ACONITASE-MEDIATED POSTTRANSCRIPTIONAL REGULATION IN *HELICOBACTER PYLORI*

by

CRYSTAL MARRIE AUSTIN

(Under the Direction of Robert J. Maier)

ABSTRACT

The gastric pathogen *Helicobacter pylori* is remarkable in that it is able to colonize the acidic environment of the stomach, a niche not chronically colonized by any other bacteria. *H. pylori* is notorious for possessing few regulatory systems. It was once thought that the stomach was a relatively stable environment, and without having competition from other bacteria, it was not necessary for *H. pylori* to possess an intricate regulatory system. However, as more evidence becomes available, it appears that *H. pylori* must be metabolically dynamic, as its response to environmental change is multifaceted and mediated by more than one regulatory system. Posttranscriptional regulation in bacteria has increasingly become recognized as playing a major role in the response to environmental stimuli. Aconitase is a bifunctional protein that acts as a posttranscriptional regulator by controlling mRNA stability. In its apo-form, aconitase binds to sequences in either the 5' or 3' untranslated regions (UTRs) of mRNA transcripts. Binding of apo-aconitase to the 5' UTR inhibits ribosome-binding, thereby decreasing translation of the downstream gene. Transcript stability is enhanced when apo-aconitase binds to the 3' UTR of transcripts, where it prevents ribonuclease degradation. The role of aconitase as a posttranscriptional regulator in *H. pylori* has not yet been explored. Here, I propose a global role

for aconitase (AcnB) in modulating expression of proteins in *H. pylori*. Putative aconitase targets for regulation include those related to oxidative stress, urease and hydrogenase activities, motility, and numerous others.

INDEX WORDS: Aconitase, *Helicobacter pylori*, posttranscriptional regulation, peptidoglycan deacetylase, oxidative stress, urease, hydrogenase, motility

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DEDICATION

This dissertation is dedicated to my mom, Carla Phillips. She has never left my heart and would be so very proud of me. I love her and miss her terribly.

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INTRODUCTION

In 1982, Drs. Barry Marshall and Robin Warren became the first to isolate *Helicobacter pylori* from gastric biopsies (1). From a self-ingestion experiment, Dr. Marshall demonstrated that *H. pylori* infection results in gastritis and contributes to the development of peptic ulcers (2). In 1991, reports appeared that correlated *H. pylori* infection with gastric cancer (3, 4). By 1994, the World Health Organization classified *H. pylori* as a Class I human carcinogen. Infection with this bacterium is also associated with non-Hodgkin's lymphomas and gastric mucosa-associated lymphoid tissue (MALT) lymphoma (5, 6). Today, it is estimated that over half of the world's population is infected with *H. pylori* (7).

Helicobacter pylori Literature Review

General microbiology and culture. *Helicobacter pylori* is a spiral-shaped, Gram-negative, microaerophilic bacterium that colonizes the gastric mucosa of humans and some primates. It is part of the ε-Proteobacteria class, which contains bacteria ranging in areas from human and animal gastrointestinal tracts to deep sea hydrothermal vents (8). In the Maier laboratory, *H. pylori* is routinely cultured on Brucella agar medium supplemented with 10% sheep's blood for 36-48 hours under microaerobic conditions (2-4% O_2, 5 % CO_2, and N_2 balance) at 37°C. It is also grown in Brain Heart Infusion broth supplemented with 0.4% β-cyclodextrin. Bottles are incubated at 37°C, shaking and initially contain 5% CO_2, 10% H_2, 75% N_2, and 10% O_2. When cultured on solid medium, *H. pylori* cells appear rod-shaped and are 2-4 μm long and 0.5-0.8 μm wide (9, 10). After prolonged incubation (starvation) or other stress conditions, cells convert to a U-shape or coccoid form that ranges from 1-4 μm in diameter (10). The coccoid form is metabolically active, but cannot be cultured *in vitro* (11). The coccoid form has been shown to replicate within the mouse host, implying that it is a strategy to survive harsh environmental conditions and continue the infection cycle (12). The bacterium has one to seven sheathed flagella originating from one pole that allow for motility and aid in colonization (9, 13). The average genome size is 1.67 Mb and the average G+C content is 39 mol% (14). Cells carry one or two cryptic plasmids depending on the strain (15). *H. pylori* strains are genetically heterogeneous, and nucleotide diversity is accomplished by mutations, substitutions, and transcriptional and translational phase variation (16, 17). The bacterium's natural DNA transformation capacity has likely contributed to diversity as well. It is thought that strain diversity contributes to adaptation of the gastric niche (18).

Epidemiology. The prevalence of *H. pylori* varies considerably for individual countries. Among adults in developing countries, *H. pylori* prevalence is more than 80% whereas in developed countries, prevalence is less than 40% (19, 20). In developing countries, *H. pylori* infection rates are high during childhood and remain constant throughout adulthood whereas in developed countries, rates of infection begin low and slowly rise during adulthood. Transmission mechanisms of *H. pylori* are unknown, but it is hypothesized that direct human to human contact, via either oral-oral or fecal-oral routes, is the likely mechanism (21).

Colonization and pathogenesis. *H. pylori* strictly colonizes the gastric mucosa of humans and primates. The use of flagella combined with its spiral-shaped morphology allow the cell to burrow into the viscous mucus layer overlaying the epithelial cell surface, where the pH is more neutral (~pH 7) and cells are protected from the acidic pH of the lumen (~pH 1-2). Urease hydrolyzes urea into ammonia and carbamate so that the surrounding microenvironment of the cell is buffered as the bacterium travels through the lumen. Most bacterial cells remain extracellular to the host cells, propelling themselves freely in the mucosa, and yet about 2% of them adhere to the epithelial cell surface (22). Adherence is partly mediated by Lewis b and Lewis x blood group antigens and *H. pylori* BabA and SabA, respectively (23, 24). The *cag* pathogenicity island (PAI) is a group of genes that encodes a type IV secretion system (T4SS) and the CagA protein (25). The T4SS injects CagA into host epithelial cells where it subsequently induces a variety of effects, including proliferation, apoptosis, cytoskeletal changes, and cellular leakage (26). Addtionally, some *H. pylori* strains produce a vaculating cytotoxin protein, VacA, which is transferred to epithelial cells resulting in the formation of vacuoles, membranous pores, and apoptosis (27).

Disease. Although infection with *H. pylori* nearly always results in gastritis, only a small percentage of people show obvious symptoms or complications (27). Among people who are infected, there is an approximately 10-20% risk for developing ulcers and a 1-2% risk for developing cancer (28, 29). Gastritis (acute or chronic) is characterized by inflammation of the gastric mucosa and infiltration of neutrophils and mononuclear cells. If left untreated, chronic gastritis can result in peptic ulcer disease or lead to gastric cancer, including non-Hodgkin's lymphoma and mucosa-associated lymphoid tissue (MALT) lymphoma (21).

Diagnosis and treatment. The majority of *H. pylori* infections are detected by histology, culture, or by the urea breath test. The use of a single antibiotic fails to clear infection and it is speculated that this may be due to the acidic environment of the gastric mucosa negating antibiotic activity. A triple therapy that combines two antibiotics and either a proton pump inhibitor or bismuth is generally administered to clear infection, but in some people re-occurrence is common (21).

Cell wall. The *H. pylori* cell wall is hydrophilic and has a negative charge (30). The outer membrane contains lipopolysaccharides (LPS) consisting of an O antigen side chain, core oligosaccharide, and lipid A (31). The LPS mimics Lewis blood group antigens that play an important role in pathogenesis (32). *H. pylori* contains several outer membrane proteins, including porin proteins, HopABCDE; adhesion proteins, BabA and HopZ; iron-related outer membrane proteins (OMPs), such as FrpB and NapA; flagella proteins, for example, FlaB and FlgE; and the hemagglutinin protein, HpaA (33).

H. pylori peptidoglycan (PG) consists of alternating *N*-acetylglucosamine (GluNAc) and *N*-acetylmuramic acid (MurNAc) residues that are connected by β-1,4 bonds and cross-linked via short peptide bridges (33, 34). PG modification is a mechanism that *H. pylori* and other bacterial

pathogens utilize in order to evade host immune detection (35-37). One such modification by peptidoglycan deacetylase (PgdA) confers resistance to lysozyme, a host-secreted antimicrobial which hydrolyzes the β-1,4 glycosidic bond between GluNAc and MurNAc (38). Hydrolysis of this bond not only decreases integrity of the cell wall, but also releases PG fragments that are then recognized by host pattern receptor proteins (39). This results in a production of proinflammatory cytokines and chemokines and recruitment of immune cells that secrete additional antimicrobials. There are two main PG modifications that confer lysozyme resistance in bacteria: *O*-acetylation and *N*-deacetylation. *O*-acetylation of PG results in acetylation of MurNAc at the C-6 position (40). *O*-acetylation has been shown to be regulated transcriptionally by the two component system, CesSR, in *Lactobacillus lactis*, and similar two-component systems in other Gram-positive bacteria (41, 42). The action of *N*-deacetylase removes an acetyl group from the C-2 of GlcNAc (43). Our laboratory has shown that *N*-deacetylation by PgdA in *H. pylori* is increased upon exposure of cells to oxidative stress and contact with macrophages (44).

Respiration and metabolism. Although other *Helicobacter* species are non-saccharolytic, *H. pylori* is capable of oxidizing glucose (45). The bacterium has enzymes for the Entner-Doudoroff pathway as well as for the pentose phosphate pathway (46, 47). Both pathways produce NADPH, which is the preferred electron donor (for *H. pylori*) over NADH (48). *H. pylori* has a branched, incomplete citric acid cycle, and data suggests it is used primarily for biosynthesis of α-ketoglutarate and succinyl-CoA as well as redox balancing (49). Instead of the typical α-ketoglutarate dehydrogenase complex found in most other bacteria, it has an α-ketoglutarate: ferredoxin oxidoreductase enzyme that converts α-ketoglutarate to succinyl-CoA (50, 51). *H. pylori* lacks succinyl-CoA synthetase, which converts succinyl-CoA to

succinate and CoA (51). It also lacks succinate dehydrogenase, which converts succinate to fumarate; instead, it uses fumarate reductase (51, 52). Furthermore, *H. pylori* has a NAD-linked malate dehydrogenase that synthesizes malate from oxaloacetate as well as a malate:quinone reductase (which serves as an electron donor) (49, 53).

In the respiratory chain of *H. pylori*, reduced substrates are oxidized via a membrane-bound NDH-1 dehydrogenase (complex I) or a hydrogen uptake hydrogenase (48, 54). Electrons are passed to menaquinone, then to cytochrome bc_1, cytochrome *c*, and finally to the terminal oxidase cytochrome cbb_3 (55, 56). Additionally, fumarate reductase can transfer electrons from menaquinone to the terminal electron acceptor fumarate, although *H. pylori* has not been shown to grow anaerobically with fumarate (52). The proton motive force generated from the electron transport chain is used to drive ATP synthase (57).

Nitrogen metabolism. Amino acids and urea serve as the major nitrogen sources for *H. pylori* (58). The bacterium is auxotrophic for alanine (some strains), arginine, histidine, isoleucine, leucine, methionine, phenylalanine, serine (some strains), and valine (59, 60). *H. pylori* can also convert amino acids into primary metabolites via fermentation (61). Urea is hydrolyzed into ammonia and carbamate via the enzyme urease, which accounts for up to 10% of total cellular protein (62). Urease is a multimeric protein that consists of UreA and UreB subunits and requires 24 nickel atoms to be active (63). *H. pylori* relies upon the ammonia produced from urease to act as a buffering agent against the acidic pH of the gastric mucosa (64). It is an important virulence factor and several studies have shown it is necessary for persistence and host colonization (65-67).

Nickel metabolism. The nickel demand for *H. pylori* is very high; in addition to supplying nickel for urease, the membrane-bound hydrogen uptake hydrogenase (HydABCD)

has a [Ni-Fe] center. Hydrogenase has been shown to be important for host colonization and for energy conservation (54, 68). The concentration of nickel is low in human serum (2-11 nM) depending on diet; and therefore, acquiring Ni^{2+} ions is a priority for this bacterium (69, 70). Outer membrane Ni^{2+} transporters include FrpB4, which is powered by the ExbB/ExbD/TonB complex, and possibly the outer membrane transporter, FecA3 (71, 72). The ExbB/ExbD/TonB system is known to use the proton motive force to transport solutes across the cytoplasmic membrane (73). NixA is a high-affinity permease transporting Ni^{2+} within the cytoplasmic membrane (74). Once inside the cell, nickel is stored and/or transferred by histidine-rich proteins termed Hpn and Hpn-like, and by HspA, a GroES chaperone homolog (75-78). Mua, the modulator for urease activity, is a protein that also binds nickel and has been found to regulate urease activity in *H. pylori* (79). Also, CeuE has recently been shown to bind nickel in the presence of a nickelophore, similar to NikA in *E. coli* (80). Furthermore, the CznABC efflux system can transport nickel out of the cell (81). Thus far, it is known that nickel homeostasis relies on a sole Ni^{2+}-dependent transcriptional regulator, NikR; however, NikR-independent mechanisms of regulation have been implied (82, 83). NikR regulates transcription of *ureAB*, *nixA*, *exbB-exbD-tonB*, *frpB4*, *fecA3*, *nikR*, *hpn*, *hpn-like*, *hspA*, *hydA*, and the ferric uptake regulator, *fur* (72, 77, 84-90).

Iron metabolism. Iron is a cofactor for many enzymes involved in basic cellular metabolism. Studying iron metabolism in *H. pylori* is of interest since infection is associated with anemia (91). In the acidic environment of the stomach, soluble iron is mostly found in the ferrous (Fe^{2+}) state and can pass freely through the outer membrane, but requires transport across the cytoplasmic membrane (92, 93). *H. pylori* possesses the cytoplasmic membrane protein FeoB, which hydrolyzes ATP to transfer Fe^{2+} into the cell (94). Most ferric (Fe^{3+}) iron at the

mucosal surface is complexed in heme or chelated by lactoferrin and requires transport across both the outer and cytoplasmic membranes (95). *H. pylori* can use ferric citrate, heme compounds, and possibly lactoferrin as sources of iron (94, 96, 97). It possesses three outer membrane heme-binding proteins with sizes of 48, 50, and 72 kDa (98). The outer membrane proteins FrpB1 and FrpB2 have both been shown to bind heme, and FrpB1 can also bind hemoglobin (99, 100). Heme imported into the cell can be used directly as a prosthetic group, for example, for activity of the antioxidant protein catalase, or degraded and used as a source of iron (101). Energy generated from the ExbB/ExbD/TonB machinery is used to transfer Fe^{3+} into the periplasmic space. From there, the periplasmic iron-binding protein, CeuE, and the ABC transporter system, encoded by *fecDE*, transport Fe^{3+} across the cytoplasmic membrane and into the cell (94, 102). *H. pylori* is not known to possess siderophores and uses only a few siderophores produced by other organisms (96). Once inside the cell, Pfr, the non-heme, iron-containing ferritin and NapA, the putative bacterioferritin, bind iron atoms for storage (103, 104).

