATTTAAAAATC
16	acxA-35Rev ²	TTATTTCAAAGTTGTTTGT

Cloning the Response Regulators

A freezer stock of M15 *E. coli* known to express *H. pylori* 26695 ArsR was obtained from Dan Hallinger (Table 2). Strains expressing FlgR, HP1021, and CrdR were created for this study by direct cloning or subcloning into the expression vector pQE30 (Qiagen) and transformation into *E. coli* M15. This vector confers ampicillin resistance, puts the inserted gene under the control of the T5 promoter, and adds a 6xHis tag at the N-terminus. The entire translated region of each of the *H. pylori* TCST response regulators was amplified from strain

26695 genomic DNA using the Expand High-Fidelity PCR Kit. A *Bam*HI restriction site was added to the 5' end of the forward primers and a *Pst*I site added to the 5' end of the reverse primers (primers 3-8 in Table 1) to facilitate ligation into the multiple cloning site of pQE30. PCR products were purified using an IBI PCR cleanup kit.

Table 2) Strains			
Name	Purpose	Source	
<i>Helicobacter pylori</i> 26695	Fully-sequenced <i>cag</i> PAI+ laboratory strain	Mark Forsyth, College of William and Mary	
<i>H. pylori</i> J99			
OneShot® MAX Efficiency® DH5α TM -T1 ^R chemically competent <i>E. coli</i>	Chemically competent <i>E. coli</i> for transformations	Invitrogen	
JM109 chemically competent <i>E. coli</i>		Promega	
M15 chemically competent <i>E. coli</i> /pREP4(kan ^R)		Dan Hallinger, Forsyth Lab, College of William and Mary	
DH5α TM <i>E. coli</i> / pCR-26695- <i>acxA</i> (amp ^R kan ^R)	Template for production of EMSA probe for <i>acxA</i> upstream regulatory region	This study	
DH5α TM <i>E. coli</i> / pCR-J99- <i>acxA</i> (amp ^R kan ^R)			
DH5α TM <i>E. coli</i> / pCR-26695 <i>crdR</i> (amp ^R kan ^R)	Stabilization and amplification of <i>H. pylori</i> response regulator genes prior to subcloning into expression vectors		
DH5α TM <i>E. coli</i> / pCR-26695HP1021(amp ^R kan ^R)			
JM109 <i>E. coli</i> / pQE30-26695 <i>crdR</i> (amp ^R)	Stabilization and amplification of <i>H. pylori</i> response regulator expression vectors		
JM109 <i>E. coli</i> / pQE30-26695 <i>flgR</i> (amp ^R)			
JM109 <i>E. coli</i> / pQE30-26695HP1021 (amp ^R)			
M15 <i>E. coli</i> / pQE30-26695HP1021 + pREP4 (amp ^R kan ^R)	Expression of putative direct regulators of <i>acxA</i> in <i>E. coli</i> for use in EMSAs		D. Hallinger
M15 <i>E. coli</i> / pQE30-26695 <i>crdR</i> + pREP4 (amp ^R kan ^R)			
M15 <i>E. coli</i> / pQE30-26695 <i>flgR</i> + pREP4 (amp ^R kan ^R)			
M15 <i>E. coli</i> / pQE30-26695 <i>arsR</i> + pREP4 (amp ^R kan ^R)			

TOPO Cloning and Subcloning

Direct cloning into pQE30 can be problematic because it is not possible to determine prior to attempting ligation whether or not the restriction digest of the intended insert has gone to completion. Therefore, TOPO cloning was performed when possible to ensure that any insert purified from a restriction digest of the TOPO plasmid would be cut at both ends. TOPO cloning of the purified *crdR* and *hp1021* PCR products was performed in the same manner as it was for *acxA*. Minipreps of the resulting transformants (DH5 α *E. coli*/pCR-26695*crdR* and DH5 α *E. coli*/pCR-26695*hp1021*, Table 2) were performed with the IBI High-Speed Mini Kit according to the manufacturer's instructions. A 20% glycerol freezer stock was also prepared from each transformant and stored at -80°C. Three double digests each of pCR-26695*crdR* and pCR-26695HP1021 were prepared with *Bam*HI and *Pst*I according to the manufacturer's protocol (Promega) and incubated 12-48h at 37°C. The products were run 1 h at 100 V on a 1.2% agarose gel and the *crdR* (641 bp) and *hp1021* (836 bp) insert bands were excised for purification. The three *crdR* insert bands were combined on one column and purified with the IBI Gel/PCR DNA Fragment Extraction Kit (Midsci) according to the manufacturer's protocol, and the same was done for *hp1021*. This process was repeated and the products were concentrated together via ethanol precipitation until an adequate DNA concentration for ligation was reached (>5 ng/ μ L).

To prepare pQE30 for subcloning, a 15 mL JM109 *E. coli*/pQE30 culture was grown overnight at 37°C in LB Amp with shaking. Four minipreps were performed with 3 mL culture each and the products were combined. Double digests of 1 μ g total pQE30 were performed with *Pst*I and *Bam*HI, with the DNA divided among as many replicate 20 μ L reactions as necessary. A 2 μ L aliquot of each product was run on a 1% agarose gel with ethidium bromide to check for uncut or nicked vector. Though uncut and cut pQE30 are

Table 3) Plasmids		
Name	Description	Purpose
pCR-26695-acxA	<i>acxA</i> regulatory region from <i>H. pylori</i> strain 26695 cloned into pCR2.1-TOPO® (Invitrogen)	Template for PCR amplification of an EMSA probe from the <i>acxA</i> promoter and putative upstream regulatory region; confers kanamycin and ampicillin resistance
pCR-J99-acxA	<i>acxA</i> regulatory region from <i>H. pylori</i> strain J99 cloned into pCR2.1-TOPO®	
pQE30-26695arsR	Entire <i>arsR</i> translated region cloned into expression vector pQE30 (Qiagen)	Production of putative direct regulators of <i>acxA</i> in <i>E. coli</i> for use in EMSAs and <i>in vivo</i> experimentation; confers ampicillin resistance
pQE30-26695crdR	Entire <i>crdR</i> translated region cloned into expression vector pQE30	
pQE30-26695flgR	Entire <i>flgR</i> translated region cloned into expression vector pQE30	
pQE30-26695HP1021	Entire <i>HP1021</i> translated region cloned into expression vector pQE30	

distinguishable on the gel, the bands run too close to each other to separate by gel purification, so digests that did not go to completion were discarded. Liquid purifications of successful digests were combined on one spin column and purified with the IBI Gel/PCR DNA Fragment Extraction Kit. The product was then treated with shrimp alkaline phosphatase (Promega) according to the manufacturer's protocol to prevent re-ligation of single-cut pQE30. (Single-cut pQE30 could not be distinguished from double-cut pQE30 on a test gel, and early trials without SAP treatment yielded mostly transformants carrying self-ligated pQE30.) SAP-treated pQE30 was purified and concentrated via ethanol precipitation until a DNA concentration of at least 10 ng/μL was obtained. The cut *crdR* and *hp1021* inserts were ligated into pQE30 with T4 DNA ligase (Promega) to create pQE30-26695crdR and pQE30-26695hp1021 (Table 2). Reactions were performed with an approximate 6:1 vector to insert ratio by mass and were incubated overnight at 14°C.

Although M15 *E. coli*/pREP4 was used as the host strain for expression of the cloned response regulators, the newly ligated plasmids were first transformed into JM109 *E. coli* as this strain is better for storage and propagation of plasmids. Strains JM109 *E. coli*/pQE30-26695crdR and JM109 *E. coli*/pQE30-26695hp1021 (Table 3) were created according to a protocol adapted from the Promega GeneEditor™ handbook: Two LB Amp agar plates per transformation were pre-warmed at 37°C. One 100 µL glycerol stock of chemically competent JM109 *E. coli* per transformation was thawed on ice and transferred to a pre-chilled 2 mL sterile tube. A 2-10 µL portion of the ligation reaction product was added to the cells and the mixture was incubated 30 minutes on ice. The cells were heat-shocked 45-50 s at 42°C, then incubated 2 min on ice. For recovery, 900 µL room temperature SOC broth was added to each vial and the cells were incubated 90 min at 37°C and shaking horizontally at 225 rpm. Two LB Amp plates, one with 100 µL transformed cells and one with 10 µL, were spread per transformation. After an overnight incubation at 37°C, 5 colonies per transformation were inoculated into 5 mL LB Amp broth each and grown overnight at 37°C with shaking. Freezer stocks with 20% glycerol were prepared from each 5 mL culture and stored at -80°C. Minipreps, restriction digests, and agarose gel electrophoresis were performed as described above to confirm successful transformation and presence of the correct size insert in pQE30. Sequencing of successfully transformed plasmids was performed as described above for *acxA* to confirm that *crdR* and *hp1021* had been inserted in frame with the *lac* promoter and 6xHis tag and were identical to the reference sequence in Genbank. Minipreps of pQE30-26695crdR and pQE30-26695hp1021 were stored at -20°C. Once the correct sequence was confirmed, pQE30-26695crdR and pQE30-26695hp1021 were transformed into M15 *E. coli* as specified in the QIAexpressionist™ manual (Qiagen) to

create M15 *E. coli*/pQE30-26695crdR + pREP4 and M15 *E. coli*/pQE30-26695hp1021 + pREP4 (Table 3).

Direct Cloning

Because the digestion of pCR®-2.1-TOPO® with PstI and BamHI produces a 1210 bp fragment that cannot be gel-purified separately from *flgR* (1145 bp), this gene was not subcloned. Double digests of the *flgR* PCR products were prepared with BamHI and PstI as described above. The products of multiple restriction digests were purified and concentrated together via ethanol precipitation until an adequate concentration for the ligation reaction was reached. Ligation into pQE30 and transformation into the host strains was performed as described above to create JM109 *E. coli*/pQE30-26695flgR, and then M15 *E. coli*/pQE30-26695flgR + pREP4 (Table 2).

Response Regulator Expression and Extraction

Soluble ArsR was expressed in and extracted from M15 *E. coli*/pQE30-26695arsR + pREP4 according to the native batch purification protocol in the QIAexpressionist manual. This protocol did not produce soluble products for the other three response regulators, and had to be optimized as follows: A freezer stock of M15 *E. coli* carrying pQE30 with the response regulator gene of interest was used to inoculate an overnight culture in 10 mL LB Amp Kan (100 µg/mL ampicillin and 50 µg/mL kanamycin) broth. The next day, this entire culture was added to 250 mL prewarmed LB Amp Kan in a sterile baffled flask. This culture was grown at 37° with shaking to an OD₆₀₀ of 0.5-0.7. A 1 mL uninduced control sample was taken from the culture, pelleted in the microcentrifuge, and resuspended in 50 µL 1× SDS-PAGE sample buffer. Expression was induced by adding IPTG to a final concentration of 1 mM, and the culture was returned to the incubator. Every hour or half hour, a 1 mL induced

control sample and a 50 mL sample for protein extraction were taken from the culture. Both samples were pelleted immediately. The induced control pellet was resuspended in 100 μ L 1x SDS-PAGE sample buffer, and the 50 mL pellet was drained and frozen overnight at -20°C . The next day, a solubility test was performed on protein extracted from each 50 mL sample taken during the time-course according to a protocol adapted from the QIAexpressionist manual: Cell pellets were thawed on ice and resuspended in 2 mL NPI-10 lysis buffer (50mM NaH_2PO_4 , 300mM NaCl, 10mM imidazole) per gram. Lysozyme was added to a final concentration of 1 mg/mL cell suspension and samples were incubated 30 minutes on ice. The resuspended cells were then sonicated at maximum power on ice in six 10 second bursts with a 10 second cooling period between each burst. Twelve units Benzonase® nuclease (Novagen) per mL expression culture was added to each sonicated sample and the mixtures were incubated 15 min on ice. The lysates were then centrifuged for 30 min at 10,000 g and 4°C to pellet the cell debris. The supernatant, which is the soluble fraction, was removed and stored at 4°C . The insoluble fraction (pellet) was resuspended in a volume of NPI-10 equal to the volume of the supernatant and stored at 4°C . A 20 μ L aliquot of each fraction was taken for SDS-PAGE. Equal volumes of the aliquots of the uninduced control, induced control, soluble fraction, and insoluble fraction from each time point were run on SDS-PAGE gels and stained with Coomassie Blue to evaluate expression levels and solubility. SDS-PAGE was performed using 12% acrylamide 0.75 mm thick gels on the BioRad Mini-PROTEAN® apparatus. The time point that produced the largest amount of the desired protein in the soluble fraction was selected as the expression time for subsequent purifications. If the protein was largely insoluble at all time points, adjustments were made to slow expression in order to avoid inclusion body formation. These included using reduced IPTG concentrations, growth at a lower temperature, and the addition of 1% glycerol to the media. Through this

optimization procedure, the following adjustments were made to the QIAexpressionist protocol for the three remaining response regulators: M15 *E. coli* expressing HP1021 were grown in 250 mL cultures and harvested 2 h post-induction. Expression cultures for FlgR were grown in a total volume of 1 L per purification and were harvested 1 h post-induction. The lysis buffer for FlgR cultures contained 0.1% Tween-20. M15 *E. coli* expressing CrdR were grown in LB Amp Kan with 1% glycerol at 18°C, were induced with 0.1 mM IPTG, and were harvested 4 h 35 min after induction.

Response Regulator Purification

ArsR, CrdR, and HP1021 were purified on 5 mL bed volume drip columns containing 3 mL 50% Ni-NTA agarose slurry according to the manufacturer's protocol (Qiagen). Up to twelve milliliters of the soluble fraction of each protein extract could be processed in one purification. For FlgR, which required more stringent conditions than the other response regulators, Tween-20 was added to all lysis, wash, and elution buffers to 0.1%, and six elutions were performed instead of four. During purification, a 20 μ L aliquot of each fraction was taken for SDS-PAGE, which was performed to check the quality of the purification and to determine which elution fractions to save for use in EMSAs. These fractions were chosen on the basis of total protein concentration as measured on the Nanodrop spectrometer (Thermo), and purity as observed on the gel. If the fractions to be reserved contained less than 2 mg/mL total protein, they were concentrated using a 10K MicrosepTM column according to the manufacturer's instructions (Pall Life Sciences). The reserved fractions were pooled and glycerol was added to 10%. Aliquots containing 60 μ g protein were prepared and stored at -20°C. Each aliquot was discarded after a maximum of three freeze-thaw cycles. A

colorimetric Western blot was performed with an α -His antibody according to the Qiagen Detection and Assay handbook to confirm the identity of the purified proteins.

Electrophoretic Mobility Shift Assays

Response Regulator Phosphorylation

ArsR, CrdR, and FlgR were phosphorylated for use in EMSAs according to the protocol employed by Loh for ArsR (37). Though there is no evidence that HP1021 can be phosphorylated, this response regulator was put through the same phosphorylation reaction for control purposes. No phosphorylation protocol existed for FlgR, so the ArsR protocol was used. Further optimization may improve EMSA results with FlgR. Though a combined phosphorylation and binding protocol exists for CrdR (27), no results were obtained when it was attempted in this study. (That is, this protocol produced a completely blank EMSA blot.)

Because the proteins are diluted in the phosphorylation reaction and because the phosphorylation buffer is different from the EMSA binding buffer, it was necessary to use 10K MicrosepTM columns to re-concentrate the phosphorylated response regulators. The proteins were concentrated to at least 1 mg/mL (1.5 mg/mL for FlgR) and glycerol was added to 10% by volume. The response regulators were split into 60 μ g aliquots as before and stored at -20°C. They were discarded after a maximum of three freeze-thaw cycles, including any that took place before phosphorylation.

Probes

EMSAs were performed with biotin-labeled DNA probes that could be visualized using the LightShift® Chemiluminescent EMSA Kit (Thermo Scientific). These probes (Table 4, Figure 2) were amplified by PCR from the templates pCR-26695*acxABC* and pCR-J99*acxABC* (or later, from existing probe stocks) using the Expand High Fidelity PCR kit and

biotinylated primers (Table 1). Unlabeled probes for competition assays were produced by the same method. Unlabeled probes were purified with an IBI PCR cleanup kit.

Because the *acxA-All** probes produced a large smear when visualized on a chemiluminescent blot, they were initially purified with an IBI gel extraction kit. The *acxA-Up** probes were co-amplified with a ~250 bp contaminant and the *acxA-Down** probes were co-amplified with a ~200 bp contaminant. These contaminants were difficult to remove by agarose gel extraction and are visible in some of the EMSA results, but acrylamide gel purification eventually yielded pure samples of all three types of probe. For purification, fresh PCR products were run on 6% acrylamide native gels containing 4 µg/mL ethidium bromide. The probe bands were then excised and purified according to the crush and soak method (44): Gel slices containing the DNA to be purified were pulverized in a

Table 4) Probes for <i>acxABC</i> EMSAs		
Probe Name	Description	Size
<i>acxA-All</i>	Spans entire putative upstream regulatory region of <i>H. pylori acxABC</i>	370 bp
<i>acxA-All*</i>	Biotinylated <i>acxA-All</i>	
<i>acxA-Up</i>	Spans end of HP0694 translation through -35 site of <i>H. pylori acxABC</i>	209 bp
<i>acxA-Up*</i>	Biotinylated <i>acxA-Up</i>	
<i>acxA-Down</i>	Starts at -35 site of <i>H. pylori acxABC</i> and ends downstream of the start of translation	175 bp
<i>acxA-Down*</i>	Biotinylated <i>acxA-Down</i>	
Glc.kin	Unlabeled non-regulatory sequence from the translated region of the <i>E.coli</i> glucokinase gene.	151 bp
EBNA	Unlabeled Epstein-Barr nuclear antigen DNA from the LightShift® EMSA Optimization kit (Thermo); includes some viral regulatory sequence.	60 bp

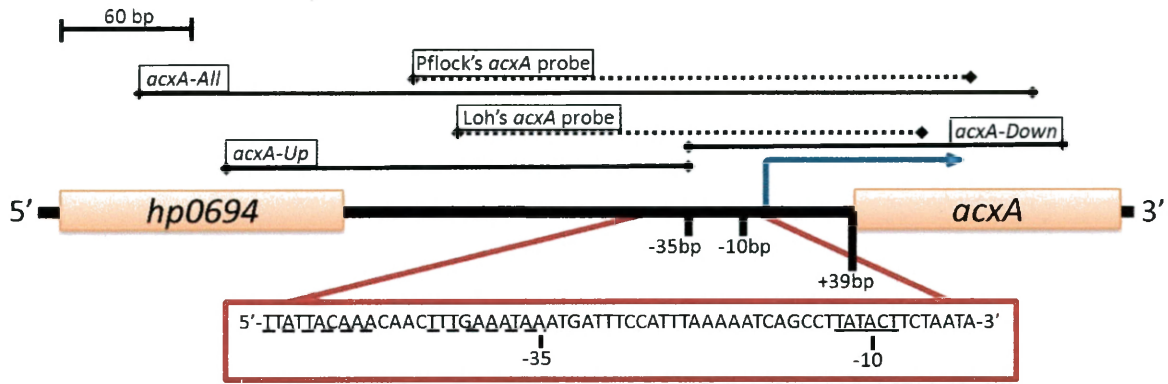


Figure 2. Sizes and locations of *acxA* EMSA probes used in this study and others. This schematic shows base pairs 745441-745920 of the *H. pylori* strain 26695 genome. My *acxA-All* probe, Pflöck's *acxA* probe, and Loh's *acxA* probe all contain the promoter region of *acxA* and extend past the start of *acxA* translation, so they are considered functionally equivalent for the purposes of this thesis (35, 37). The *acxA-Up* probe was designed to include potential proximal binding sites for transcriptional activators of *acxA*, and *acxA-Down* was expected to include binding sites for *acxA* repressors. The magnified region shows the promoter sequence, which resembles the typical *H. pylori* housekeeping σ^{80} promoter including the extended Pribnow box (tgnTAtaAT) at -10 bp (underlined) and AT-rich region between the -10 and -35 sites (41, 42). Dashed underlines indicate inverted repeats, a potential binding site for response regulators.

microcentrifuge tube, mixed with three volumes elution buffer (300 mM sodium acetate, 1mM EDTA pH 8.0), and shaken 24-72 hours at room temperature and 200 rpm. The samples were centrifuged 10 minutes at 10,000 g to pellet the gel fragments, and the probe was purified from the supernatant by ethanol precipitation.