Fur, the ferric uptake transcriptional regulator protein, regulates iron uptake and storage in *H. pylori* (105). In its iron-loaded form, Fur binds to the promoter regions of *fecA1*, *fecA2*, and *frpB1*, thereby repressing transcription of those genes in response to high iron levels (106, 107). In its apo-form (low iron levels), it binds to *pfr* and to the iron-containing superoxide dismutase, *sodB* (108, 109). Fur also binds to the promoter regions of *fur*, *ceuE, and exbB2* (89, 110, 111). Additionally, Fur has been found to bind to various genes involved in stress, redox metabolism, flagellar synthesis, and acid tolerance. Some targets include *ureA* and *flaB*; *nifS*, a cysteine desulfurase involved in the synthesis of [Fe-S] clusters; *amiE*, an amidohydrolase that

participates in nitrogen metabolism; *oorDABC*, the α-ketoglutarate oxidoreductase; and numerous others (112-116).

Oxidative stress. Reactive oxygen species (ROS) are generated by polymorphonuclear leukocytes during the oxidative burst and are byproducts of the electron transport chain during aerobic respiration and other chemical processes (117). The host immune system defends itself against bacterial infections by producing ROS that damage macromolecules, such as DNA and proteins. Superoxide (O_2^-) and hydrogen peroxide (H_2O_2) are the major sources of ROS (118). Both can attack proteins containing Fe-S centers, thereby releasing iron into the cell. Ferrous iron (Fe^{2+}) can combine with H_2O_2 to produce ferric iron (Fe^{3+}) and the toxic hydroxyl radical, ($OH^{\cdot}$) (119). In order to persist, *H. pylori* utilizes enzymes that directly detoxify ROS and repair damaged macromolecules. The bacterium possesses the iron-containing superoxide dismutase, SodB, which breaks down O_2^- into H_2O_2 and O_2 (103, 120). Catalase (KatA) is an important protein involved in oxidative stress defense (121, 122). It requires heme to be active and detoxifies toxic H_2O_2 into H_2O and O_2 (123). *H. pylori* also has alkyl hydroperoxide reductase, AhpC, that reduces several different hydroperoxides, including hydrogen peroxide and *tert*-Butyl hydroperoxide (124).

Many ROS-detoxifiying enzymes need to first be reduced in order to be functional. *H. pylori* accomplishes this by using the thioredoxin system, which consists of NADPH, thioredoxin (Trx), and thioredoxin reductase (TrxR) (124-126). Both Pfr and the neutrophil-activating protein, NapA, protect *H. pylori* from oxidative damage by sequestering free iron, and the later perhaps by interacting with DNA (127-129). To repair damaged DNA, *H. pylori* employs a few different enzymes, including endonuclease III, which can excise lethal lesions; MutS, which binds to oxidatively-damaged DNA; and RuvC, an endonuclease that mediates homologous

recombination (130-132). Protein damage can be reversed by Msr, the methionine sulfoxide reductase, which reduces oxidized methionine residues (133). Some of Msr's repair targets are themselves oxidative stress combating enzymes.

It is not yet clear how *H. pylori* mediates the response to oxidative stress since it lacks homologs of other bacterial regulators that are activated by the presence of ROS, including SoxRS, OxyR, RpoS, LexA, and PerR (57). However, it does possess the carbon storage regulator, CsrA, which functions as a global posttranscriptional regulator to modulate expression of numerous genes, including those involved in combating oxidative stress (134).

Gene regulatory mechanisms. *H. pylori* has few regulatory proteins and networks as compared to other Gram-negative bacteria, for example, *Escherichia coli* (57, 135). There are 4 two-component regulatory systems that operate at the transcriptional level, including FlgRS, which regulates motility; CheA-CheY, which mediates chemotaxis; ArsRS, which modulates several genes, including those involved in acid adaptation; and CrdRS, which regulates copper resistance genes (136-139). In addition, there are three sigma factors present: RpoD (σ^{80}), RpoN (σ^{54}), and FliA (σ^{28}) (140-142). *H. pylori* lacks homologs of the stress-induced sigma factors, RpoS (σ^{38}) and RpoH (σ^{32}), that are found in other bacteria. There are a few other transcriptional regulators, including Fur, a global regulator for genes involved in iron and nickel metabolism, motility, stress, and various others; NikR, which regulates numerous genes, including *fur* and those involved in nickel metabolism; HspR, which regulates chaperone, heat shock, and stress genes; HrcA, that mediates heat shock and stress genes; FlhA, which regulates genes involved in motility, urease, adherence, and others; and two orphan response regulators (135, 136, 143-145). There are also more than 60 small RNAs identified in *H. pylori*, among which is the *cis*-encoded antisense 5' *ureB* which regulates expression of *ureAB* via transcription termination (146).

Aconitase: Posttranscriptional Regulator

Aconitases are dual-functioning proteins that convert citrate to isocitrate via *cis*-aconitate in the tricarboxylic acid (TCA) cycle and act as posttranscriptional regulators by binding mRNA transcripts to affect stability (147). Under iron-limiting or high oxygen conditions, the [4Fe-4S] center of aconitase becomes disassembled and the protein loses enzymatic activity. Aconitase then undergoes domain rearrangements and functions as a posttranscriptional regulator, which can bind to either the 5' or 3' untranslated regions (UTRs) of mRNA transcripts (147-149). If apo-aconitase binds to the 5' UTR, it blocks ribosome binding, thus inhibiting translation of the corresponding mRNA. If apo-aconitase binds to the 3' UTR, it prevents ribonuclease degradation, resulting in enhanced translation. In eukaryotes, there is an established consensus sequence known as the iron responsive element (IRE). Iron regulatory protein 1 (IRP1; or cytosolic aconitase, cAcn) has been shown to bind to the IRE. The IRE is a ~30 nucleotide stem-loop structure consisting of a six-membered loop sequence "CAGUGN" and an unpaired "C" in the 5' region of the stem (148). The structure of IRP1 complexed with ferritin H revealed that the defining features of the IRE-IRP1 interaction are the –AGU– triplet, the unpaired "N", and the "C" buldge of the IRE (149). Binding studies with alternative IRE ligands have shown varying binding affinities for iron regulatory proteins (150).

In bacteria, several aconitase-IRE regulatory systems have been studied; however, the consensus IRE sequence is not always present in target transcripts. The binding affinity of eukaryotic IRP1 to IRE is higher (K_D = 5-50 pM) than bacterial aconitases to their targets (K_D = 1-8 μM) (150). In *E. coli*, AcnA and AcnB bind to the 3'UTRs of the *acnA* and *acnB* transcripts, respectively, despite the lack of a consensus IRE (151). Also, *E. coli* AcnA and AcnB both bind to the *sodA* transcript to enhance and decrease stability, respectively (152). Furthermore, AcnB

in *Salmonella enterica* serovar Typhimurium LT2 binds to the protease, *ftsH*, to indirectly regulate the flagellum protein, FliC (153). Among the aforementioned transcript targets, there is no consensus IRE, but they all share the sequence "ACGCG" which may be involved in binding-recognition (151, 153). Additionally, *Bacillus subtilis* aconitase (CitB) binds to the transcriptional regulator *gerE*, which has a stem-loop structure that is similar to the consensus IRE (154). Lastly, *Mycobacterium tuberculosis* aconitase binds to the thioredoxin (*trxC*) transcript and to an iron-dependent transcriptional regulator, *ideR*, both of which contain the consensus IRE (155).

Scope of Study

H. pylori has relatively few regulatory systems as compared to other Gram-negative bacteria, for example *E. coli* (57). It has been hypothesized that gene regulation is either not as elaborate or operates differently than in other microorganisms (135). Since studies in other bacteria show that aconitase plays a significant a role in the regulation of important genes, we explored how aconitase may posttranscriptionally regulate expression of different genes in *H. pylori*, particularly in regards to genes involved in oxidative stress and metal-containing enzymes.

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CHAPTER 2

ACONITASE-MEDIATED POSTTRANSCRIPTIONAL REGULATION OF *HELICOBACTER PYLORI* PEPTIDOGLYCAN DEACETYLASE[1]

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Abstract

Some bacterial aconitases are bifunctional proteins that function in the citric acid cycle and act as posttranscriptional regulators in response to iron levels and oxidative stress. We explore the role of aconitase (AcnB) in *Helicobacter pylori* as a posttranscriptional regulator of the cell wall-modifying enzyme peptidoglycan deacetylase, PgdA. Under oxidative stress, PgdA is highly expressed and confers resistance to lysozyme in wild-type cells. PgdA protein expression as well as transcript abundance is significantly decreased in an *acnB* mutant. In the wild-type, *pgdA* mRNA half-life was 13 min, whereas the half-life for the *acnB* strain was 7 min. Based on electrophoretic mobility shift assays and RNA footprinting, the *H. pylori* apo-AcnB binds to the 3'-untranslated region of the *pgdA* RNA transcript. Some of the protected bases (from footprinting) were localized in proposed stem-loop structures. AcnB-*pgdA* transcript binding was abolished by the addition of iron. The *acnB* strain is more susceptible to lysozyme-mediated killing and was attenuated in its ability to colonize mice. The results support a model whereby apo-AcnB directly interacts with the *pgdA* transcript to enhance stability and increase deacetylase enzyme expression, which impacts *in vivo* survival.

Introduction

Helicobacter pylori is a Gram-negative, microaerophilic bacterium that infects over 50% of the world's population and is the etiological agent for gastritis, peptic ulcer disease, and most gastric cancers (1). During colonization of the human gastric mucosa, *H. pylori* induces a strong inflammatory response resulting in the production of large amounts of reactive oxygen species (ROS). *H. pylori* can thrive in the gastric mucosa by employing a battery of diverse antioxidant enzymes that detoxify oxidants and repair essential biomolecules (2, 3). Additionally, *H. pylori* utilizes other mechanisms to persist in the host, including peptidoglycan (PG) modification (4).

H. pylori PG consists of alternating *N*-acetylglucosamine (Glc*N*Ac) and *N*-acetylmuramic acid (Mur*N*Ac) residues connected by β-1,4 bonds and cross-linked via short peptide bridges (5, 6). The β-1,4 bonds are susceptible to hydrolysis by the muramidase lysozyme, which results in decreased cell wall integrity and cell lysis. Lysozyme, an important component of the host innate immune system, is abundant in the mucosal surface and is present in the granules of professional phagocytes (7, 8). Interestingly, bacteria have evolved mechanisms to modify their PG around the lysozyme cleavage site to prevent hydrolysis (9). *H. pylori* is equipped with a peptidoglycan deacetylase (PgdA) that confers both pure PG and whole bacterial resistance to lysozyme degradation (10). PgdA expression is significantly increased in *H. pylori* cells when they are exposed to oxidative stress and when in contact with macrophages (11). Furthermore, *pgdA* mutants have an attenuated ability to colonize the mouse stomach and permit a stronger host immune (cytokine) response (11). This previous work led us to question how PgdA expression is regulated especially considering that *H. pylori* lacks many of the oxidative stress response regulators found in most other Gram-negative bacteria. We hypothesized that aconitase may be

playing an important role during oxidative stress by serving as a posttranscriptional regulator for peptidoglycan deacetylase (10).

Aconitases are [4Fe-4S] proteins that catalyze the reversible isomerization of citrate to isocitrate in the citric acid cycle. In eukaryotes, there are two types of aconitase proteins; mitochondrial aconitase (m-Acn) and cytosolic aconitase (c-Acn), also referred to as iron regulatory protein 1 (IRP1). IRP1 is bifunctional, having enzymatic activity when the [4Fe-4S] cluster is intact and acting as a posttranscriptional regulator when the cluster is disassembled (12). Iron deprivation and oxidative stress are known to cause disassembly of the cluster, resulting in the apo-form of IRP1, which undergoes domain rearrangements that allow it to bind to iron responsive elements (IREs) (12, 13). IREs are approximately 30-nt-long sequences that form stem-loop structures in the untranslated regions (UTRs) of mRNA transcripts. The eukaryotic consensus IRE contains a C bulge in the stem and the sequence CAGUGN in the loop (14). If the IRE is located in the 5' UTR, binding of IRP1 will inhibit translation; if the IRE is located in the 3' UTR, binding of IRP1 will increase transcript stability and result in enhanced translation (12, 14).

Several bifunctional bacterial aconitases have been studied thus far, including *Escherichia coli* AcnA and AcnB, which have been found to enhance and decrease superoxide dismutase (SodA) expression, respectively (15). In *Salmonella enterica* serovar Typhimurium LT2, AcnB was shown to indirectly regulate the flagellum protein, FliC (16). *Bacillus subtilis* aconitase (CitB) binds to the 3' UTR of *gerE*, a transcriptional activator involved in sporulation (17). Furthermore, *Mycobacterium tuberculosis* aconitase (Acn) binds to the thioredoxin (*trxC*) transcript and to the *ideR* transcript, an iron-dependent activator and repressor (18). *H. pylori* possesses one copy of aconitase, *acnB*, whose gene product is known to have aconitase activity

in the citric acid cycle (19), but it has not yet been studied as a posttranscriptional regulator. Here, we present evidence that apo-AcnB acts as a posttranscriptional regulator for PgdA, which is an important PG modification enzyme conferring lysozyme resistance, and that it contributes to survival in the mouse host.

Materials and Methods

Bacterial strains and growth conditions. *H. pylori* X47 and 43504 wild-type and *acnB* strains were grown on Brucella agar (Difco) supplemented with 10% defibrinated sheep blood (BA plates) and chloramphenicol (50 μg/ml) at 37°C under constant microaerophilic conditions (2% O_2). For mRNA half-life determinations, wild-type and mutant strains were grown in Brain Heart Infusion (BHI) broth with 0.4% β-cyclodextrin. Sealed bottles initially contained 5% CO_2, 10% H_2, 75% N_2, and 10% O_2 and were incubated at 37°C, shaking. *H. pylori* wild-type and *acnB* strain growth patterns were similar. *E. coli* BL21 RIL cells were grown at 37°C aerobically on Luria-Bertani agar or broth (shaking) supplemented with ampicillin (100 μg/ml) and chloramphenicol (50 μg/ml).