Electrophoretic Mobility Shift Assays

Binding Reactions

EMSAs were carried out according to a procedure adapted from the LightShift® EMSA Optimization kit protocol, the Sigma EMSA Optimization kit protocol, and the ArsR EMSAs performed by Loh et al (37). Each experiment comprised 5-7 binding reactions which were run on native PAGE gels and blotted. The following is a typical set of reactions:

- 1) Labeled probe
- 2) Labeled probe + Unphosphorylated protein
- 3) Labeled probe + Unphosphorylated protein + 500x Unlabeled probe
- 4) Labeled probe + Unphosphorylated protein + 500x Unlabeled nonspecific DNA
- 5) Labeled probe + Phosphorylated protein
- 6) Labeled probe + Phosphorylated protein + 500x Unlabeled probe
- 7) Labeled probe + Phosphorylated protein + 500x Unlabeled nonspecific DNA

In early experiments, unphosphorylated response regulators were not expected to produce a shift, so reactions 3 and 4 were not included. These reactions were added to each EMSA after ArsR was observed to produce a shift without phosphorylation. The amount of unlabeled probe in reactions 3, 4, 6, and 7 was the same for all samples in one EMSA, but was varied between experiments in an effort to obtain more information about binding specificity. In each EMSA, the amount of protein in reactions 2-4 was the same, as was the amount in reactions 5-7.

Each binding reaction in an EMSA had a total volume of 30 μ L. Reagents were combined in this order: 6 μ L 5x binding buffer, 2 μ L poly dI-dC (1 mg/mL), unlabeled probe (if applicable), 0.2-0.4 nmol protein (if applicable), and dI H₂O to 29 μ L. This mixture was incubated 10 minutes at room temperature. Next, 1 μ L (1-2 ng) labeled probe was added to each binding reaction and incubated 20 minutes at room temperature. Reactions were electrophoresed immediately after this incubation was complete.

The binding buffers for ArsR and HP1021 were based on previous studies. For ArsR, the 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 (37). The 5x HP1021 binding buffer contained 50 mM Tris-HCl, 50 mM KCl, 5 mM DTT, 5% glycerol, and 25 mM EDTA (35).

There was no existing EMSA protocol for FlgR when this study began, so two binding buffers were tested and the ArsR binding buffer was found to be superior to the buffer specified by the LightShift® base protocol. Because the combined phosphorylation

and binding protocol for CrdR described above yielded no results, the 5x ArsR binding buffer was used for CrdR as well. Further optimization may improve EMSA results for FlgR and CrdR.

Electrophoresis

7.5 μ L loading buffer (Thermo) was added to each binding reaction in preparation for gel electrophoresis, and 18 μ L of each binding reaction was loaded onto a 6% acrylamide native PAGE gel. (Though 4% acrylamide gels are traditionally used for EMSAs, 6% acrylamide was the minimum concentration at which the especially thin gels required by our electrophoresis cell could be transferred to the blotting apparatus intact.) Gels were pre-electrophoresed at least 30 minutes at 100 V before loading. The binding reactions, along with 0.5 μ g 2-log biotinylated DNA ladder (New England Biolabs) in the first and last lanes, were electrophoresed in 0.5x TBE at 100 V until the unbound biotinylated probe was ~5 mm from the bottom edge of the gel. Gels containing EMSAs performed with the larger “All” probes were run 2 h 40 min, and those performed with the “Up” or “Down” probes were run until the light blue xylene cyanol loading dye band reached the bottom of the gel.

Blotting and Detection

Tank blotting onto Zeta-Probe GT® nylon membranes was performed immediately after electrophoresis according to the manufacturer’s protocol for the BioRad Mini Trans-Blot® cell. The fiber pads, filter paper, and membranes were soaked in 0.5x TBE at least 10 minutes before the blotting cartridges were assembled. Transfers were performed in chilled 0.5x TBE for 30 minutes at 380 mA. Blots were crosslinked while still damp in a Stratalinker® UV crosslinker and were stored dry at room temperature until the detection procedure could be performed (Stratagene).

Detection was carried out according to the LightShift® EMSA kit protocol. The finished blots were exposed immediately to Thermo Scientific CL-XPosure® X-ray film for 10 s. The film was developed in a Konica automated film processor. If the blot was too bright, shorter exposures were performed. If no band appeared where one was expected, longer exposures were performed up to and including a “burnout” exposure in which the blot and film were left in the exposure cassette much longer than the 30 minutes for which the luminescence of the blot was expected to persist.

Results and Discussion

ArsR Mobility Shift Assay

All ArsR electrophoretic mobility shift assays (EMSAs) were performed using DNA probes amplified from the cloned upstream regulatory region of *Helicobacter pylori* strain 26695 *acxA* unless stated otherwise (Table 4, Figure 2). The results in Figures 3-5 show that response regulator ArsR from *H. pylori* strain 26695 binds the *acxA* upstream regulatory region both upstream and downstream of the -35 site of the promoter, whether or not the protein has been phosphorylated *in vitro*. (Phosphorylated ArsR is abbreviated as ArsR~P.) This is in agreement with previous EMSA results obtained using both the DNA-binding domain (DBD) of ArsR and the full-length protein from *Helicobacter pylori* strain J99 using a probe that included the entire *acxA* promoter region (Figure 2) (37). In Loh's study, a shift occurred in a binding reaction with full-length ArsR~P (3 μ M) and labeled *acxA* probe (100 pM), and specific competition was observed in a reaction that contained ArsR-DBD (2 μ M), labeled probe (100 pM), and a 20-fold excess of unlabeled probe. No competition reactions were performed in this study with full-length ArsR. A recent review contends that the DNA-binding domains of response regulators tend to have greater affinity for their target DNA than their full-length counterparts, but my results combined with Loh's suggest that this may not be true for ArsR, as specific competition appears to occur more readily in EMSA reactions with the ArsR DBD than with full length ArsR (8).

Binding Specificity of ArsR to *acxA* Probes

In each EMSA, competition reactions are performed to examine binding specificity between the response regulator and its target sequence. Unlabeled probe identical to the labeled probe is added to one binding reaction to test for specific competition, and the same

amount of an unlabeled probe amplified from an unrelated gene (containing either no regulatory sites or regulatory sequence from a different species) is added to another binding reaction to test for nonspecific competition. In order for this assay to produce the most meaningful data regarding binding specificity, at least some specific competition must be observed. In the ArsR EMSAs exemplified by Figure 3, specific competition was only demonstrated once in a binding reaction with the *acxA-All* probe despite the competition reaction being performed seven times with up to 1200x unlabeled probe. It is not certain why specific competition was so difficult to demonstrate in this binding reaction, especially when competition occurred in Loh's competition reactions performed with ArsR-DBD at a 20-fold

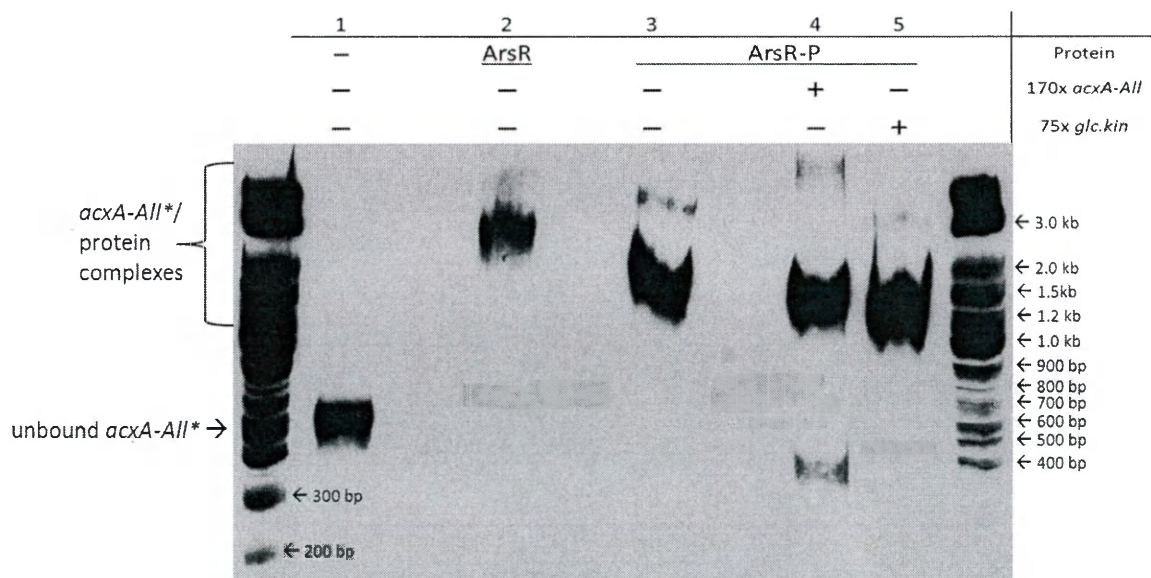


Figure 3. ArsR EMSA with *acxA-All* probe. An asterisk indicates biotinylated probe, and “~P” following the name of a protein indicates that it has been phosphorylated. See Table 4 for probe descriptions. First and last lanes: 2-log biotinylated DNA ladder (New England Biolabs). All reactions contain 2 ng *acxA-All** amplified from *Helicobacter pylori* strain 26695, and reactions 2-5 contain 10 µg protein. Reactions 2 and 3 were performed to test for the formation of probe/protein complexes, and reactions 4 and 5 were performed to assess binding specificity. The gel was accidentally stretched upwards at lanes 1-3 while being transferred to the blot apparatus. In six similar (with the exception of the amount of *acxA-All**; 1 ng) assays with up to 1200x specific competitor, the same *acxA-All**/protein complexes were observed but the results for specific competition were negative (data not shown). Similar results (not including specific competition) were obtained when the same EMSA was performed three times using *acxA-All* probe amplified from pCR-J99-*acxA* (Table 2).

excess of unlabeled probe (37). Attempts to replicate specific competition results in this study by decreasing the amount of protein in the reaction and increasing the amount of unlabeled probe were unsuccessful (data not shown). Upon review of experimental records, it was discovered that 2 ng of labeled *acxA-All* was added to the binding reactions in Figure 3 instead of 1 ng as for all other EMSAs with this probe. It is possible that the amount of competition that occurred in the ArsR and *acxA-All* EMSAs was so small that it could only be visualized when the amount of labeled probe in the reaction was accidentally doubled. The reaction conditions necessary to demonstrate specific competition, and therefore binding specificity, with full-length ArsR and probes containing the whole upstream regulatory region of *acxA* will require optimization.

However, considering the molar ratios of the binding reaction components, it is surprising that competition occurred in the EMSA in Figure 3 or in Loh's EMSA at all. When the binding reactions are assembled, the protein and unlabeled probe are co-incubated, and then the labeled probe is added. If there is enough free protein still available after the first incubation for the labeled probe to bind, then all of the labeled probe will be shifted and no evidence of competition will appear on the blot. Because the affinity of the protein for the labeled and unlabeled probe should be equal, competition should occur only if there is a shortage of protein available for binding. Loh's binding reactions that produced competition contained as much as 1000 times more ArsR protein than total probe. Given this ratio one might expect that all of the *acxA* probe, labeled and unlabeled, would have been shifted and no visible competition for ArsR-DBD would have occurred. A possible explanation is that although there was a 1000-fold excess of total protein in the binding reactions, only a small percentage of this protein was ArsR-DBD in a state that was capable of binding its *acxA* target sequence. Purity of the ArsR solutions used in EMSAs is not discussed in Loh's study,

but the ArsR content of the protein extracts produced for this thesis work using a similar protocol was visually estimated to be >75% based on SDS-PAGE results. Even then, Loh's binding reactions would still have contained at least a 750-fold excess of ArsR. In this case, competition only could have occurred if the majority of this ArsR was in a state or conformation that was unable to bind to the probe.

The Effect of Potential Active and Inactive States of Response Regulators on Competition Assays in EMSAs

The hypothesis that TCST response regulators, whether phosphorylated or unphosphorylated, exist in an equilibrium between active and inactive states is discussed in Bourret's review of two component signal transduction (Figure 4) (8). According to this theory, the active state of a response regulator is able to bind DNA and regulate gene expression and the inactive state binds its target sequence with very low affinity if at all. Bourret contends that phosphorylation shifts the equilibrium toward activation, but it is possible that only a very small fraction of the response regulator is active at a time whether it is phosphorylated or not. Assuming this is true of ArsR, it could be that competition occurred in Figure 3 and in Loh's ArsR-DBD EMSAs because despite the large excess of total protein over total probe, there was actually an excess of total probe over *active* ArsR. (This is not necessarily representative of the activity of ArsR *in vivo*; it could be an artificial effect of the experimental phosphorylation or binding conditions.)

Potential Multiple Binding Sites for ArsR in the *acxA* Upstream Regulatory Region

Reactions 2-5 of the EMSA in Figure 3 also produced a minor band that was less mobile than the major ArsR/*acxA*-All complex. This may represent a supershift that occurs when ArsR bridges two probe molecules by binding them simultaneously, and if so would be

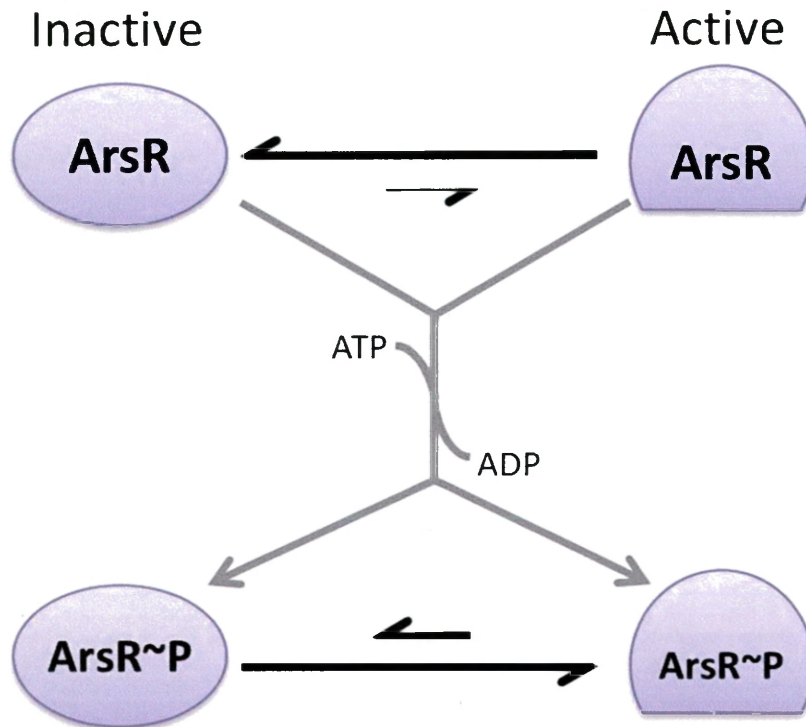


Figure 4. Proposed model for activity of ArsR with respect to *acx4* regulation. ArsR may exist in an equilibrium between inactive and active states. The active state is able to bind DNA, and the inactive state cannot. Phosphorylation of ArsR increases its ability to repress *acx4* transcription by shifting the equilibrium toward the active state.

an artifact of the EMSA. (The term “supershift” refers to a larger than normal shift observed in an EMSA, and its specific definition varies with the parameters of the assay. For example, one recombinant protein tagged with a 6xHis epitope binds to one DNA probe and generates a shift in a hypothetical EMSA. A supershift could be obtained by adding an α -His antibody to the binding reaction, which would increase the molecular weight and size of the DNA/protein complex.) It is also possible that there are multiple binding sites for ArsR in the *acx4* upstream regulatory region, and that this less-mobile band represents an ArsR/*acx4*-All complex with a higher molecular weight due to the involvement of additional ArsR molecules bound to distinct sites on the DNA probe. The results in Figure 3 do not indicate how many ArsR molecules are involved in each of the two potential complexes. Repetition of

this assay using a gel with longer lanes may allow detection of additional ArsR/*acxA-All* complexes, which could aid in determining the number of ArsR binding sites in the *acxA* upstream regulatory region.

ArsR EMSAs performed with shorter *acxA* regulatory region probes, *acxA-Up* and *acxA-Down* (Table 4), provide more direct evidence of multiple binding sites for ArsR. ArsR and ArsR~P EMSAs performed with probes containing only the portion of the putative *acxA* regulatory sequence upstream of the -35 site (*acxA-Up*) were positive for specific binding (Figure 5). No full competition was observed in reactions with 5 μ g protein and up to a 290-fold excess of specific competitor, but an intermediate band that migrated between the free probe and the fully-shifted probe/protein complex was observed in specific competition

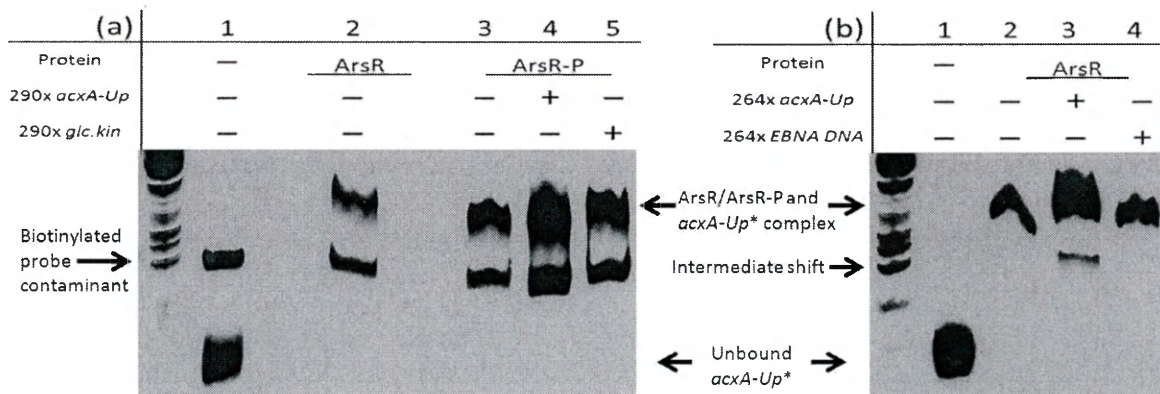


Figure 5. ArsR EMSAs with *acxA-Up* probes. (a) ArsR and *acxA-Up* EMSA/ArsR~P and *acxA-Up* EMSA with competition test. Leftmost lane: 2-log biotinylated DNA ladder (New England Biolabs). Reactions 1-5 contain 1 ng *acxA-Up** and reactions 2-5 contain 5 μ g protein. The *acxA-Up** probe stock used in this EMSA contained a larger contaminant, a biotinylated nonspecific PCR product that was not resolved separately from the probe in agarose gel purification and which may have interfered with the competition assay. Acrylamide gel purification eliminated this contaminant from future EMSAs, including Figure 3b. This image represents results obtained in two replicate assays. Similar results were obtained when the same EMSA was performed using *acxA-Up* probe amplified from pCR-J99-*acxA* (Table 2). **(b) ArsR and *acxA-Up* EMSA with competition test.** Leftmost lane: 2-log biotinylated DNA ladder (New England Biolabs). Reactions 1-4 contain 0.7 ng *acxA-Up** and reactions 2-4 contain 5 μ g ArsR. The band labeled “intermediate shift” is the result of competition, not the contaminant.

reactions. These results point to two ArsR binding sites on the *acxA-Up* probe, one with higher affinity and one with lower affinity for ArsR and ArsR~P. I hypothesize that in reactions without the specific competitor, there is enough ArsR to bind both sites on all of the *acxA-Up* probes, producing only the full shift. In the specific competition reactions (Figure 5b, lane 3), there is a limited amount of unbound ArsR available when the labeled probe is added. ArsR binds the higher-affinity site on every *acxA-Up* probe first, then begins to bind the low-affinity sites. The *acxA-Up* probes with ArsR only bound to the higher-affinity site account for the intermediate shift.

ArsR and ArsR~P EMSAs performed with probes containing the *acxA* regulatory sequence downstream of the -35 site (*acxA-Down*) were also positive for specific binding (Figure 6). In competition reactions performed with 10 μ g ArsR~P and 50x unlabeled *acxA-Down*, both full and intermediate shifts were observed. In competition reactions performed with 5 μ g ArsR, a 30-fold excess of specific competitor eliminated the full shift and produced