Mutant construction. The 2.6 kb *acnB* gene (*hp0779*) has been determined to be the first gene of an operon containing 3 additional genes - *hp0780*, *hp0781*, and *hp0782* (20). Ninety-eight percent of the gene was deleted in *H. pylori* strains X47 and 43504 using overlap extension PCR and was replaced with the chloramphenicol (*cat*) cassette, which was inserted in the same direction of transcription as that of the native gene. The *cat* cassette has its own promoter, lacks a transcription terminator, and is routinely used in our laboratory without any polar effect (21, 22). Primers acnB1 (5'-CCCCGCATCAATACGCC-3') and acnB2 (5'-ATCCACTTTTCAATCTAT ATCCTAAAAA ATCTTTCATCAT-3') were used to amplify a 327-bp DNA sequence that contained the beginning of *hp0778*, the intergenic region between *hp0778* and *hp0779*, the

beginning of *hp0779*, and a portion of the *cat* cassette. Primers acnB3 (5'-CCCAGTTTGTCGC ACTGATAAGGAGAATTTCAGGCTCTAG-3') and acnB4 (5'-CTAGCGCCAATTATAGAT ATAAGG-3') were used to amplify a 388-bp sequence containing part of the *cat* cassette, the end of *hp0779*, the intergenic region between *hp0779* and *hp0780*, and *hp0780*. Primers acnB1 and acnB4 were used in the final PCR step to fuse together the product of acnB1/acnB2, the *cat* cassette, and the product of acnB3/acnB4. This final step yielded a 1.5-kb PCR product that was then cloned into the pGEM-T vector (Promega) to generate pGEM*acnB*::*cat.* This plasmid was introduced into the wild-type strain via natural transformation and homologous recombination. *acnB* mutants were selected on BA plates with chloramphenicol (50 μg/ml) at 2% O_2. Insertion of the *cat* cassette and absence of the *acnB* gene were confirmed by PCR and sequencing using primers acnB1, acnB4, and primers specific for the *cat* cassette (5'-GATATAGATTGAAAAGT GGAT-3' and 5'-TTATCAGTGCGACAAACTGGG-3'). Also, RT-PCR was performed on the downstream gene, *hp0781*, to rule out any polar effect due to the *cat* cassette. Since *hp0780* is only 273-bp in length, we instead designed primers for *hp0781* (1.3-kb). Total RNA was extracted (Aurum Total RNA Mini Kit; Bio-Rad), treated with DNase (Turbo DNA-free kit; Ambion), and used as a template for cDNA synthesis (iScript cDNA Synthesis kit; Bio-Rad). The generated cDNA was then used as a template for PCR and the product of the expected size was obtained for both the wild-type and *acnB* strains (data not shown).

Western blotting. *H. pylori* wild-type and *acnB* strains were each separately grown under 2% O_2 for 36 h and 12% O_2 for 72 h. The time for cells grown under 12% O_2 was extended because cell growth is slower at this higher oxygen level. Cell-free extracts were subjected to SDS-PAGE and the proteins were electroblotted onto a nitrocellulose membrane. The membrane was then incubated with anti-PgdA (1:500) (Antagene, Inc.) followed by exposure to goat anti-

rabbit IgG alkaline phosphatase-conjugated secondary antibody (1:1000) (Bio-Rad). Anti-UreA (1:10,000) (Santa Cruz Biotechnology) was used as an internal loading control. ImageJ (http://rsweb.nih.gov/ij/) was used to analyze dried membranes. Student's *t* test was used for statistical comparisons.

Real-time quantitative PCR. The Aurum Total RNA Mini Kit (Bio-Rad) was used to extract total RNA from *H. pylori* wild-type and *acnB* cells grown under 2% and 12% O_2. The Turbo DNA-free kit (Ambion) was used as an additional measure to degrade any remaining DNA. cDNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad) according to the kit instructions. The iQ SYBR Green Supermix (Bio-Rad) kit was used for real-time PCR according to the manufacturer's protocol. Primers specific for *pgdA* (5'-GGATTCGCCTGATG ATATTTCG-3' and 5'-CCTGCATCCACGATCATTTTC-3') were used along with primers specific for *gyrA* (5'-GCTAGGATCGTGGGTGATGT-3'and 5'-TGGCTTCAGTGTAACGCAT C-3'), the internal control. Relative transcript abundance was calculated using the $2^{-\Delta CT}$ formula (23). For mRNA half-life determinations, wild-type and *acnB* strains were grown in BHI for 24 h (early exponential phase) and subjected to 21% O_2 for 2 h. Rifampin (500 μg/ml) was added, and after 70 s ($t = 0$), time points were established (2, 5, 10, 15, 20, and 30 min). For each time point, RNA was extracted as described above and cDNA was synthesized using primers specific for *pgdA*. The quantitative (qPCR) data was used to calculate the *pgdA* mRNA half-life as described (24). Prism (GraphPad, San Diego, CA) and the equation $Y = (Y_0)e^{-kt}$ was used to find first-order decay constants (k) by nonlinear regression analysis. Half-lives were calculated using $t_{1/2} = \ln 2/k$ (24).

Overexpression and purification of AcnB. The *H. pylori acnB* gene was PCR amplified using wild-type DNA as the template with primers acnBHisF (5'-CGCACCCATATGATGAAA

GATTTTTTAGAAG-3') and acnBHisR (5'-GAAGACCTCGAGGAGCCTGAAATTCTCCAT TAAG-3'). The PCR product was cloned into the pET-21b vector (Novagen) and overexpressed as a hexahistidine-tagged protein in *E. coli* BL21 RIL. Cells were grown in LB broth to an A_{600} of 0.5 and induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside for 4 h. After harvesting by centrifugation (5,000 x *g*, 10 min, 4°C) and resuspending and washing the pellet with 50 mM NaH_2PO_4, 300 mM NaCl, pH 8 (buffer A supplemented with 10 mM imidazole), cells were resuspended in the same buffer and lysed by three passages though a French pressure cell (22). Following centrifugation of the extract (16,000 x *g*, 30 min), the supernatant was collected and applied to a nickel-nitrilotriacetic acid column (Qiagen). The column was washed with buffer A supplemented with 20 mM imidazole and the protein was eluted with buffer A supplemented with 250 mM imidazole. Purified protein was analyzed by SDS-PAGE and estimated to be approximately 95% pure. Apo-AcnB was prepared by incubating the purified native protein with dipyridyl (0.5 mM) for 30 min and then dialyzing overnight against 20 mM Tris-Cl pH 8, 100 mM NaCl, 3% glycerol (18). As expected, the UV-visible absorption spectrum of native AcnB showed absorbance in the 300-600 nm range indicative of Fe-S clusters, while absorbance in this range was significantly less for the dipyridyl-treated protein (data not shown). Protein concentration was determined using the bicinchoninic acid assay kit (Thermo Scientific).

Electrophoretic mobility shift assays. The entire *pgdA* mRNA 3'UTR (45-nt) was synthesized (Integrated DNA Technologies) and radiolabeled at the 5' end with T4 polynucleotide kinase (Invitrogen) according to the manufacturer's instructions. 2.5-µl of [γ-^{32}P] ATP (10 µCi/µl, 3000 Ci/mmol) was incubated with 5 pmol of *pgdA* RNA, 1X forward reaction buffer, T4 polynucleotide kinase, and water for 10 min at 37°C. Labeled RNA was purified by phenol extraction and ethanol precipitation. Size and purity were checked on a denaturing urea

polyacrylamide gel. Purified *pgdA* RNA (50 nM) was incubated with either increasing apo-AcnB (0, 600, 1,200, and 3,000 nM) or BSA (3,000 nM) in binding buffer (10 mM Tris pH 8.3, 20 mM KCl, and 10% glycerol) with 130-fold nonspecific yeast tRNA for 15 min at room temperature. Apo-AcnB (3,000 nM) was incubated with either 1 mM ammonium iron (II) sulfate plus 10 mM dithiothreitol or 0.5 mM dipyridyl. Unlabeled specific *pgdA* competitor (50X and 100X molar excess) was added to apo-AcnB (3,000 nM) in some reactions prior to the addition of radiolabeled *pgdA* RNA and allowed to incubate for 5 min to maximize effectiveness (recommended by Thermo Scientific). As a positive control for the binding reactions, the 5' UTR human ferritin sequence (5'-GTGAGAGAATTCGGGAGAGGATTTCCTGCTTCAACAGTGC TTGGACGGAACTTTGTCTTGAAGCTTGGAGAG-3') was synthesized (Integrated DNA Technologies) and cloned into the pGEM-3Z vector (Promega). HindII was used to generate the linearized plasmid, which was then used as a template for *in vitro* transcription. T7 RNA polymerase (Promega) was used to synthesize radiolabeled RNA using [α-^{32}P] UTP (10 µCi/µl, 800 Ci/mmol) according to the manufacturer's instructions. RNA was purified by phenol extraction and ethanol precipitation. As a negative control, the pGEM-3Z vector alone was linearized and subjected to *in vitro* transcription. The positive- and negative-control reactions were performed using 3,000 nM apo-AcnB. Reaction products were resolved on a 6% non-denaturing polyacrylamide gel. The gel was incubated overnight in a phosphor screen cassette (Molecular Dynamics) and the screen was scanned using the Typhoon Imager (GE Healthcare).

RNA footprinting. 5'-End-labeled *pgdA* probe was prepared as described for electrophoretic mobility shift assays. *pgdA* probe (50 nM) was incubated for 15 min at room temperature with (1 µM) and without apo-AcnB, 1X RNA structure buffer (Ambion), 1 µg of yeast tRNA, and either RNase A (1 ng) or RNase V_1 (0.001 U). Digestion products were purified

by phenol extraction and ethanol precipitation. Samples were resolved on a 20% denaturing polyacrylamide 7 M urea gel in 1X Tris-borate-EDTA (TBE). The gel was vacuum dried, incubated overnight in a phosphor screen cassette (Molecular Dynamics), and scanned using the Typhoon Imager (GE Healthcare).

Lysozyme sensitivity assays. Wild-type and *acnB* strains were grown to late exponential phase under microaerophilic conditions, harvested, and resuspended in phosphate buffered saline (PBS) to an optical density at 600 nm (OD_{600}) of 1, and then subjected to incubation for 8 h with lysozyme (50 mg/ml). After incubation, cells were serially diluted and plated, and colonies were counted after 4 days. Significant differences between the wild-type and mutant were determined using Student's *t* test.

Mouse colonization. All procedural work was approved by the Institutional Animal Care and Use Committee at the University of Georgia, Athens, GA. *H. pylori* wild-type and *acnB* strains were grown on BA plates under microaerophilic conditions for 48 h. Cells were suspended in PBS to an OD_{600} of 1.7 and 3 X 10^8 cells were administered to each C57BL/6J mouse. Three weeks after inoculation, the mice were sacrificed after withholding food and water for 1.5 to 2 h, and the stomachs were removed, weighed, and homogenized. Stomach homogenate dilutions were made in PBS and plated onto BA plates supplemented with amphotericin B (10 µg/ml), bacitracin (100 µg/ml), and vancomycin (10 µg/ml). After 5 days of incubation under microaerophilic conditions, cells were enumerated and the data expressed as log (CFU/g) of stomach. The Wilcoxon signed-rank test was used to determine significant differences in colonization between the wild-type and mutant strains.

Results

PgdA expression, transcript abundance, and mRNA half-life in the strains upon oxidative stress exposure. We began this work by investigating the effect of the aconitase deletion on expression of peptidoglycan deacetylase (PgdA). Western blots were performed using anti-PgdA antibody and cell extracts from wild-type and *acnB* strains grown under both 2% O_2 for 36 h and 12% O_2 for 72 h. A significant difference in PgdA expression was found between the wild-type at 2 versus 12% O_2 (Fig. 2.1), similar to the previous finding that reported PgdA is increased upon subjection of cells to oxidative stress (10). No significant difference in PgdA levels was found in the *acnB* mutant between 2 and 12% O_2. There was a decrease in PgdA expression in the *acnB* strain under 2% O_2 as compared to the wild-type under the same condition. Interestingly, there was 4-fold less PgdA expression in the *acnB* strain under 12% O_2 as compared to the wild-type under 12% O_2.

pgdA transcript abundance was assessed in the *acnB* strain using quantitative real-time PCR (Fig. 2.2). Wild-type and *acnB* strains were subjected to the same growth conditions as described for the Western blot experiments. There was a 2-fold increase in relative *pgdA* transcript abundance for the wild-type under 12% O_2 as compared to the 2% O_2 condition, which was expected. *pgdA* transcript levels in the *acnB* mutant under 12% O_2 was 2-fold decreased as compared to the 2% O_2 incubation. A 3-fold decrease in *pgdA* abundance was found in the *acnB* strain under 2% O_2 versus the wild-type under the same condition. Most interestingly, a 9-fold decrease was observed for the *pgdA* transcript in the *acnB* strain under 12% O_2 versus the wild-type under 12% O_2.

To directly determine if AcnB was functioning as a post-transcriptional regulator to stabilize the *pgdA* message, we used qPCR to calculate the half-life of the *pgdA* mRNA in the

wild-type and *acnB* strains. Cells were grown under microaerophilic conditions and then exposed to 21% O_2 for 2 h. Our results revealed that upon high oxygen exposure, the wild-type *pgdA* mRNA half-life is 13 min whereas the *acnB* mutant *pgdA* mRNA half-life is 7 min.

In summary, these findings suggested that apo-AcnB directly interacts with the *pgdA* transcript. The model is that under oxidative stress, the [4Fe-4S] cluster of AcnB is likely to be disassembled and apo-AcnB binds to the *pgdA* transcript, stabilizing the message and resulting in increased transcript abundance and expression.