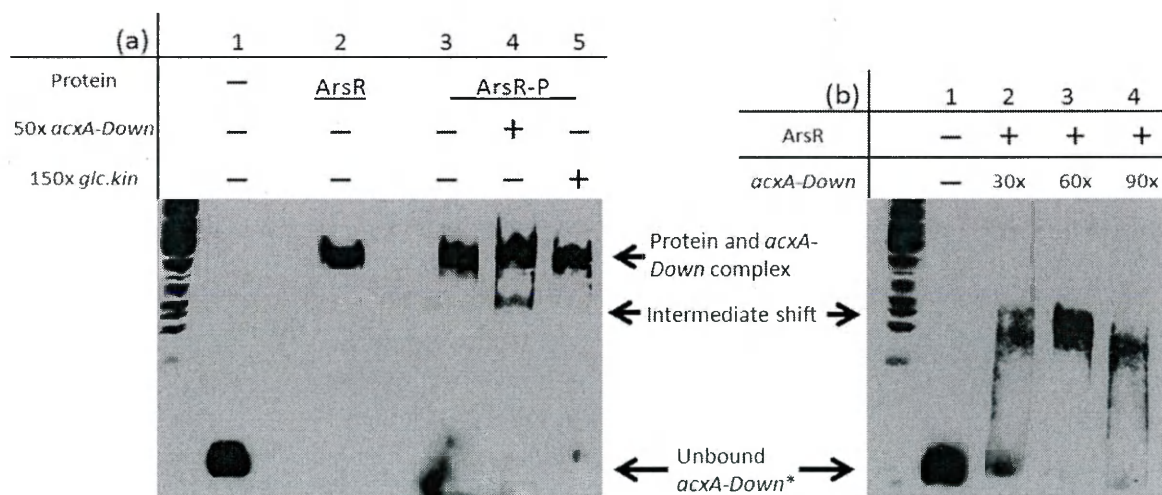


Figure 6. ArsR EMSAs with *acxA-Down* probes. (a) ArsR and *acxA-Down* EMSA/ArsR~P and *acxA-Down* EMSA with competition test. Reactions 1-5 contain 1 ng *acxA-Down and reactions 2-5 contain 10 μ g protein. Leftmost lane: 2-log biotinylated DNA ladder (New England Biolabs). (b) ArsR and *acxA-Down* EMSA with increasing amounts of specific competitor. Reactions 1-4 contain 1 ng *acxA-Down** and reactions 2-4 contain 5 μ g ArsR. Leftmost lane: 2-log biotinylated DNA ladder.**

an intermediate shift and free probe. In conclusion, ArsR~P appears to bind *acxA-Down* with slightly greater affinity than ArsR, and the DNA sequence within the probe *acxA-Up* may contain higher-affinity binding site(s) for ArsR and ArsR~P than *acxA-Down*. I hypothesize that the intermediate shift seen in the ArsR EMSAs indicates that *acxA-Down* also contains at least two ArsR binding sites, one with greater affinity for ArsR than the other. Thus the combined results of the EMSAs shown in Figures 3 and 4 support a model of regulation of *acxA* expression involving four binding sites for ArsR in the *acxA* upstream regulatory region. In Loh and Cover's study, intermediate shifts were observed in ArsR EMSAs performed with probes amplified from the promoter regions of the target genes *rocF* and *arsR*, suggesting that multiple ArsR binding sites may be part of a consistent mechanism for this response regulator (37).

A Model for Repression of *acxA* Transcription by ArsR

Figure 7 is a model of ArsR activity based on the results of this study and other currently available data. I hypothesize that ArsR, a repressor of *acxA* transcription according to qRT-PCR and microarray data from two independent studies (37, 6), binds to the *acxA* promoter at two sites upstream and two sites downstream of the -35 position. ArsR binds to the upstream sites with higher affinity than the downstream sites. In this model, transcription of *acxA* is repressed when ArsR binds to all four sites, but otherwise *acxA* transcription may proceed. (Depending on the positions of the two binding sites downstream of the -35 site, it is also possible that *acxA* transcription may also be repressed when only three ArsR molecules are bound.) As unphosphorylated ArsR is mostly inactive, it would be unlikely to form a stable *acxA* repression complex but may bind transiently to any site. This could lead to "leaky" repression of *acxA* transcription by unphosphorylated ArsR, which may have an advantage over no repression at all in terms of resource conservation. When ArsR is

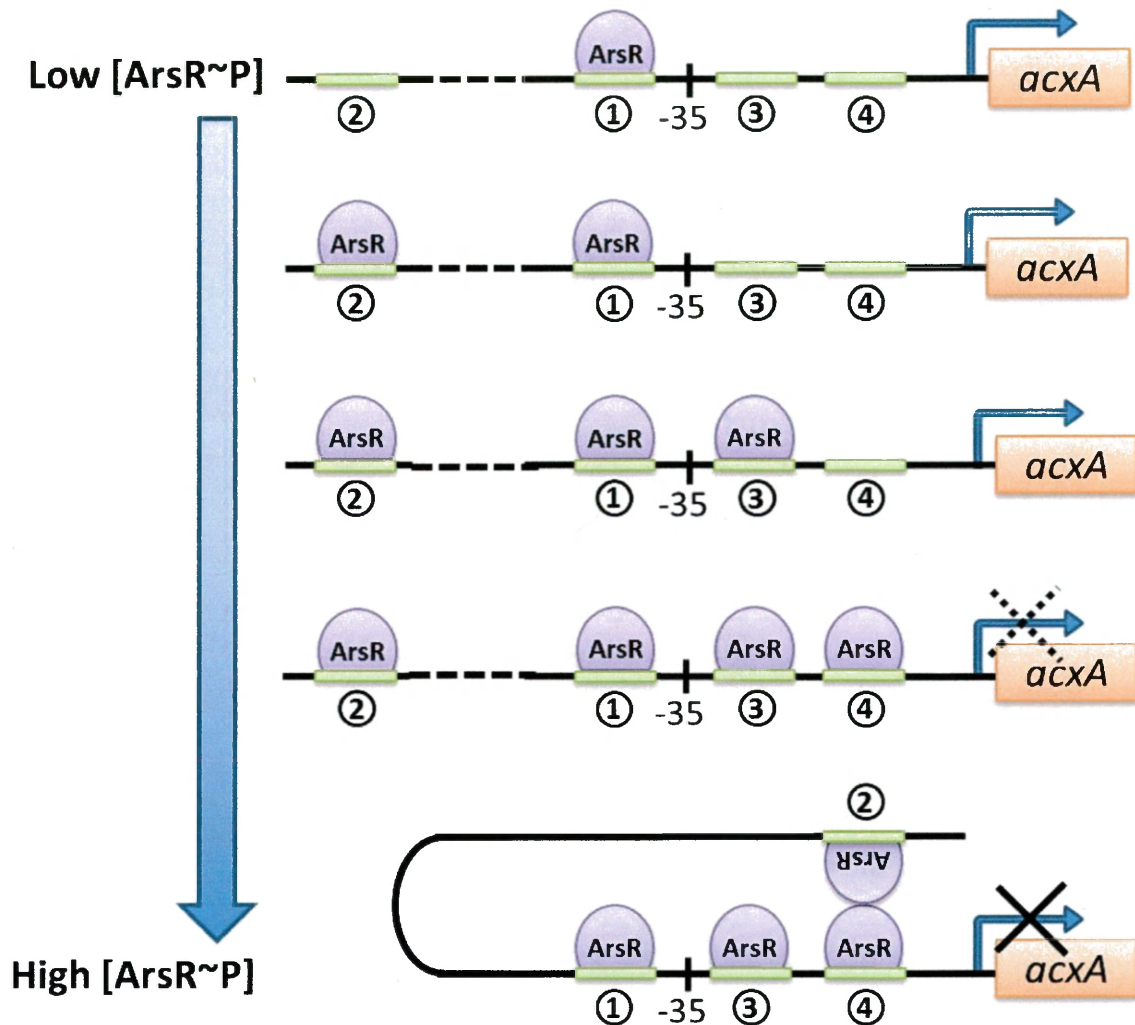


Figure 7. Proposed model for the function of multiple binding sites in transcriptional regulation of *acxA* by ArsR. ArsR binding sites are labeled 1-4, from highest to lowest affinity. Sites 1 and 2 are both upstream of the -35 site, and sites 3 and 4 are both downstream of the -35 site. The locations of these sites are otherwise unknown, and the positions of sites 3 and 4 may be reversed. In this model, due to existing primarily in the inactive state (see Figure 4), unphosphorylated ArsR is most likely to be unbound or bound only to the high-affinity upstream *acxA* regulatory sites, which allows for *acxA* transcription. Phosphorylation of ArsR causes the majority of this response regulator to be in the active state, which is more likely to bind all four sites simultaneously and repress *acxA*. Protein-protein interactions between the bound ArsR molecules could stabilize the *acxA* repression complex.

phosphorylated in response to a signal from ArsS, the equilibrium would shift so that ArsR~P was mostly in the active state, and would therefore be more likely to bind at all four of its sites in the *acxA* regulatory region. If the ArsR~P molecule bound to the lowest-affinity site interacts with another one of the ArsR~P molecules in the complex while they are both bound to the DNA, this could serve to stabilize the repression of *acxA*. The possibility of protein-protein interaction between the bound ArsR~P molecules was included in this model due to the structural similarity of ArsR to OmpR (20).

The placement of the binding sites in Figure 7 is partially informed by the DNA sequence and EMSA results, and partially arbitrarily determined: The highest-affinity site (#1) is placed directly upstream of the -35 site because this is the location of a set of inverted repeats. Though a consensus binding site for ArsR has not been identified, the helix-turn-helix motif in the ArsR DBD indicates that ArsR binding sites are likely to contain repeats. Site #2 is therefore placed further upstream, though its position could be anywhere between 59 bp and 244 bp before the start of *acxA* transcription. Though two ArsR binding sites are likely to exist between the -35 site of the *acxA* promoter and the end of the *acxA-Down* probe (140 bp downstream of the transcription start site), the position of these sites relative to each other and the promoter is unknown.

The requirement for four ArsR molecules to bind the *acxA* regulatory region simultaneously to repress transcription would dampen the signal transduced by ArsRS. This could keep *acxA* repression by ArsR~P from occurring in response to transient acid exposure, which combined with leaky repression by ArsR would allow *acxABC* to respond to low pH in a less sensitive manner than other members of the ArsRS regulon. This could represent two slightly different regulatory mechanisms carried out by the same TCST system, which if

correct could lead to greater understanding of how *H. pylori* compensates for its relatively small number of transcriptional regulatory systems.

CrdR Mobility Shift Assay

While the results of the EMSA shown in Figure 8 are somewhat less than clear and the current CrdR EMSA protocol developed in this study requires further optimization, some information may be gleaned from this experiment. First, while excess glycerol in the binding reactions may have resulted in smearing, it is not known to retard the motion of DNA through the gel (45). Therefore, the position of the labeled probe in lanes 2-5 is almost certainly due to interaction with purified *H. pylori* recombinant CrdR. It also appears that unphosphorylated CrdR produces a larger shift than phosphorylated CrdR. This could indicate that unphosphorylated CrdR has a greater affinity for the probe, or that the probe contains multiple CrdR binding sites. If there are multiple binding sites for CrdR in the *acxA* regulatory region, it is possible that CrdR can bind to more of these sites than CrdR~P, and that the CrdR/probe complexes therefore have a higher molecular weight than the CrdR~P/probe complexes. It is also possible that CrdR and CrdR~P each bind the probe at the same number of sites, but form protein/DNA complexes with different shapes resulting in different mobilities.