Apo-AcnB binds to the *pgdA* 3' UTR. Since it is known that aconitase can function as a posttranscriptional regulator and the above results suggested aconitase was interacting with the *pgdA* transcript, we examined the 5' and 3' untranslated regions (UTRs) of the *pgdA* gene for possible IRE-like sequences. The *pgdA* 5' UTR contained only 10-nt (known IREs are approximately 30-nt in length); therefore, the *pgdA* 3' UTR was chosen for further investigation. The secondary structure of the 3' UTR was predicted using the STAR program (25) (Fig. 2.4B), revealing two stem-loops that may be involved in aconitase binding. To test if apo-AcnB could bind to the transcript, electrophoretic mobility shift assays were performed. Radiolabeled *pgdA* probe (50 nM) was incubated with 0, 600, 1,200, and 3,000 nM apo-AcnB protein. As apo-AcnB concentration increased, more *pgdA* probe was observed to shift (Fig. 2.3A). The addition of ammonium iron (II) sulfate and dithiothreitol resulted in no shift while the addition of dipyridyl promoted binding (Fig. 2.3B). These results agreed with other bacterial studies (18, 26) that demonstrated the RNA-binding ability of aconitase is diminished upon the addition of Fe^{2+} and restored when iron is not present or the specific iron chelator dipyridyl is added to the reaction. The presence of the [4Fe-4S] cluster in aconitase determines whether it can bind to RNA transcripts (12). Additionally, binding reactions were performed using 50 and 100X molar excess

of specific cold competitor (Fig. 2.3C). The radiolabeled *pgdA* probe is effectively outcompeted when unlabeled *pgdA* 3' UTR is added at 100X molar excess. When bovine serum albumin, rather than apo-AcnB, was incubated with the radiolabeled *pgdA* probe, no shift was observed (Fig. 2.3D). Furthermore, binding reactions were conducted with apo-AcnB (3,000 nM) using a vector-only probe as a negative control, which did not result in a shift, and with the human ferritin IRE as a positive control, which resulted in a shift similar to that of *pgdA* (data not shown).

RNA footprinting was conducted to elucidate where apo-AcnB (dipyridyl-treated; see Materials and Methods) was binding to the *pgdA* 3' UTR. 5' End-labeled *pgdA* probe was incubated with and without apo-AcnB and subjected to cleavage using two different ribonuclease enzymes (Fig. 2.4A). RNase A cleaves the 3' end of single-stranded C and U residues, and RNase V_1 cleaves base-paired or stacked nucleotides. RNase A cleavage of nucleotides 18, 29, 32 to 34, and 41 and 42 occurred less frequently in the presence of apo-AcnB. Incubation with RNase V_1 revealed that the majority of the *pgdA* probe is protected when incubated with apo-AcnB, especially nucleotides 9 to 12, 20 to 23, and 27 (Fig. 2.4B). Nucleotides 3 to 7 were cleaved more frequently by RNase V_1 in the presence of apo-AcnB.

Deletion of *acnB* confers lysozyme sensitivity in *H. pylori* mutants. Lysozyme hydrolyzes the β-1,4 bonds connecting Glc*N*Ac and Mur*N*Ac residues in bacterial PG, leading to decreased cell wall integrity and subsequent cell lysis. Previously, our lab showed that an *H. pylori pgdA* strain is more sensitive to lysozyme degradation than the wild-type (10). Thus far, our findings suggested apo-AcnB regulates PgdA expression. Therefore, we speculated that the *acnB* strain may be more sensitive to lysozyme killing, and this sensitivity was tested over an 8-h period (Fig. 2.5). The *acnB* strain is significantly more sensitive to lysozyme killing than the

wild-type after 4, 6, and 8 h ($P < 0.01$) as determined by Student's *t* test. The kill curve for the *acnB* strain closely resembles that previously published for the *pgdA* strain (10).

The *acnB* strain has an attenuated ability to colonize the mouse stomach. We wanted to characterize the physiological role of aconitase in *H. pylori* by comparing the colonization abilities of the *acnB* strain to that of the wild-type. Each strain was individually inoculated into eight C57BL/6J mice, and after 3 weeks, stomach colonization was examined. The mean colonization for the wild-type was 2.1 X 10^6 CFU/g, whereas the mean for the *acnB* strain was 4.6 X 10^5 CFU/g (Fig. 2.6). Thus, there was a 4.5-fold decrease in mouse colonization by the *acnB* strain. Using the Wilcoxon signed-rank test, the range of mutant colonization values is significantly smaller than that of the wild-type at greater than 95% confidence ($P < 0.05$). These results demonstrate aconitase contributes to *H. pylori* survival and colonization in the mouse stomach.

Discussion

During *H. pylori* colonization, host immune cells mount a strong inflammatory response resulting in the production of large amounts of ROS, including hydrogen peroxide (H_2O_2) and the superoxide anion (O_2^-). These can be detrimental to *H. pylori*, causing damage to DNA, proteins, and lipids. Despite containing a diverse repertoire of antioxidant enzymes (3), the bacterium lacks homologues of the oxidative stress response regulators found in other bacteria, such as OxyR, SoxRS, and the RpoS sigma factor. It has thus been hypothesized that posttranscriptional regulation in *H. pylori* may play a role in the regulation of genes in response to stress (27). We propose a model for the role of aconitase in *H. pylori* colonization (Fig. 2.7). Bacterial aconitases are subject to oxidation and Fe/S cluster loss, but the apo-form can still play regulatory roles (16, 18). PgdA has been shown to be upregulated in response to oxidative stress

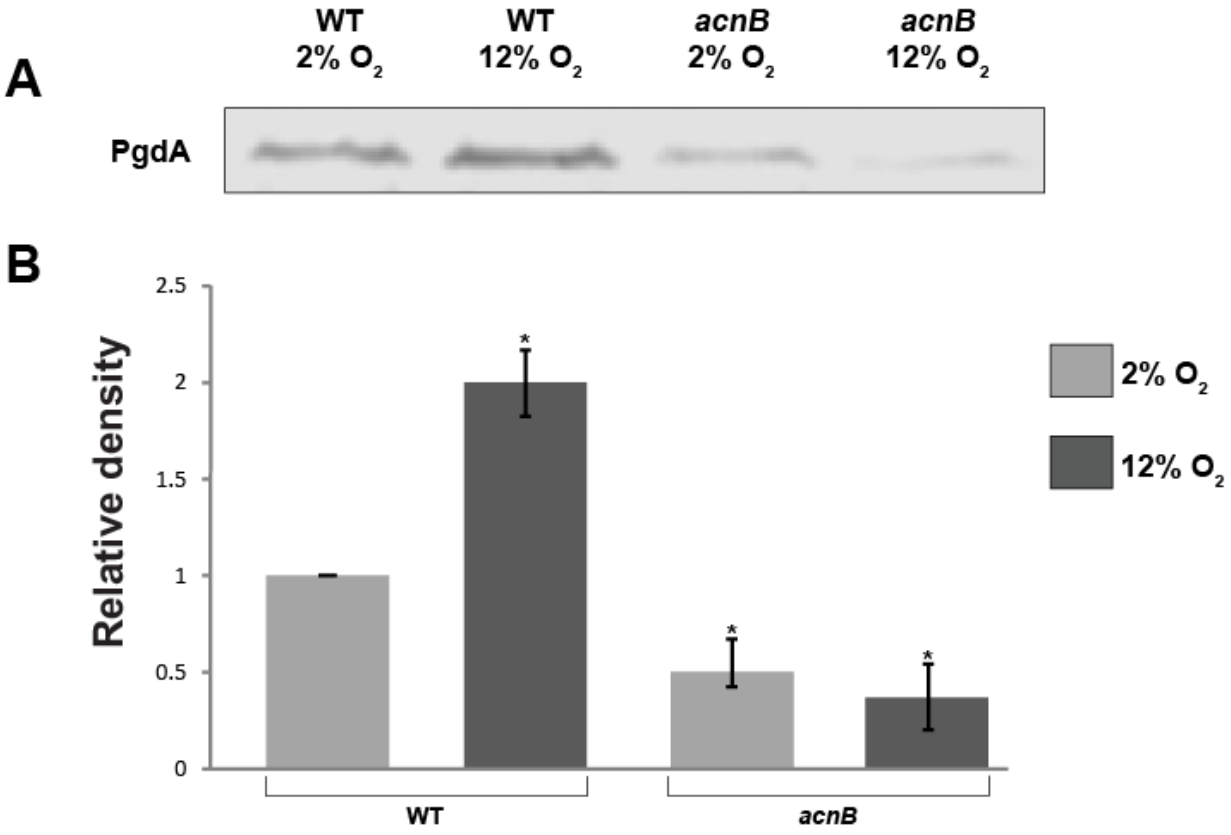
and upon contact with neutrophils (10, 28). Failure of the *acnB* strain to upregulate PgdA expression under 12% O_2 conditions is in contrast to the wild-type, and led us to hypothesize aconitase was playing a role in deacetylase regulation (Fig. 2.1). Decreased transcript abundance and *pgdA* mRNA half-life in the *acnB* strain supported our hypothesis that aconitase is involved in the post-transcriptional regulation of *pgdA*. Electrophoretic mobility shift assays and RNA footprinting data demonstrated that the regulatory effect of aconitase results from direct interaction between apo-AcnB and the transcript. Furthermore, the *acnB* strain was more sensitive to lysozyme degradation and was attenuated in its ability to colonize the mouse stomach, so an AcnB role *in vivo* is proposed (Fig. 2.7).

Aconitase-mediated transcript stabilization is a previously described regulatory mechanism. In vertebrates, IRP1 binds to IREs involved in maintaining iron homeostasis (12). The consensus IRE has a C bulge in the stem and the sequence CAGUGN in the loop (12), but the precise structure or sequence recognized by bifunctional bacterial aconitases is less defined. Some aconitase transcript targets contain the eukaryotic consensus sequence, such as thioredoxin (*trxC*) in *M. tuberculosis* (18); however, the *E. coli acnA*, *acnB*, *sodA*, and *ftsH* transcripts as well as the *B. subtilis gerE* transcript do not have the consensus sequence (15-17, 29). The *pgdA* 3’ UTR also does not contain this consensus sequence, but gel shift data indicate binding to apo-AcnB (Fig. 2.3). RNA footprinting revealed which nucleotides of the *pgdA* 3’ UTR were protected by apo-AcnB (Fig. 2.4), but it is still unclear which specific nucleotides or sequences are necessary for binding. This appears to be the case for other bacterial aconitase targets as well (16). It is of interest to learn the total repertoire of mRNA targets and thus the complete role of this tricarboxylic acid (TCA) cycle enzyme.

Acknowledgements

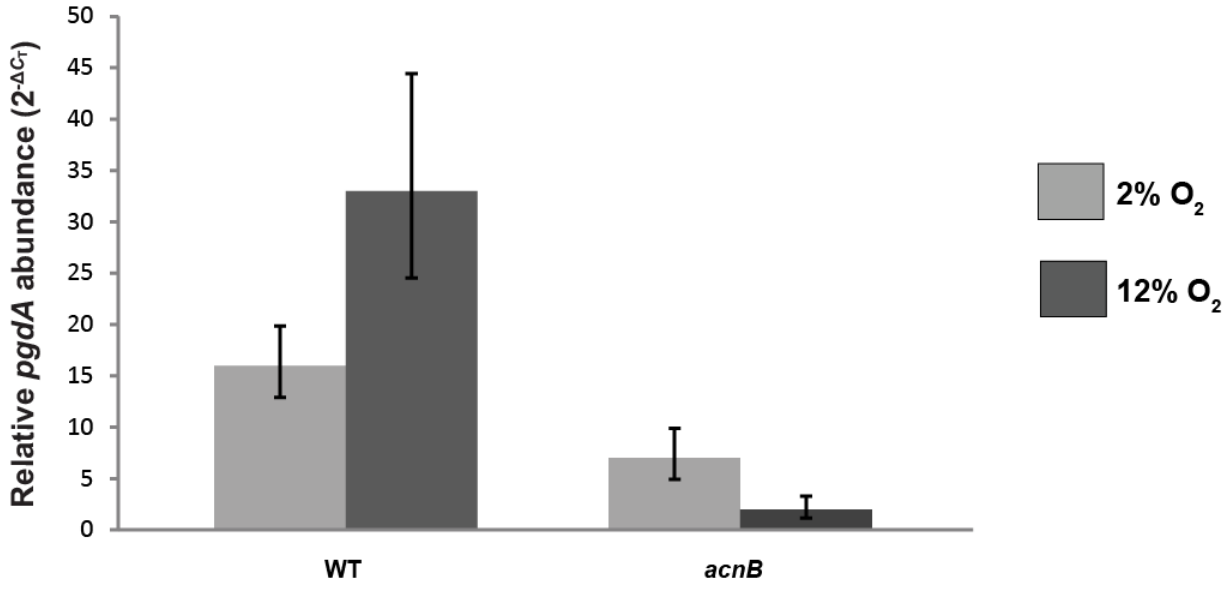
We would like to thank Bijoy Mohanty for all of his guidance and expertise with the RNA footprinting experiments. We also thank Stéphane Benoit for many helpful discussions and Sue Maier for her help with the mouse colonization experiments.

FIG 2.1 Expression of the PgdA enzyme at two oxygen levels



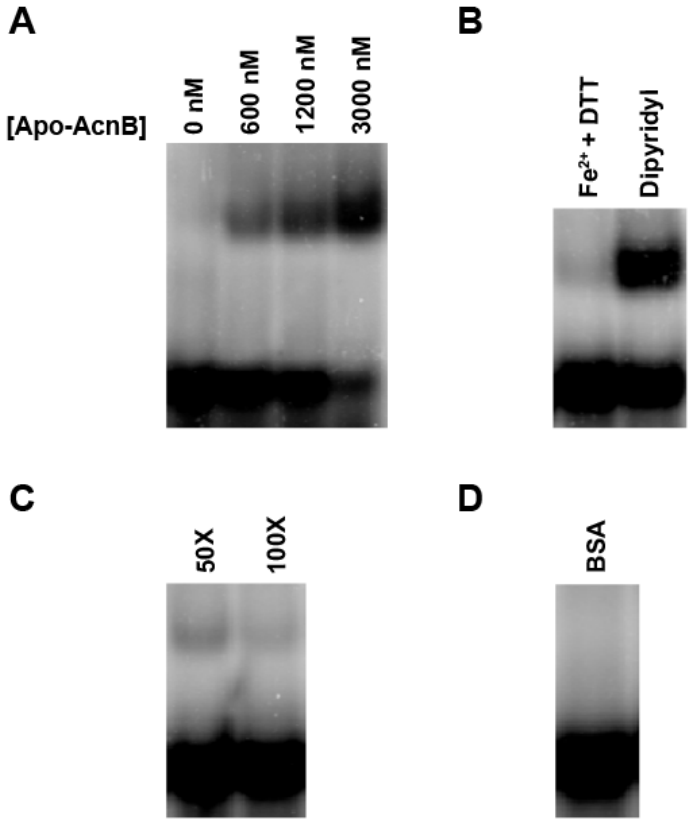
Western blot (A) and densitometry (B) analysis of PgdA expression from the Western blot are shown. Cell extracts from 43504 wild-type and *acnB* strains were subjected to SDS-PAGE followed by immunoblotting using specific anti-PgdA antibody. Dried blots were analyzed using ImageJ. Wild-type (2% O_2) expression was used as the reference (adjusted to unit of 1.0 U). The experiment included an internal loading control, UreA (not shown), that verified equal amounts of protein were loaded. Data shown are averages and standard deviations (SD) from three independent experiments. Asterisks indicate a significant difference compared to the reference. $P < 0.01$ as determined by Student's *t* test.