Previous results have indicated that expression of *acxA* increases in *crdR*- and *crdS*-strains of *H. pylori*, suggesting that CrdR~P is a repressor of *acxA* transcription (43). Because CrdS responds to environmental stress (either by sensing excess Cu^{2+} or reactive oxygen species), the repression of acetone carboxylase expression by CrdR may follow a pattern similar to the acid stress response carried out by ArsR. Expression of *acxABC* may be repressed when CrdS senses oxidative stress so that more resources can be devoted to the oxidative stress response, and multiple CrdR binding sites in the *acxA* upstream regulatory

region could dampen the sensitivity of this response as proposed above for ArsR. It is also possible that CrdR represses *acxA* expression in response to another unidentified environmental signal. Additional EMSAs with an optimized binding protocol and probes designed to elucidate the CrdR binding site(s), at least one of which is expected to be on or downstream of the promoter due to CrdR being a putative repressor of *acxA*, will be necessary to provide meaningful insight into this function of CrdR.

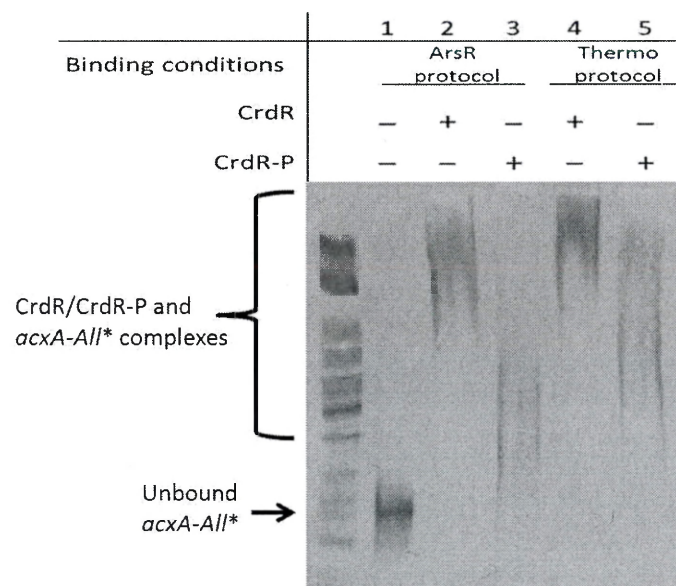


Figure 8. CrdR and *acxA-All* EMSA optimization. This assay was performed to compare several EMSA binding protocols following an unsuccessful EMSA that was performed with 2 μ g CrdR/CrdR-P per reaction and the ArsR protocol. Leftmost lane: 2-log biotinylated DNA ladder (New England Biolabs). Reactions 1-5 contain 1 ng *acxA-All**, and reactions 2-5 contain 6 μ g protein. The CrdR-P used in this assay was phosphorylated according to the ArsR protocol. Two reactions performed concurrently using a published CrdR phosphorylation and EMSA protocol produced no results (data not shown) (27). Smearing is likely due to excess glycerol in the binding reactions.

FlgR Mobility Shift Assay

In an EMSA with *acxA-All* probe, results for both FlgR and FlgR~P were positive for binding (Figure 9). However, binding appeared to be weaker than that of ArsR and may be

nonspecific. Complete competition occurred in FlgR~P EMSAs with a 300-fold excess of specific competitor, and near-complete competition occurred with a 300-fold excess of nonspecific competitor that contained no regulatory sequence (Figure 9b). A third set of competition assays for both FlgR and FlgR~P was attempted with higher protein concentrations and smaller amounts of unlabeled probe, but results were unreadable due to smearing that most likely resulted from an excess of glycerol as described above for CrdR. FlgR stocks were exhausted in this EMSA, but this assay should be repeated with an appropriate glycerol concentration when more FlgR can be purified.

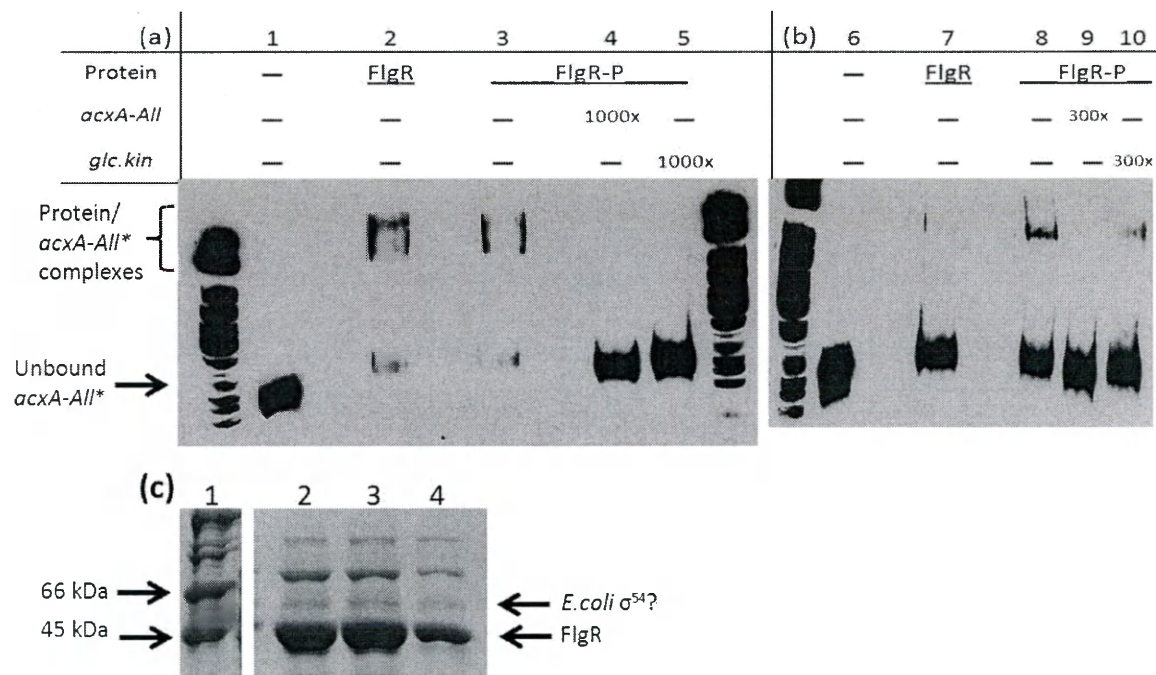


Figure 9. FlgR EMSAs with *acxA-All* probes. (a) FlgR and *acxA-All* EMSA/FlgR~P and *acxA-All* EMSA with competition test. First and last lanes: 2-log biotinylated DNA ladder (New England Biolabs). Reactions 1-5 contain 1 ng *acxA-All and reactions 2-5 contain 10 μ g protein. This image represents the results of two replicate assays. Two apparent protein/*acxA-All** complexes are present, with the less mobile complex dominant in figure 9a and the more mobile complex dominant in figure 9b. (b) FlgR and *acxA-All* EMSA/FlgR~P and *acxA-All* EMSA with competition test. Leftmost lane: 2-log biotinylated DNA ladder. Reactions 6-10 contain 1 ng *acxA-All** and reactions 7-10 contain 5 μ g protein. (c) SDS-PAGE of FlgR purification. Lanes: 1) Bio-Rad High Range Standards, 2) Elution 2, 3) Elution 3, 4) Elution 4. The pictured elution fractions were pooled and used in EMSAs. It is possible that FlgR was unintentionally co-purified with *E. coli* σ^{54} , as a band of the correct molecular weight is present.**

It is possible that these results indicate that FlgR binds to the upstream regulatory region of *acxA* independently, but more weakly than ArsR. However, according to the NCBI Conserved Domains Database, *Helicobacter pylori* FlgR lacks a known DNA-binding or response regulator effector domain. A study of FlgR and its flagellar regulon has identified this response regulator as an enhancer-independent σ^{54} -binding transcriptional activator (30). In EMSAs, FlgR did not bind directly to probes generated from its known flagellar regulatory targets. However, FlgR was able to activate transcription of these target genes by interacting with both *H. pylori* and *E. coli* σ^{54} -RNA polymerase. A possible explanation for the results obtained in Figure 9 is that *E. coli* σ^{54} associated with FlgR in the expression cultures and was unintentionally co-purified with FlgR. SDS-PAGE performed to evaluate the purity of the FlgR elution fractions shows a band that ran to the midpoint of the 45 kDa and 66 kDa bands of the ladder, which may be the *E. coli* σ^{54} subunit (Figure 9c). It is possible that the shift seen in figure 9a is actually a complex of the *acxA-All* probe and *E. coli* σ^{54} that may or may not include FlgR. The *acxA* promoter region does not contain a good match to the consensus sequence for *E. coli* σ^{54} binding sites, so if interaction of this sigma factor with *acxA-All* is responsible for the shift then the weak specificity that was observed is not surprising. Furthermore, if this explanation is true, the results in Figure 9 are an artifact of protein purification and do not yield any information about *in vivo* regulation of *acxA* by FlgR in *H. pylori*. Performing additional EMSAs with *acxA* probes and purified *E. coli* σ^{54} could reveal whether the shift is an artifact due to interference from this sigma factor. If results are positive, FlgR could be expressed in *rpoN*- M15 *E. coli* for use in future EMSAs to investigate the possibility that FlgR interacts directly with *acxA* despite its lack of an obvious DNA-binding motif.

Helicobacter pylori FlgR activates transcription of its flagellar regulon through interaction with σ^{54} and in response to low environmental pH. However, *acxA* does not have a σ^{54} promoter, and its transcription is down-regulated in *flgS*- *H. pylori* mutants in a pH-independent manner (31). This suggests that FlgR responds, via FlgS, to at least two types of signals which may lead to two distinct active states of FlgR with separate regulons. Because *acxA* has a housekeeping σ^{80} promoter and because FlgR is already known to interact with one sigma factor, it would make sense to investigate potential interaction of FlgR and *H. pylori* σ^{80} through co-immunoprecipitation or by performing an EMSA with *acxA* probes and both FlgR and *H. pylori* σ^{80} to look for a supershift. It is also possible that FlgR activates *acxA* expression by interacting directly with a currently unidentified transcription factor that binds the *acxA* promoter. If so, EMSAs including the *acxA* probe and FlgR along with this transcription factor would produce a supershift. A third possibility is that the activation of *acxA* by FlgR is indirect, and is mediated by one or more protein-protein and/or protein-DNA interactions.