FIG 2.2 Relative *pgdA* transcript abundance



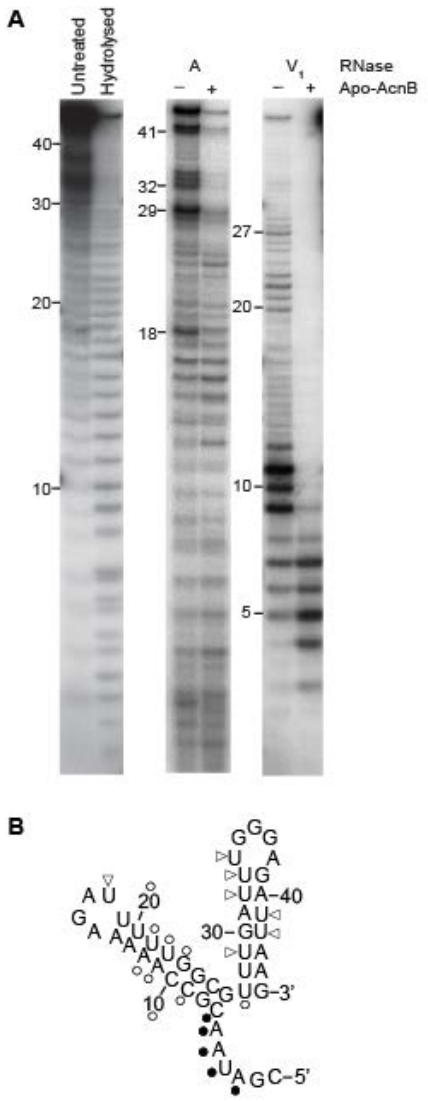
Transcript levels were determined by quantitative real-time PCR after growth of each strain under either 2 or 12% O_2. The *gyrA* housekeeping gene was used as an internal control. Results shown are representative data from one experiment performed in triplicate. The entire experiment was repeated with similar results. Error bars represent $2^{-(\Delta CT-SD)}$-$2^{-\Delta CT}$ and $2^{-\Delta CT}$-$2^{-(\Delta CT+SD)}$.

FIG 2.3 Electrophoretic mobility shift assays



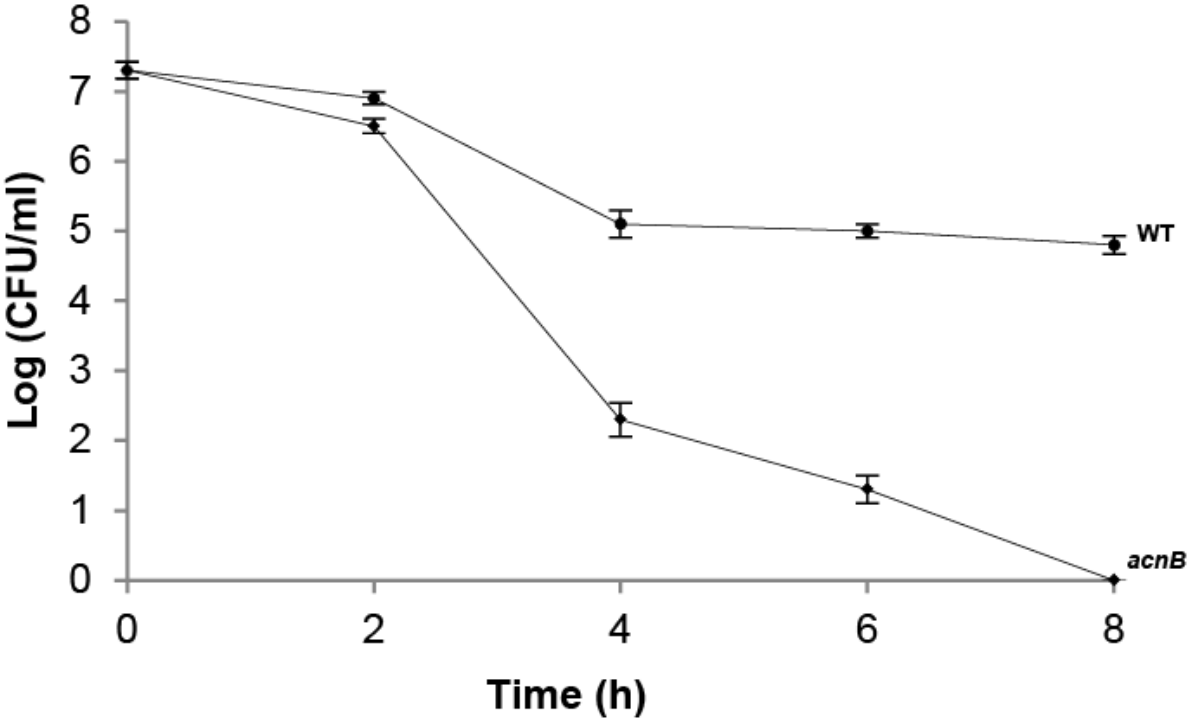
(A) More *pgdA* transcript (50 nM) bound to apo-AcnB with increasing protein concentration. (B) Addition of iron and reductant abolished binding, whereas the iron chelator dipyridyl promoted binding. (C) Radiolabeled *pgdA* probe was outcompeted by specific unlabeled competitor RNA at 100X molar excess. (D) Use of nonspecific protein bovine serum albumin (3,000 nM) instead of apo-AcnB did not result in a shift.

FIG 2.4 RNA footprinting



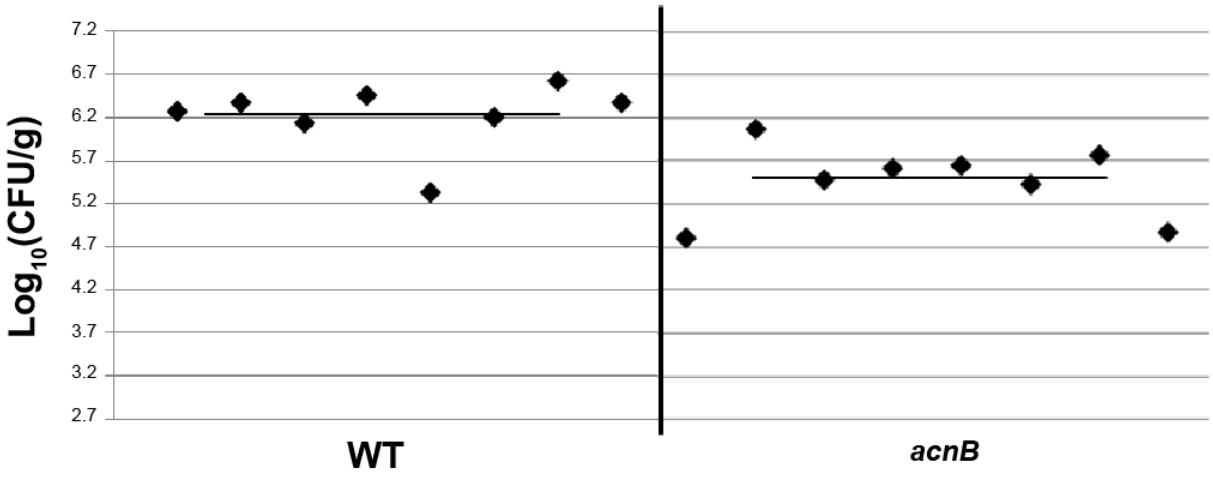
(A) 5'-End-labeled 45-nt *pgdA* RNA was incubated with (+) and without (-) apo-AcnB and subjected to ribonuclease digestion. (B) RNase A (triangles) and RNase V_1 (circles) cleavage sites are mapped on the predicted *pgdA* 3' UTR structure. Open symbols indicate nucleotides that were more protected from RNase cleavage in the presence of apo-AcnB, whereas filled symbols indicate those nucleotides that were less protected.

FIG 2.5 Susceptibility of strains to lysozyme killing



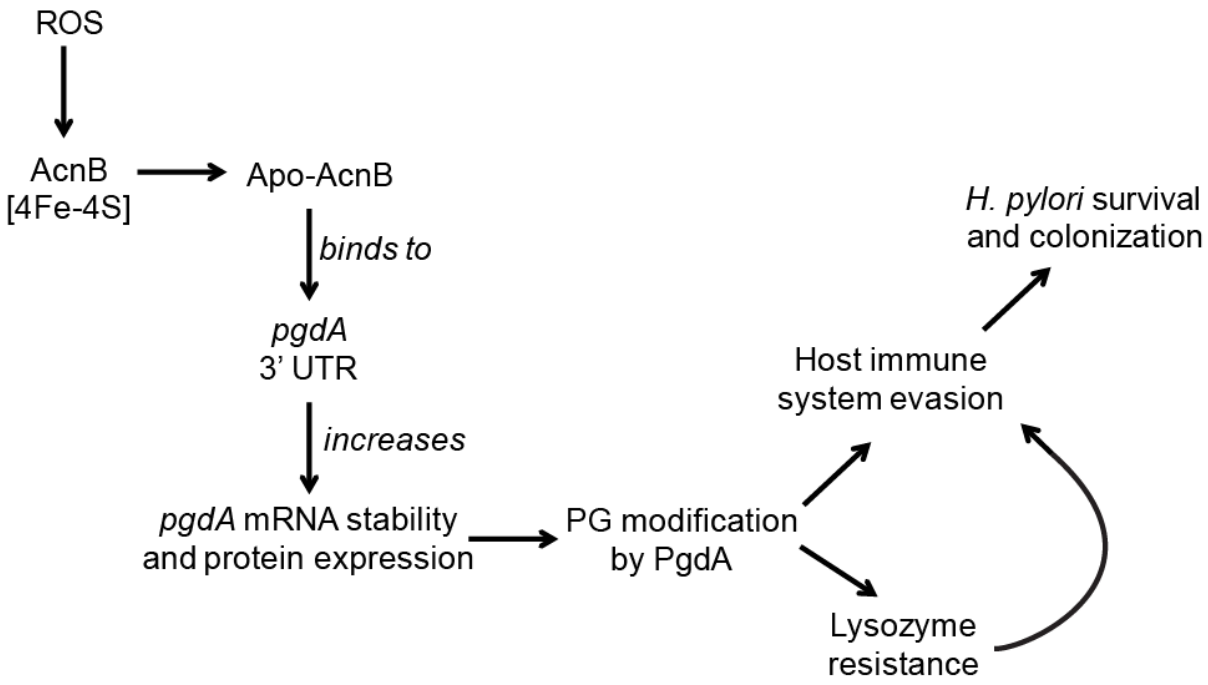
Strains were treated with 50 mg/ml lysozyme and samples were taken over an 8-h period for comparative viability (CFU determination). Each data point represents the average of combined replicates from two independent experiments (each performed in triplicate). Error bars represent standard deviations. The mutant strain values are significantly less than the wild-type at the 4-, 6-, and 8-h time points at $P < 0.01$ (Student's *t* test).

FIG 2.6 Mouse colonization by strains



Wild-type and *acnB* strains were separately injected into 8 mice each, and colonization ability (CFU recovered per gram of stomach) was examined after 3 weeks. Each point represents the CFU count from one stomach, and solid horizontal lines represent the means of colonization for each strain. The baseline (log_{10}[CFU/g] = 2.7) is the limit of detection. The experiment was repeated with similar results.

FIG 2.7 Model of *pgdA* posttranscriptional regulation and its effects on *H. pylori*



ROS oxidize the [4Fe-4S] cluster of AcnB, rendering apo-AcnB, which then binds to the *pgdA* transcript. Increased transcript stability leads to increased expression of the PG-modifying enzyme, PgdA. PG modification is a mechanism *H. pylori* utilizes to circumvent host immune system detection and resist lysozyme degradation (which further mitigates host immune system recognition), both of which contribute to long-term survival and colonization.

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CHAPTER 3

PUTATIVE TARGETS FOR POSTTRANSCRIPTIONAL REGULATION BY *HELICOBACTER PYLORI* ACONITASE[1]

[1] **Austin, C., D. Johnson, R. Orlando, and R. J. Maier.** To be submitted to *J. Proteom.*

Abstract

The Gram-negative, gastric pathogen *Helicobacter pylori* has few regulatory systems as compared to *Escherichia coli* and lacks homologues of the commonly known regulators that respond to reactive oxygen species (ROS). During host colonization, *H. pylori* induces a strong inflammatory response consisting of toxic ROS that cause damage to bacterial macromolecules. The precise mechanisms that *H. pylori* utilizes to regulate expression of ROS-combating proteins are not fully understood. In this study, we investigated the contribution of aconitase as a posttranscriptional regulator in *H. pylori*, particularly under oxidative stress conditions. Using a proteomics approach combined with bioinformatics, we have identified several differentially expressed proteins that are putative targets for aconitase-mediated regulation in *H. pylori*. As a result of our findings, we propose that aconitase functions as a global posttranscriptional regulator, mediating expression of proteins involved in the oxidative stress response, urease and hydrogenase activities, motility, and numerous others.

Introduction

Helicobacter pylori is a Gram-negative, gastric pathogen that infects about half of the world's population and is the etiological agent for gastritis, peptic ulcer disease, and gastric cancer (1). *H. pylori* is remarkable in that it is able to successfully colonize the unique niche of the stomach, where it encounters low pH and oxidative stress. *H. pylori* possesses one copy of aconitase (encoded by *acnB*), the product of which is known to be bifunctional in both eukaryotes and bacteria (2). The enzymatically active form of aconitase has an intact [4Fe-4S] cluster that participates in the tricarboxylic acid cycle. The [4Fe-4S] cluster is oxidized under high oxygen or low iron conditions rendering apo-aconitase, which is able to bind to mRNA transcripts and function as a posttranscriptional regulator. In eukaryotes, aconitase (or iron regulatory protein 1; IRP1) is highly studied. IRP1 binds to iron regulatory elements (IREs) that consist of stem-loop structures containing the consensus sequence "CAGUGN" in the loop and an unpaired "C" in the 5' end of the stem. The IRP/IRE system is known to posttranscriptionally regulate numerous genes involved in iron metabolism [for a review see (3)]. IRP1 binds IREs in the untranslated regions (UTRs) of mRNA transcripts to posttranscriptionally affect translation. If IRP1 binds to the 5' UTR, it inhibits ribosome binding, and therefore, decreases translation of the downstream gene. If IRP1 binds to the 3' UTR, it prevents ribonuclease degradation of the transcript, thereby providing stability and resulting in increased translation.

Bacterial aconitases have been studied in a variety of microorganisms. In *E. coli*, AcnA and AcnB have been found to bind to and regulate expression of the *acnA* and *acnB* transcripts, respectively (4). Also, superoxide dismutase (*sodA*) was found to be positively regulated by AcnA and negatively regulated by AcnB (5). In *Salmonella enterica* serovar Typhimurium, aconitase was shown to indirectly modulate flagellar synthesis by interacting with the protease

transcript, *ftsH* (6). Aconitase in *Bacillus subtilis* (CitB) binds to the *gerE* transcript, a gene involved in sporulation (7). *Streptomyces viridochromogenes* Tu494 AcnA binds to the recombinase A (*recA*) transcript and to *ftsZ*, a cell division gene involved in sporulation. Several additional targets for AcnA have been identified in *S. viridochromogenes* Tu94 when cells were exposed to oxidative stress (8). Additionally, Acn in *Mycobacterium tuberculosis* binds to the thioredoxin (*trx*) transcript and to *ideR*, an iron-dependent regulator (9). Our laboratory has demonstrated that in *H. pylori*, apo-AcnB binds to the 3' UTR of the peptidoglycan deacetylase (*pgdA*) transcript, resulting in transcript stability and increased expression (10). We seek to further characterize the role of aconitase as a posttranscriptional regulator in *H. pylori* by identifying differentially expressed proteins in an *acnB*-deletion strain combined with a bioinformatics approach.

Materials and Methods

Bacterial strains and growth conditions. *H. pylori* X47 and 43504 wild-type and *acnB* strains [for mutant construction see (10)] were grown either on Brucella agar (Difco) supplemented with 10% defibrinated sheep blood (BA plates) at 37°C under constant microaerobic conditions (2-4% O_2) or in Brain Heart Infusion (BHI) broth (pH 6.5 for atomic absorption assays) with 0.4% β-cyclodextrin. Sealed bottles initially contained 5% CO_2, 10% H_2, 75% N_2, and 10% O_2 and were incubated at 37°C, shaking. To maintain the mutant strain, *acnB* was cultured on BA plates with chloramphenicol (25 µg/ml) every other passage.

Protein separation and identification. *H. pylori* 43504 wild-type and *acnB* strains were each separately grown on BA plates under 2% O_2 for 36 h and 12% O_2 for 72 h (oxidative stress condition). Cells were harvested, resuspended in phosphate buffered saline (PBS) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF), and lysed via three passages of the French

pressure cell. Samples were then centrifuged at 6,000 x *g* for 10 min, and the cell-free extract was ultracentrifuged at 100,000 x *g* for 3 h. The supernatant (containing soluble proteins) and pellet (containing membrane proteins) were separated, and the pellet was resuspended in PBS supplemented with 1 mM PMSF. Protein concentrations were determined using the bicinchoninic acid assay kit (Pierce). Either 10 or 15 µg of soluble or membrane proteins were loaded onto a 4-20% Mini-PROTEAN TGX Gel (Bio-Rad) and separated using SDS-PAGE. For each lane, all protein bands were excised and digested with 0.5 µg trypsin. Digested samples were then sent for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis and samples were searched against the *H. pylori* 26695 database (Mascot Daemon).

Search for potential aconitase-binding patterns. Known aconitase-binding patterns, including CAGUG, CAGCG, GAGAG, GGGAG, CAGGG, CUGUG (8, 10, 11) as well as the *pgdA*-like (10) pattern, CNNNNAGAUNNNNG, were used as input sequences for the Pattern Locator program (12). The program was set to search the *H. pylori* 26695_uid57787 DNA sequence within the intergenic regions of the plus (default) and minus DNA strands.

Air survival assay. *H. pylori* X47 wild-type and *acnB* strains were grown on BA plates, harvested, and resuspended in PBS to an optical density at 600 nm (OD_{600}) of 1.3. Cell suspensions were incubated at 37°C, shaking, under atmospheric conditions (21% O_2). Every two hours (2, 4, 6, 8, 10 h) samples were removed, serially diluted, and spread onto BA plates. Colonies were counted after 3-4 days of incubation under microaerobic conditions (4% O_2).

Disk sensitivity assay. *H. pylori* X47 wild-type and *acnB* strains were grown on BA plates, harvested, and resuspended in PBS to an OD_{600} = 0.8. 100 µL of the bacterial suspension was spread evenly onto a BA plate and a sterile paper disk was placed in the center. 10 µl of 0.4 M *t*-butyl hydroperoxide (*t*-BOOH) was pipetted onto the center of the disk. After 3 days of

incubation under microaerobic conditions (4% O_2), the diameter of the zone of inhibition (mm) was measured.

Urease assay. *H. pylori* X47 wild-type and *acnB* cells were grown with and without nickel supplementation (10 μM $NiCl_2$), harvested, washed three times with 50 mM HEPES buffer (pH 7.5), and lysed by sonication. Protein content was determined using the bicinchoninic acid assay kit (Pierce). Urease assays were carried out as previously described (13, 14).

Hydrogenase assay. *H. pylori* wild-type and *acnB* cells were grown for 24 h on BA plates incubated in sealed jars with the BBL CampyPak system which generated a microaerobic environment. Hydrogen uptake measurements were determined amperometrically on whole cells with O_2 as the final electron acceptor as previously described (15).

Measurement of intracellular nickel levels. *H. pylori* X47 wild-type and *acnB* cells were grown in BHI broth with 0.4% β-cyclodextrin supplemented with 100 μM $NiCl_2$. Cells were harvested, washed three times with ddH_2O, lysed via sonication, and protein content was determined using the bicinchoninic acid assay kit (Pierce). Nickel measurements in cell-free extracts were determined using graphite furnace atomic absorption spectrophotometry (Shimadzu AA-6701F).

Real-time quantitative PCR. The Aurum Total RNA Mini Kit (Bio-Rad) was used to extract total RNA from *H. pylori* wild-type and *acnB* cells grown in liquid BHI medium with and without the addition of 10 μM $NiCl_2$. The Turbo DNA-free kit (Ambion) was used as an additional measure to degrade any remaining DNA. cDNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad) according to the kit instructions. The iQ SYBR Green Supermix (Bio-Rad) kit was used for real-time PCR according to the manufacturer's protocol. Primers specific for genes of interest (see Table 3.1 for primer sequences) were used along with primers

specific for *gyrA*, the internal control. Relative fold change was calculated using the $2^{-\Delta\Delta CT}$ formula (16).

Motility assay. *H. pylori* X47 wild-type and *acnB* strains were grown on BA plates under microaerobic conditions (4% O_2). Cells scraped from the plate were used to inoculate motility agar (Mueller Hinton Broth supplemented with 10% serum and 0.4% noble agar) and incubated for 1 week. The diameter (mm) of the motility halo was measured for each strain.

Results and Discussion

Identification of differentially expressed proteins. Previously, our laboratory studied the role of aconitase as a posttranscriptional regulator for the oxidative stress-induced peptidoglycan deacetylase (PgdA) (10). In bacteria, apo-aconitase binds to different targets related to oxidative stress, including *sodA* in *E. coli* and *trx* in *M. tuberculosis* (5, 9). It is also known that in *H. pylori*, AcnB is induced under oxidative stress conditions (17). We wanted to further characterize the *acnB* strain as it relates to oxidative stress in effort to identify new targets for aconitase-mediated regulation. *H. pylori* cells were each separately grown under 2% O_2 for 36 h (control) and 12% O_2 for 72 h (oxidative stress). Cell-free extracts were separated using SDS-PAGE and the protein bands were excised, digested with trypsin, and sent for protein identification using liquid chromatography-tandem mass spectrometry (LC-MS/MS). We used the Pattern Locator program (12) to identify putative aconitase-binding patterns (See Materials and Methods for search parameters) among the DNA sequences of identified proteins from LC-MS/MS as well as for proteins of interest that were not identified by proteomics. Putative targets for direct aconitase-mediated regulation (i.e., targets that were identified by a proteomics approach and by recognition parameters) are listed in Supplementary Table 3.1 (Table S3.1). Putative targets for indirect regulation by aconitase (i.e., targets that were identified by

proteomics but contain no aconitase-binding patterns) are listed in Supplementary Table 3.2 (Table S3.2). Gene locus numbers for *H. pylori* strain 26695 are used throughout the manuscript.

Aconitase contributes to oxidative stress defense. *H. pylori* possesses a diverse repertoire of antioxidant proteins [for a review see (18)] to combat toxic reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI) that are produced from neutrophils and macrophages as part of the host inflammatory response (19, 20). ROS and RNI can cause damage to bacterial macromolecules, including DNA, proteins, and lipids. Among *H. pylori* antioxidant proteins is the thioredoxin-dependent alkyl hydroperoxide reductase (AhpC). Along with Trx1 and TrxR, AhpC detoxifies lipid hydroperoxides (LOOH) as well as hydrogen peroxide (H_2O_2) and *t*-butyl hydroperoxide (*t*-BOOH) (21). Our proteomic study revealed that, under oxidative stress, AhpC is upregulated 1.3-fold in the wild-type strain and downregulated in 1.3-fold the *acnB* strain (Table 3.2). There are two putative aconitase-binding motifs in the 5' and 3' UTRs of the *ahpC* transcript, suggesting that apo-AcnB may be directly regulating expression of this protein, particularly under oxidative stress (Fig. 3.1).

In addition to AhpC, there are expression differences in superoxide dismutase (SodB) and catalase (KatA) (Table S3.2). SodB catalyzes the dismutation of superoxide (O_2^-) into hydrogen peroxide and oxygen, and for *H. pylori*, this enzyme has been shown to protect against DNA damage and aids in colonization (22). KatA detoxifies hydrogen peroxide into water and oxygen, and contributes to *H. pylori* colonization and survival in the presence of professional phagocytes (20, 23). Both SodB and KatA proteins are upregulated 1.7- and 1.0-fold in the wild-type strain under oxidative stress whereas both proteins are decreased 1.6- and 1.3-fold, respectively, in the *acnB* mutant under the same condition. There are no binding patterns for

aconitase on either of these transcripts, indicating that the differences may be indirectly related to aconitase. Methionine sulfoxide reductase (Msr) is upregulated 1.5-fold in the wild-type under oxidative stress, but downregulated 1.6-fold in *acnB* under the same condition. Msr is an important antioxidant enzyme in *H. pylori* that can repair oxidized methionine residues, and along with the chaperone protein, GroEL, has been shown to repair HOCl-treated KatA (24). The *msr* transcript does not contain any aconitase-binding patterns, and therefore, may also be an indirect effect of the *acnB* deletion.

Moreover, the thiol-peroxidase (Tpx) is upregulated 1.2-fold in the wild-type strain under oxidative stress, but is downregulated 1.9-fold in *acnB*. Deletion of *tpx* in *H. pylori* renders cells that are more sensitive to chemical oxidants and are attenuated in mouse colonization (25, 26). A putative aconitase-binding pattern was located in the 3' UTR of *tpx*, suggesting that aconitase may stabilize this transcript under oxidative stress (Fig. 3.1).

To further characterize the effect of oxidative stress on the *acnB* strain, we performed an air sensitivity assay by exposing cells to atmospheric oxygen (21% O_2) for 10 h. Every two hours, cells were removed, diluted, and plated (Fig. 3.2A). No *acnB* cells were recovered after 8 h of air exposure, whereas wild-type cells were recovered after 10 h. Additionally, we performed a disk assay with 0.4 M *t*-BOOH (Fig. 3.2B). The *acnB* strain exhibited a greater zone of inhibition than the wild-type, which was found to be statistically significant ($P < 0.05$) according to the Student's *t* test. These results correlate with our proteomic data that demonstrate decreased expression of some of the antioxidant proteins in *acnB*.

Urease and hydrogenase activities in the *acnB* strain. *H. pylori* successfully colonizes the acidic environment of the stomach by utilizing the nickel-containing enzyme urease to maintain a neutral pH in the cell (27). Urease has been shown to be essential for *H. pylori*

virulence in several studies (28-30). From the proteomic study, it was found that the UreA and UreB subunits of urease were upregulated in the wild-type and downregulated in the *acnB* strain (Table S3.2). UreA has previously been shown to be upregulated in *H. pylori* in response to oxidative stress (17). Urease activity was measured in cell-free extracts of wild-type and *acnB* strains grown with and without nickel supplementation. Under microaerobic conditions without nickel supplementation, the *acnB* strain had 5-fold less urease activity than the wild-type (Fig. 3.3). When nickel was supplemented in the growth medium, the *acnB* strain had approximately 7-fold less urease activity than the wild-type strain.

To test if activity of another nickel-dependent enzyme was affected by the aconitase deletion, we measured hydrogenase activity in both strains under microaerobic conditions. The *acnB* strain had 2-fold less hydrogenase activity than the wild-type (Table 3.3). Since both urease and hydrogenase enzymes require nickel for activity, we speculated that intracellular nickel was decreased in the *acnB* strain. A measurement of nickel levels using atomic absorption showed that the *acnB* strain had nearly 2-fold less intracellular nickel as compared to the wild-type under the same conditions (Table 3.4).

qPCR analysis on some of the genes involved in nickel metabolism revealed transcript level differences between the wild-type and *acnB* strains grown with or without added nickel (Fig. 3.4A-C). Interestingly, under conditions without supplemented nickel, the nickel transporters *frpB4*, *fecA3*, and *nixA* are downregulated in the *acnB* strain 6-, 2.5-, and 2-fold, respectively, as compared to the wild-type strain. Under nickel-supplemented conditions, transcript levels of *frpB4*, *fecA3*, and *nixA* between the wild-type and *acnB* strain are all similarly downregulated. Surprisingly, the *hpn* transcript is upregulated 1.6- and 2.6-fold in *acnB* under

both nickel and no nickel supplemented conditions, respectively. Hpn is the *H. pylori* nickel protein that sequesters nickel for storage (31, 32).

Collectively, these data show that in the *acnB* strain, there are decreased nickel levels leading to decreased urease and hydrogenase activities. This may be partly due to a decrease of nickel import in *acnB*, as shown by qPCR analysis of *frpB4*, *fecA3*, and *nixA*. Also, the nickel-sequestering activity of Hpn may contribute to decreased intracellular nickel levels (31). The 5' UTR of the *hpn* transcript was found to contain the consensus IRE sequence, suggesting a role for aconitase in mediating expression of this protein (Fig. 3.5). Aconitase may bind to the 5' UTR to decrease the activity of Hpn, leading to increased intracellular nickel in wild-type cells.