HP1021 Mobility Shift Assay

The results in Figure 10 indicate that HP1021 binds the promoter region of *acxA* whether or not it has been phosphorylated. (Although there is no evidence that HP1021 is activated by phosphorylation, HP1021 treated with phosphorylation buffer was used in the EMSA reactions in lanes 6-8 for control purposes.) This assay was performed in order to confirm previous results obtained with HP1021 and *acxA* probes including the entire promoter region from strain 26695 (35). Pflock's EMSA produced four shifted bands of various sizes, the middle two of which are visible in lanes 2-7 of Figure 10. (The smallest complex was very faint in Pflock's assay and if present here would not be visible above the

background. The largest complex, if it formed during the assay in Figure 10, did not run out of the wells.) It is possible that the high isoelectric point of HP1021 (pI = 8.09) as predicted from its sequence caused low overall mobility in this assay, and that the complexes would be better visualized if the pH of the gel and buffer was raised for the native PAGE portion of the EMSA. No specific competition was observed, so future experiments should include more tests for binding specificity of HP1021 to the *acxA* regulatory region. EMSAs should also be performed with the *acxA-Up* and *acxA-Down* probes to detect potential multiple HP1021 binding sites.

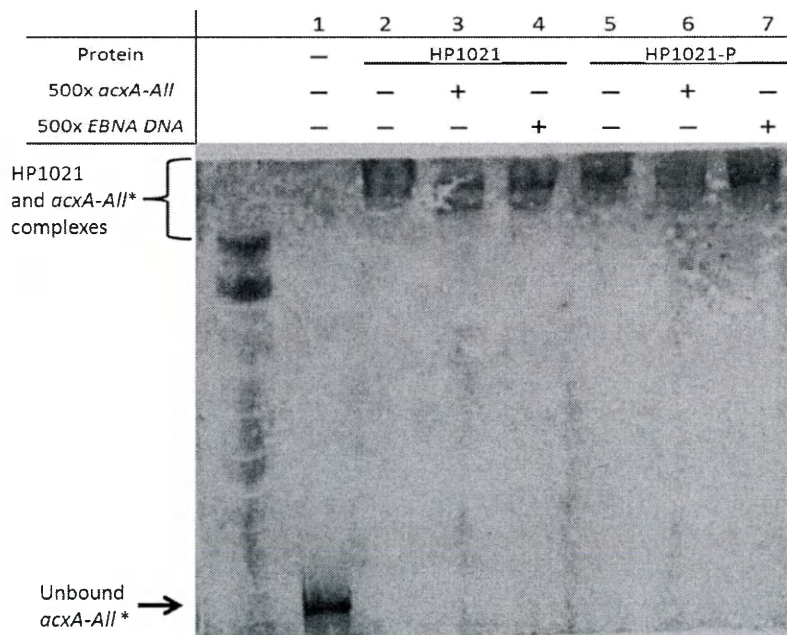


Figure 10. HP1021 and *acxA-All* EMSA with competition test. HP1021~P is HP1021 that has been treated with the ArsR phosphorylation protocol for control purposes. There is no evidence that HP1021 can be phosphorylated. All reactions contain 1 ng *acxA-All** and reactions 2-7 contain 10 µg protein. Leftmost lane: 2-log biotinylated DNA ladder (New England Biolabs). This image represents the results of two replicate assays.

Concluding Remarks

These results, combined with previous studies of ArsR and HP1021, support direct regulation of *acxABC* transcription by binding of ArsR, CrdR, and HP1021 to the *acxA* upstream regulatory region (37, 35). Furthermore, EMSA results for ArsR suggest that the degree of repression of *acxA* transcription by this response regulator may be modulated by binding of ArsR to four distinct sites in the *acxA* upstream regulatory region. EMSA results for CrdR suggest that this response regulator interacts directly with the *acxA* upstream regulatory region in at least two different conformations depending on the phosphorylation state of CrdR. These results demonstrate the importance of the *acxABC* operon as a model for multilayered gene regulation by a variety of mechanisms at a single locus.

Future Directions

Optimization of the Response Regulator Purification and EMSA Protocols

Obtaining Clearer and More Consistent EMSA Results

Of the three response regulators that bind directly to the *acxA* upstream regulatory region, the EMSA protocol for CrdR requires the most optimization. Despite being the only published EMSA protocol for CrdR, Waidner's combined phosphorylation and binding protocol produced no results in our lab. It is possible that this is due to a lack of a DNA carrier, which was not mentioned in Waidner's methods but may have been omitted in error (27). For optimization purposes, one could re-try this protocol and add poly dI-dC to the same concentration specified by the ArsR protocol. First, however, more CrdR EMSAs with the ArsR and Thermo base protocols should be attempted with an appropriate amount of glycerol in the reaction. In the successful ArsR EMSAs shown in Figures 3, 5, and 6, the concentration of glycerol in the binding reactions after addition of the loading buffer was 9-12% by volume. A study of the effect of glycerol on acrylamide gel electrophoresis indicates that the clearest results may be obtained when the glycerol concentration in a DNA sample to be electrophoresed is twice the concentration of acrylamide in the gel, which would set the ideal glycerol concentration for the EMSA samples in this thesis work at 12% (45). However, the samples in lanes 2-5 of Figure 8 were at least 20% glycerol. The excess glycerol stems from the relatively low CrdR concentration has been obtained in every purification performed so far. Since all protein stocks have at least 10% glycerol added for preservation, the volume of a low-concentration protein stock that is added to a binding reaction will be larger and will therefore contain more glycerol than that of a more concentrated protein stock. Smearing of

the CrdR binding reactions could be minimized by omitting glycerol from the binding buffer and by obtaining more concentrated CrdR.

The ArsR EMSAs performed in this thesis work produced consistent shifts, but inconsistent results in tests for specific binding. In a more recent EMSA performed in our lab, increasing the amount of labeled *acxA-All* probe to 14 fmol per reaction allowed for visualization of minor bands produced by competition with unlabeled probe (46). It may be that the use of more labeled probe in each EMSA reaction is all that is necessary to visualize all of the ArsR/probe complexes and free probe bands that are present on the blot. It is also possible that the difficulty of demonstrating binding specificity of ArsR to *acxA-All* is atypical, and that specific binding of ArsR to probes used in future experiments may be demonstrated as easily as it was for the *acxA-Up* and *acxA-Down* probes. If binding specificity of ArsR cannot be demonstrated reliably in future *acxA* EMSAs even after the amount of labeled probe is increased, then optimization of the binding buffer and/or reaction conditions may be required.

HP1021 interacts with the *acxA-all* probe to produce an extraordinarily large shift (Figure 10). In order to perform EMSAs with HP1021 to locate the binding site(s) of this response regulator in the *acxA* upstream regulatory region, it will first be necessary to optimize the HP1021 EMSA protocol so that all four potential HP1021/*acxA-All* complexes, as seen in Pflock's study, may be visualized. This could be accomplished by using a larger gel apparatus, but this solution requires development of a new blot protocol (the blotting tank used for the experiments described in this thesis only accommodates mini-gels, which are 8 cm wide and 6 cm high excluding the wells) and increasing the size of the blots would add considerable expense due to increased consumption of chemiluminescent EMSA reagents (Thermo). A potential work-around involves electrophoresing the HP1021 binding reactions

in a large gel electrophoresis cell until the distance between the largest known HP1021/probe complex and the free probe is slightly less than 8 cm. A 6 cm wide and 8 cm high section of the gel containing the electrophoresed EMSA reactions is excised, rotated 90°, and placed in the tank-blotting apparatus. The EMSA would be completed as described in the methods section. This method would require some optimization, but effectively adds 2 cm vertical distance to each lane, which may be enough additional room for all of the HP1021/probe complexes to appear on the blot.

An alternate approach to optimization of the HP1021 EMSAs is to increase the mobility of the HP1021/*acxA-All* complexes. The isoelectric point of HP1021 as predicted from its amino acid sequence is 8.09, and the pH of the running buffer and the gel used for electrophoresis of the HP1021 binding reactions was 8.3. The HP1021 EMSA protocol was developed with the assumption that the probe, being negatively charged and more than seven times the molecular weight of HP1021, would “carry” bound HP1021 through the gel. However, the results of the EMSA shown in Figure 10 indicate that the effect of the IEP of HP1021 on the mobility of these complexes may be greater than expected. In a Blue Native-PAGE assay performed with a gel and running buffer at pH = 9.0, HP1021 migrated approximately 3 cm in 3.5 hours (data not shown). Performing the electrophoresis portion of the HP1021 EMSA at $\text{pH} \leq 9$ may therefore increase the mobility of the HP1021/probe complexes so that all of them appear on an 8 x 6 cm mini-blot.

Increasing Response Regulator Purification Yields

The current CrdR expression protocol developed in this study produces low concentrations of soluble CrdR, limiting downstream applications and leading to unreliable and unclear EMSA results. Though it is a cytoplasmic protein, *H. pylori* CrdR is mostly

insoluble when extracted after being overexpressed in *E. coli*, most likely due to the formation of inclusion bodies. Soluble CrdR can only be purified from *E. coli* cultures with expression levels that are too low to yield an adequate CrdR concentration after purification. More concentrated CrdR stocks may be obtained by cloning just the CrdR DNA-binding domain (DBD), or by creating a CrdR-MBP (maltose-binding protein) fusion. Steps have already been taken in our lab to clone the CrdR-DBD, though it has not yet been purified. In previous studies in which protein solubility was a concern, individual domains have been less likely to form inclusion bodies than full-length proteins, so it is possible that CrdR-DBD will remain soluble when expressed in larger amounts than full-length CrdR (8).

Though EMSAs performed with CrdR-DBD could yield limited information about the locations of CrdR binding sites, full-length CrdR is necessary to obtain the most useful data about binding mechanisms and networking between *H. pylori*'s TCST systems. Since phosphorylation occurs on the receiver domain of CrdR, CrdR-DBD could not be used for further study of the effect of phosphorylation status on the binding of CrdR to the upstream regulatory region of *acxA* (Figure 8). Also, it is possible that direct interactions occur between CrdR and other regulatory proteins, and if so it is likely that these interactions involve the CrdR receiver domain. Fusion with *E. coli* maltose-binding protein is known to promote solubility of recombinant proteins when they are expressed in *E. coli*, and the bond between the protein of interest and MBP can be cleaved after purification to avoid interference of MBP in downstream experiments (47). Creation of a CrdR-MBP fusion may allow purification of adequate concentrations of full-length CrdR for future experiments. If necessary, this technique could be applied towards obtaining more concentrated stocks of the other response regulators as well.

Investigation of FlgR Protein-Protein Interactions

As discussed in the Results, it is possible that recombinant *H. pylori* FlgR associated with an *E. coli* transcription factor during purification, and that nonspecific binding of this *E. coli* protein to the *acxA-All* probe caused the shift observed in the EMSA (Figure 9).