Aconitase regulates expression of genes related to motility. Motility is an essential factor for *H. pylori* colonization (33). The structure of bacterial flagella is complex and has a highly ordered assembly process [for a review see (34)]. *H. pylori* regulates transcription of flagellar genes using all three of its sigma factors: RpoD (σ^{80}), RpoN (σ^{54}), and FliA (σ^{28}). The role of aconitase as a posttranscriptional regulator for *H. pylori* flagellar-related genes has not yet been explored. In *S.* Typhimurium, deletion of *acnB* results in decreased motility and flagella production (6). It was found that apo-AcnB binds to the *ftsH* transcript, a cellular division protein, to indirectly regulate expression of the flagellum protein, FliC. In our proteomic study, *H. pylori* FtsH levels were found to be similar in both the wild-type and *acnB* strain under 2% O_2. Under 12% O_2, FtsH was upregulated 1.4-fold in the wild-type and downregulated 1.3-fold in *acnB*. No aconitase-binding patterns were found in the *H. pylori ftsH* transcript. Although the precise role of FtsH in *H. pylori* remains unknown, it has been shown to be required for cell viability and is part of an operon whose transcription is induced under heat shock and the presence of copper (35, 36).

Transcription of RpoN-dependent genes, including *flgBC*, *flgE*, and *flaB*, requires activation by the two-component system, FlgRS (33). Phosphorelay from the sensor kinase, FlgS, to the response regulator, FlgR, activates expression the RpoN regulon (34). Three putative aconitase-binding motifs (Fig. 3.6) were found in the *flgR* transcript that, upon binding of apo-AcnB, could influence transcription of RpoN-dependent flagellar genes. The RpoN-dependent proteins FlaB and FlgE were differentially expressed in *acnB* as compared to the wild-type (Tables S3.1 and S3.2). FlaB levels were increased 4-fold in *acnB* under 2% O_2 as compared to the wild-type. Under 12% O_2, FlaB was upregulated in the wild-type 6-fold, but remained constant in *acnB*. FlgE was downregulated 2.8- and 1.1-fold under 2% and 12% O_2, respectively, in the *acnB* strain as compared to the wild-type under the same conditions. Because of the expression differences of flagellar-related proteins in *acnB*, we assessed motility of the mutant strain and found that the deletion of *acnB* results in a non-motile strain (Fig. 3.7).

In conclusion, this study revealed that deletion of *acnB* results in pleiotropic effects in the cell, supporting the hypothesis that aconitase may be functioning as a global posttranscriptional regulator in *H. pylori*. Other studies support posttranscriptional regulation in *H. pylori* may play a major role in regulating the response to environmental change (37-39). Our proteomic study revealed expression differences in proteins related to oxidative stress, urease and hydrogenase, motility, and numerous others. Our bioinformatics search demonstrated overlap between some of the differentially expressed proteins and aconitase-binding patterns. It is possible that aconitase posttranscriptionally regulates multiple targets both directly and indirectly, perhaps simultaneously or in a hierarchal manner. Alternatively, some of the phenotypes we have observed may be a downstream effect due to the role of aconitase in posttranscriptionally regulating expression of proteins involved in replication, transcription, and protein synthesis and

folding (Table S3.1 and S3.2). Further experiments, including confirmation of direct targets for aconitase, are necessary to gain a more comprehensive knowledge of how aconitase influences these proteins at the posttranscriptional level.

TABLE 3.1 Primers used for qPCR

Name	Sequence (5' - 3')
HP1512 RT F (frpB4)	TCTAGCTGCAACGACTGGGAATGT
HP1512 RT R (frpB4)	AGCGCGAGAGCCAGGTTAGGATA
HP1400 RT F (fecA3)	TTGAGCGATCGCATTGAAGCTTGG
HP1400 RT R (fecA3)	AAGCCAATGTTGGTTGAGGGCATC
HP1077 RT F (nixA)	CGTTTGATGCGGATCACATCGCTT
HP1077 RT R (nixA)	TCTTCTAGCATCGGCGTGTGTTCT
hpn Forward qPCR	ATCACCACACACACCACCA (40)
hpn Reverse qPCR	GACTGTCGCTAGTGCTGCAA (40)

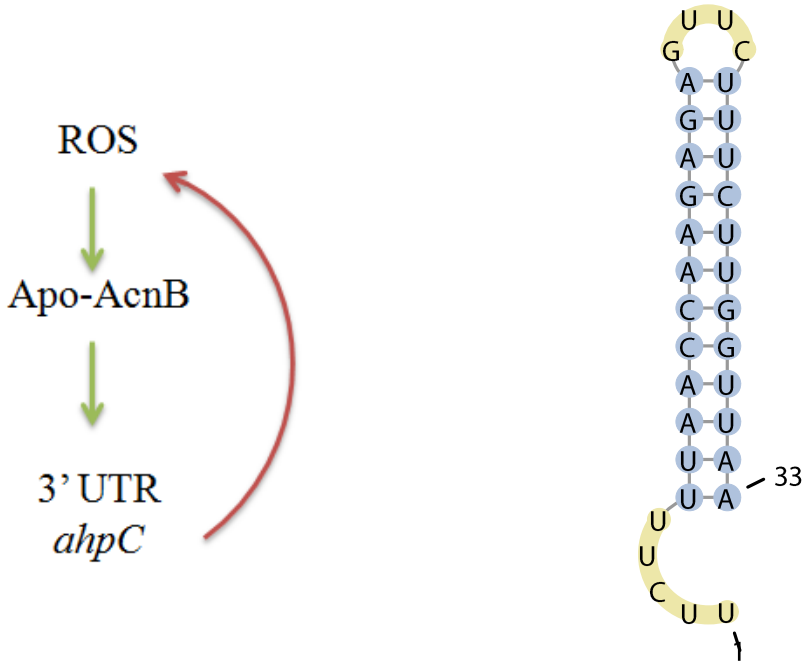
TABLE 3.2 Putative antioxidant proteins for aconitase-mediated regulation

Locus	**Description**	**WT 2% NSC***	**WT 12% NSC**	***acnB* 2% NSC**	***acnB*1 2% NSC**	**Pattern**	**Location**	**Distance****
HP0390	adhesin thiol peroxidase (Tpx)	15	18	17	9	GAGAG	3'	93
HP1563	alkyl hydroperoxide reductase (AhpC)	262	343	275	219	CAGCG; GAGAG	5'; 3'	87; 23

***** NSC = Normalized spectral count from proteomic approach

****** = bp from start or stop codon

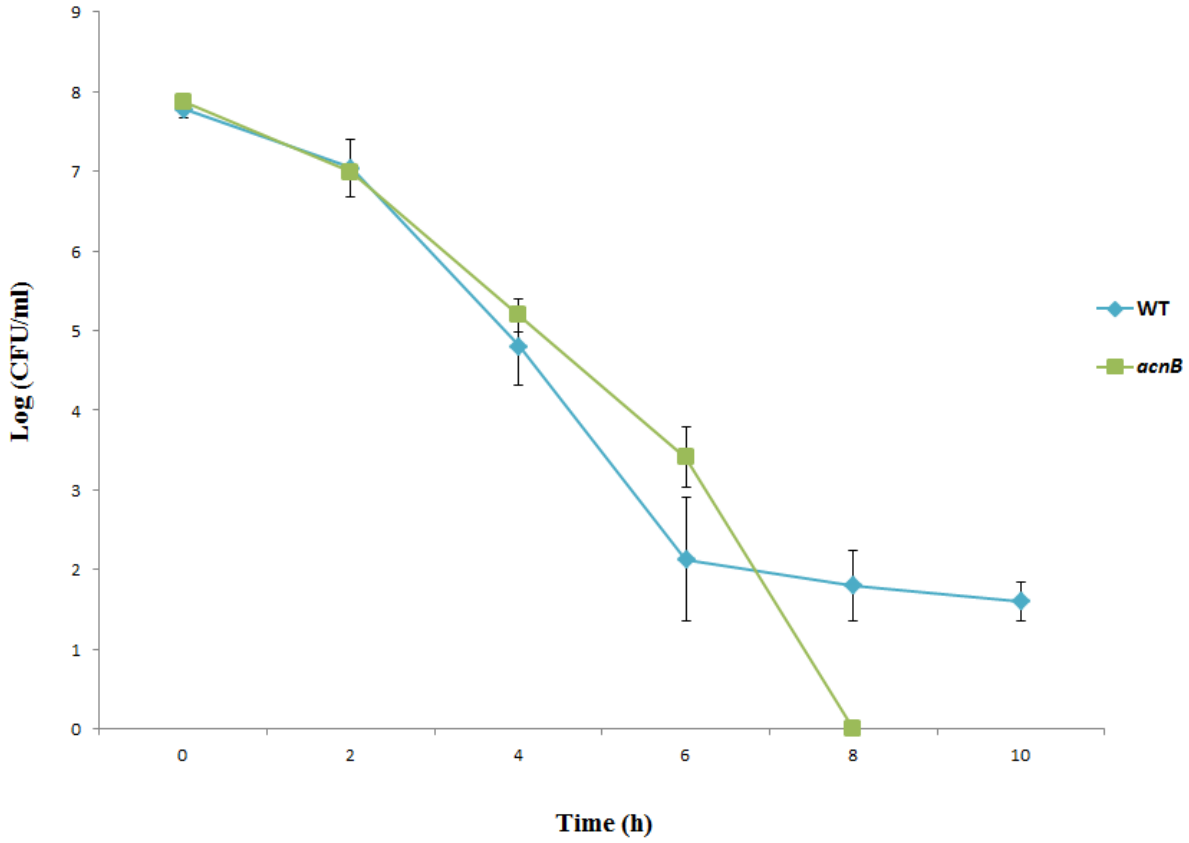
FIGURE 3.1 Putative model for aconitase-mediated regulation of *ahpC* and predicted stem-loop structure



In the presence of ROS, apo-AcnB protein is increased, which may bind to the 3' UTR of *ahpC* leading to decreased levels of ROS. The STAR program (41) was used to fold the sequence and PseudoViewer (42) was used to visualize the secondary structure.

FIGURE 3.2 Susceptibility to oxidative stress

A Air survival assay

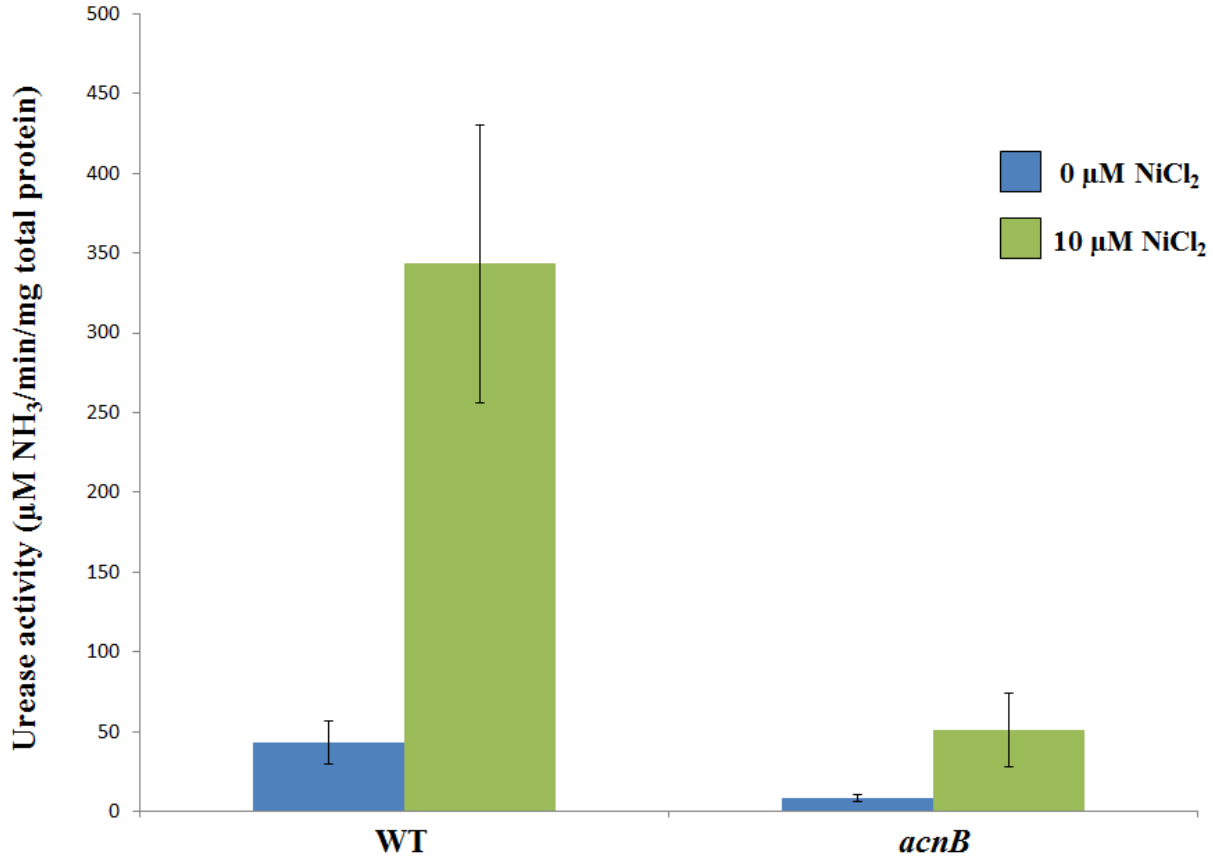


B Disk sensitivity assay

	Zone of inhibition (mm)
WT	32.4 ± 4.6
acnB	43.6 ± 4.8

The two values are statistically significant at $P < 0.05$ as determined by Student's *t* test.

FIGURE 3.3 Urease activity assay



The two values are statistically significant at $P < 0.01$ as determined by Student's *t* test.