Because the ability to purify FlgR without contamination by other transcription factors is important for future experiments, it may be helpful to know which *E. coli* protein is being co-purified with FlgR. Recombinant FlgR could then be expressed in M15 *E. coli* that cannot express the contaminant assuming that it is not an essential gene. Since FlgR activates transcription by interacting with *H. pylori* σ^{54} RNA polymerase holoenzyme and is known to associate with *E. coli* σ^{54} *in vitro*, one possible solution is to create a *rpoN*- M15 *E. coli* mutant and express recombinant *H. pylori* FlgR in this strain (30). However, this will only work if FlgR associates with σ^{54} and no other *E. coli* transcription factors during purification.

Since a large number of genes in the FlgR regulon do not have σ^{54} promoters, it is possible that FlgR interacts with other *H.pylori* transcription factors as well. It seems likely that if the *E. coli* DNA-binding protein co-purified with FlgR was not *E. coli* σ^{54} , it was the *E. coli* homolog of another *H. pylori* protein with which FlgR interacts with *in vivo*. A yeast two-hybrid screen performed with an expression vector carrying *H. pylori* FlgR as the bait and a library of prey plasmids constructed from *H. pylori* total cDNA could reveal interactions between FlgR and other *H. pylori* proteins. However, this assay does not allow for FlgR phosphorylation, which may be critical to its protein-protein interactions. Since *H. pylori* is only known to express 12 transcription factors including FlgR and sigma factors, cloning and adding FLAG-tags to these transcription factors and performing co-immunoprecipitation with FlgR would be feasible. (The ability to express and purify every *H. pylori* transcription factor would also be very useful in investigation of regulatory networks.)

M15 *E. coli* mutants lacking orthologs of the *H. pylori* transcription factors(s) that associated with FlgR could then be used for FlgR expression. If FlgR associates with an essential protein, it may be possible to adjust the purification protocol to disrupt this interaction.

Identification of Binding Sites for ArsR, CrdR, and HP1021

Based the EMSA results shown in figures 3, 4, and 6, I hypothesize that there are at least four ArsR binding sites in the upstream regulatory region of *acxABC*; two upstream and two downstream of the -35 site of the promoter. The CrdR EMSA results obtained in this study indicate that CrdR interacts with at least one binding site in the *acxA* regulatory region (Figure 8). I also hypothesize that there are multiple HP1021 binding sites within this regulatory sequence based on the four HP1021/probe complexes observed in Pflock's EMSA (35). Locating the binding sites for all of these response regulators is the logical next step toward characterizing the mechanisms by which ArsR, CrdR, and HP1021 regulate transcription of *acxABC*. This could be accomplished with DNA footprinting, but if it is necessary to avoid the use of radioactive reagents then binding sites could be located with additional chemiluminescent EMSAs. Waidner was able to locate the CrdR binding site in the upstream regulatory region of *crdA* by performing EMSAs with a series of DNA probes containing different parts of this regulatory sequence (27). A similar set of probes could be constructed for the *acxA* upstream regulatory region and used in EMSAs with ArsR, CrdR, and HP1021 (Figure 11).

Investigation of TCST Networking in Regulation of *acxABC*

The degree of overlap between the binding sites of ArsR, CrdR, and HP1021 in the *acxABC* upstream regulatory region will be fundamental for building models of competition

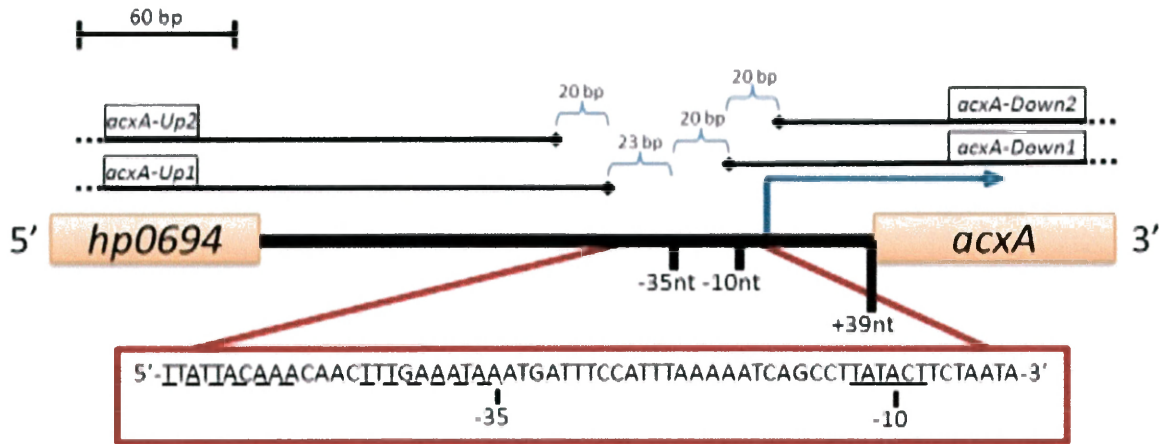


Figure 11. Proposed method for location of response regulator binding sites. The proposed *acxA-Up1* probe ends 23 bp upstream of the -35 site, and excludes the inverted repeats (marked with a dashed underline). If a response regulator binds specifically to *acxA-Up* and not *acxA-Up1*, then these inverted repeats are likely to be the binding site. New incrementally spaced *acxA* probes could be constructed and used in EMSAs, with the probes being moved upstream 20 bp at a time until all of the response regulator binding sites in the *acxABC* upstream regulatory region have been identified. A series of incrementally spaced *acxA-Down* probes could be created and used in EMSAs in the same manner. The *acxA-Down1* probe could be used to determine the significance of the -10 to -35 region of the *acxA* promoter in direct regulation via TCST, and the *acxA-Down2* probe could be used to test for binding of response regulators to the -10 site of the *acxA* promoter.

between these TCST response regulators for transcriptional control of *acxABC*. However, it is also possible that two or more different response regulators may act on the *acxA* upstream regulatory region simultaneously. A combination of modified EMSAs and co-immunoprecipitation assays could be used to detect protein-protein interactions that may occur between TCST response regulators while they are bound to the *acxA* upstream regulatory region. As noted above, these techniques will be particularly important in identifying whether or not FlgR interacts directly with another transcription factor to activate *acxA* transcription. Because FlgR is already known to interact with at least one *H. pylori* sigma factor and *acxA* has a σ^{80} promoter, I hypothesize that FlgR may be an activator of *H. pylori* σ^{80} as well as σ^{54} (30). Once recombinant *H. pylori* σ^{80} has been cloned and purified as described above, a series of EMSAs could be performed with FlgR and σ^{80} in the same

binding reaction. If a binding reaction containing both FlgR and σ^{80} with the *acxA-All* probe produces a larger shift than the same reaction without FlgR, this would suggest that FlgR interacts directly with σ^{80} to initiate *acxA* transcription. This test could also be used to detect interaction of FlgR with any of the other *H. pylori* transcription factors at the *acxA* regulatory region.

Similar techniques could be applied to look for direct interactions between any of the *H. pylori* response regulators. If separate binding sites for two different response regulators are found, then an EMSA could be performed with both response regulators and a DNA probe including both binding sites in a single reaction. When compared with results for binding reactions containing this probe and only one of the proteins, a supershift would indicate simultaneous binding of both response regulators to the probe. Co-immunoprecipitation could then be used to investigate whether or not these response regulators may interact directly with each other while bound to DNA upstream of *acxABC*. The possibility of direct physical interaction between members of different TCST systems is especially interesting in the case of the acid response. ArsRS, FlgS, and HP1021 are all known to respond to low environmental pH, and CrdS is required for survival of *H. pylori* in acidic conditions (25). The integration of the environmental acidity signal by the acid-responsive TCST systems is not yet fully understood. As the regulatory target of all three TCST response regulators and HP1021, *acxABC* may be an ideal model for study of the global transcriptional response of *H. pylori* to acid exposure.

Investigation of Environmental Influences on *acxABC* Expression

The expression level of *acxA* is altered when any one of *H. pylori*'s two-component signal transduction systems is deleted or made non-functional by mutation, and also has been shown to change with changes in environmental pH levels. However, acidity cannot be the

only environmental signal that affects *acxA* expression, because CrdRS and HP1021 do not respond to changes in pH. Additionally, the *acxABC* operon is a member of the pH-independent regulon of FlgS, and the signal(s) sensed by FlgS in addition to acidification of the cytoplasm are unknown (31).

The relative change in expression of *acxABC* in response to various chemical environmental signals can be measured *via* qRT-PCR. In this assay, *H. pylori* is grown overnight in liquid culture, exposed to an environmental signal suspected to alter expression of *acxA*, and then cells are harvested for RNA extraction and subsequent qRT-PCR with *acxA* primers. Potential environmental signals for *acxA* regulation include acidity, copper, oxidative stress, acetone, acetoacetate, and β -hydroxybutyrate: Previous results suggest that the repression of *acxABC* in acidic conditions may be gradual, so a range of pH levels should be included in this assay. Copper and oxidative stress are included because CrdS is known to sense increased environmental Cu^{2+} concentration and is a potential oxidative stress sensor, but the effect of these two environmental conditions on *acxABC* expression has not yet been measured (27). Hydrogen peroxide could be added to the *H. pylori* growth media to simulate the release of reactive oxygen species by the host immune system. Finally, though no sensor kinase is currently known to sense ketone bodies, it would be an advantage for *H. pylori* to express acetone carboxylase only when enough acetone is available to compensate for the energy used to construct this enzyme. Therefore, one of the signals sensed by TCST systems that regulate *acxABC* expression may be acetone or one of the other two ketone bodies that are produced with acetone; acetoacetate and β -hydroxybutyrate. Acetoacetate decomposes spontaneously to acetone above room temperature and therefore cannot be used in this experiment, but acetone and β -hydroxybutyrate could be added separately to *H. pylori* cultures to test their individual effects on *acxABC* expression.

If environmental signals that activate or repress *acxA* expression in addition to acidity are identified through qRT-PCR, then the next step is to identify which TCST systems regulate *acxA* in response to the newly discovered signals. This necessitates the creation of *crdS*-, *flgS*-, and *arsS*- *H. pylori* mutants for use in additional qRT-PCR assays. (A *hp1021*-mutant could be created as well, but due to the small-colony phenotype it may not be feasible to grow this mutant in liquid culture as is necessary for this assay.) If any one of these mutant strains has lost its ability to alter expression of *acxA* in response to one of the previously identified signals, then it is likely that the signal in question is detected by the sensor kinase that is deleted in this strain.

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