TABLE 3.3 Hydrogenase activity [nmoles H_2 consumed min^{-1} $(10^9$ cells$)^{-1}$]

WT	***acnB***
5.5 $\pm$ 0.5	2.2 $\pm$ 0.4

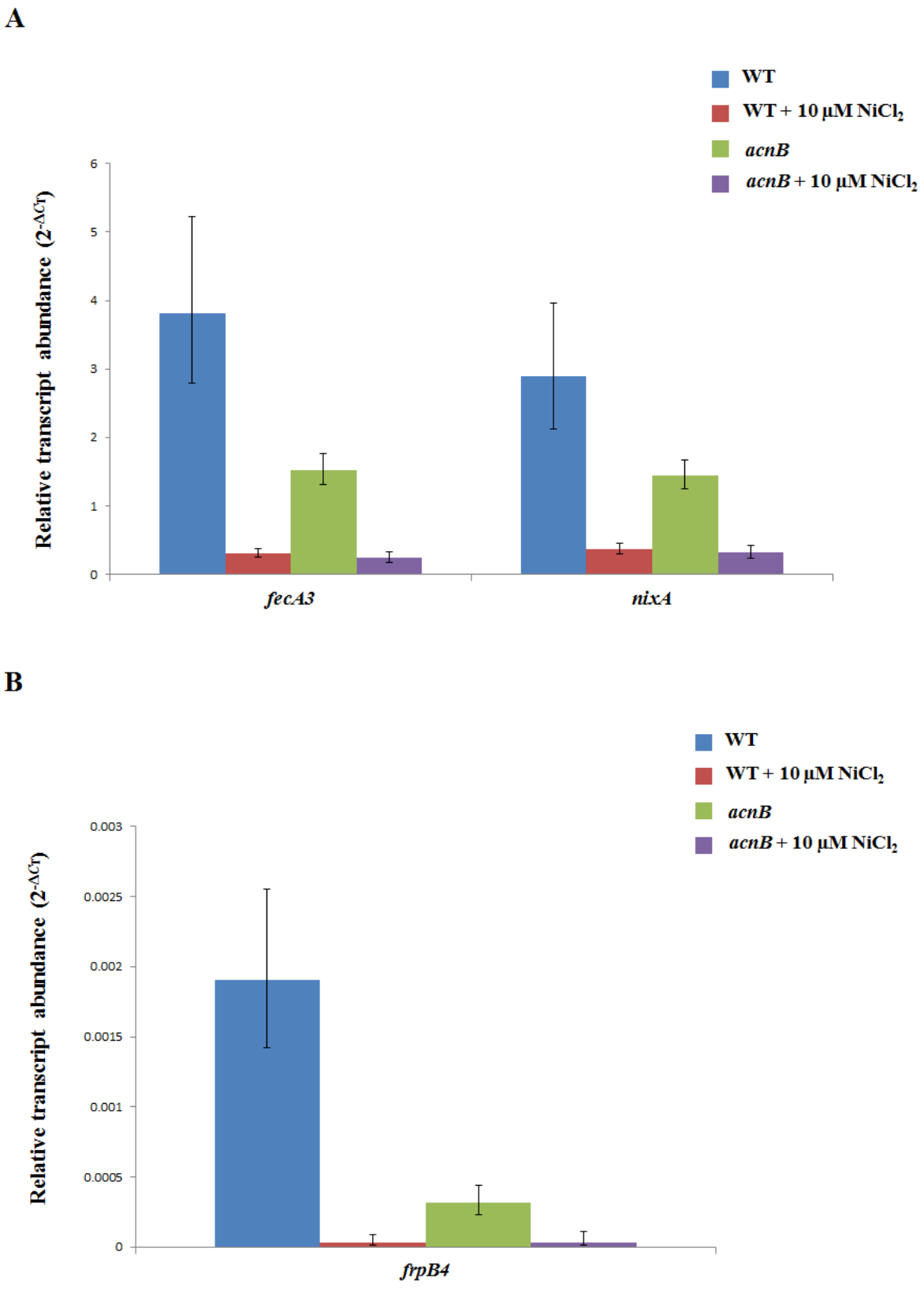
The two values are statistically significant at $P < 0.01$ as determined by Student's *t* test.

TABLE 3.4 Intracellular free nickel levels [ng Ni^{2+} (mg total protein)$^{-1}$]

WT	58.4 ± 0.1
acnB	32.0 ± 0.1

The two values are statistically significant at $P < 0.01$ as determined by Student's *t* test.

FIGURE 3.4 Effect of the *acnB* deletion on transcript levels



C

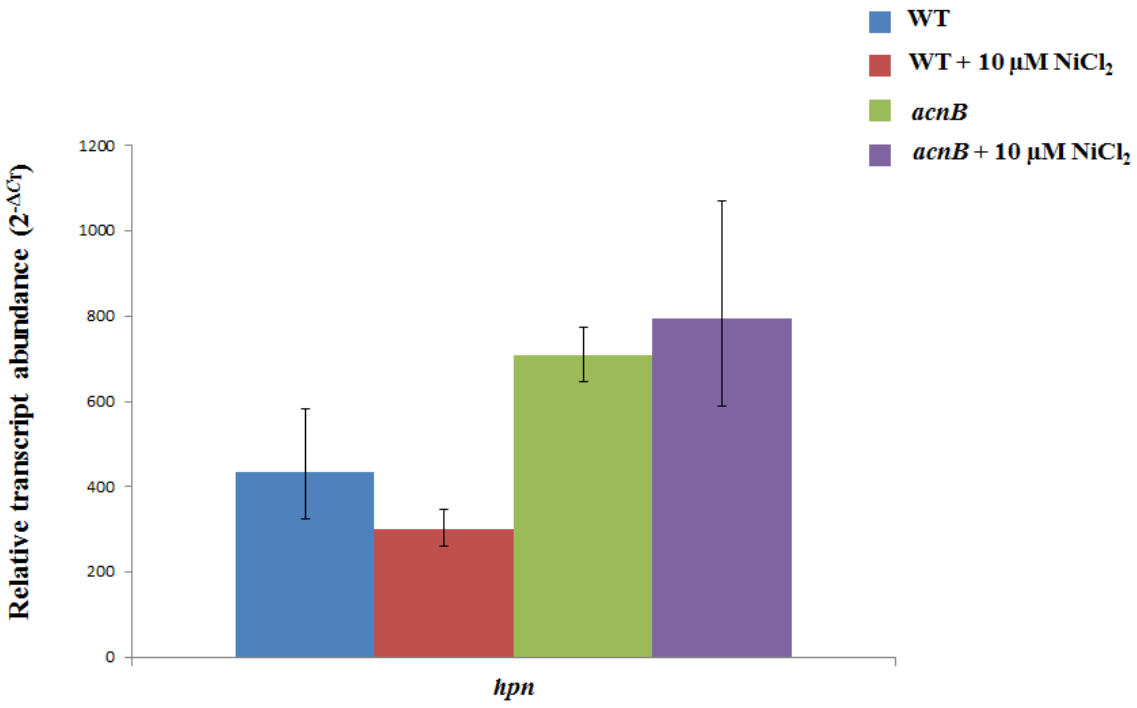
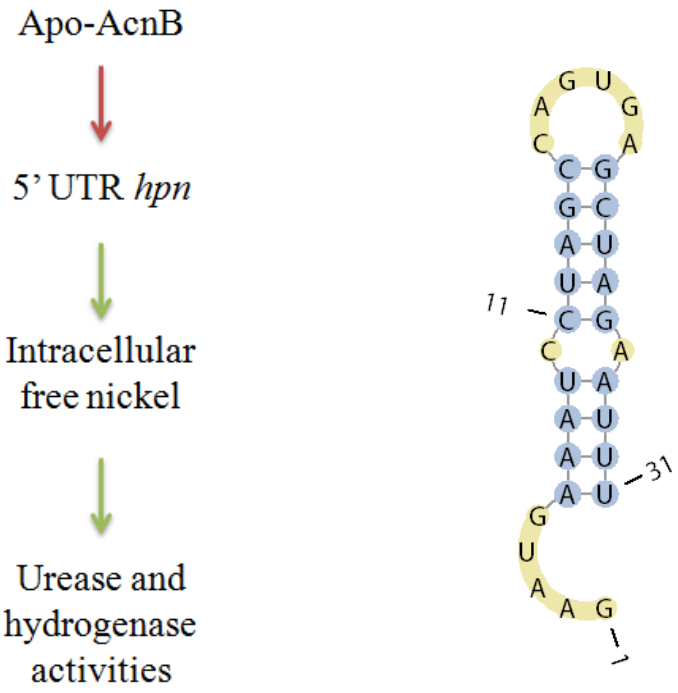
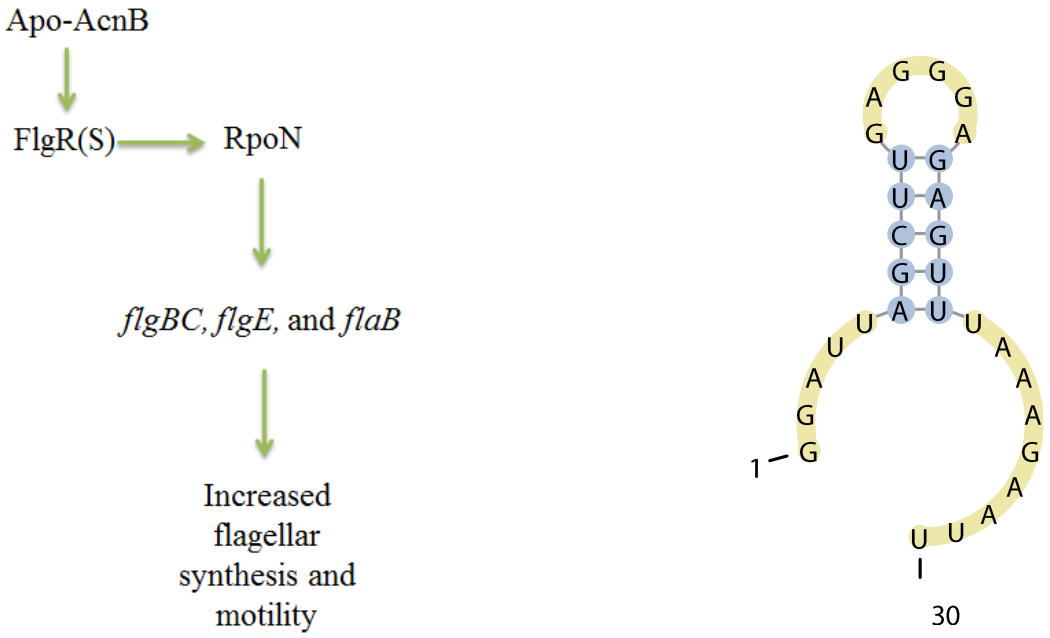

WT
WT + 10 µM NiCl2
acnB
acnB + 10 µM NiCl2
Relative transcript abundance (2-ΔCT)
1200
1000
800
600
400
200
0
hpn

FIGURE 3.5 Putative model for aconitase-mediated regulation of *hpn* and predicted stem-loop structure



Apo-AcnB may bind to the 5' UTR of *hpn*, leading to increased intracellular free nickel available for incorporation by urease and hydrogenase. The STAR program (41) was used to fold the sequence and PseudoViewer (42) was used to visualize the secondary structure.

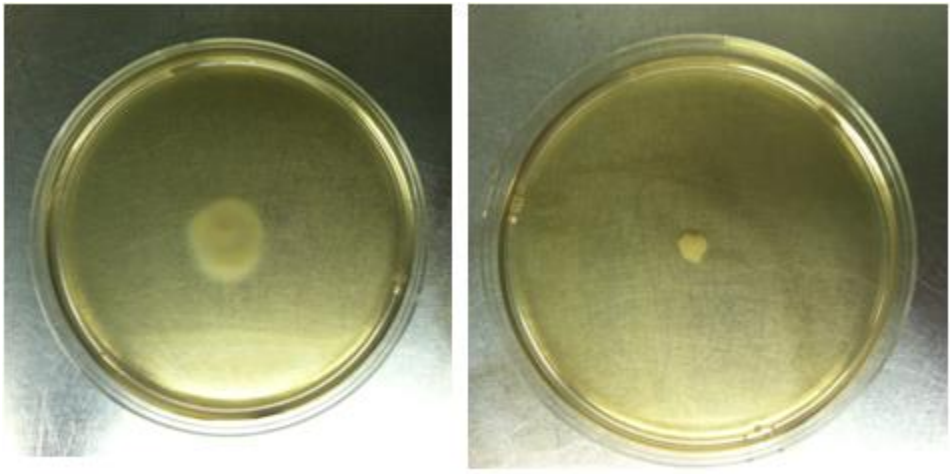
FIGURE 3.6 Putative model for aconitase-mediated regulation of *flgR* and predicted stem-loop structure



Apo-AcnB may bind to the 3' UTR of *flgR*, providing stability to the transcript and increasing transcription of the RpoN regulon, which contains flagellar-related genes. The STAR program (41) was used to fold the sequence and PseudoViewer (42) was used to visualize the secondary structure.

FIGURE 3.7 Motility assay

	Motility halo (mm)
WT	7.0 ± 0.8
acnB	N/A



WT ***acnB***

SUPPLEMENTARY TABLE 3.1 (Table S3.1) Putative targets for aconitase-mediated regulation based on proteomics approach and sequence patterns

Category	Locus	Description	WT 2% NSC*	WT 12% NSC	*acnB* 2% NSC	*acnB* 12% NSC	Pattern	Location	Distance**
Amino acid biosynthesis									
	HP0183	serine hydroxyl methyl-transferase (GlyA)	126	172	161	191	GGGAG	3'	1
	HP0512	glutamine synthetase (GlnA)	44	44	52	44	GAGAG; CAGUG	3'; 3'	209; 268
Biosynthesis of cofactors, prosthetic groups, and carriers									
	HP0163	delta-amino levulinic acid dehydratase (HemB)	5	2	0	1	GAGAG	5'	4
	HP1118	gamma-glutamyltranspeptidase (Ggt)	78	69	74	73	GAGAG; GAGAG	5'; 5'	69; 67
	HP1458	thioredoxin (Trx2)	29	28	27	36	CUGUG; CAGUG; GGGAG	3'; 3'; 3'	292; 215; 354
Cell envelope									
	HP0115	flagellin B (FlaB)	1	6	4	4	CAGCG	5'	69
	HP0127	outer membrane protein (Omp4)	3	2	0	0	GAGAG	5'	153
	HP0175	cell binding factor 2	97	124	120	164	CUGUG	5'	81
	HP0410	neuraminyllactose-binding hemagglutinin (HpaA)	15	25	26	24	GAGAG; GGGAG	3'; 3'	32; 70
	HP0752	flagellar hook-associated protein 2 (FliD)	35	45	20	38	GAGAG	3'	38
	HP0913	outer membrane protein	13	8	21	24	GAGAG; GAGAG	3'; 3'	107; 704

		(Omp21)							
	HP1243	outer membrane protein (Omp28)	30	25	27	47	GAGAG; GGGAG	3'; 3'	559; 481
	HP1501	outer membrane protein (Omp32)	10	11	15	30	CUGUG; CUUUCA UCUCUA AG	3'; 3'	83; 25
	HP1564	outer membrane protein	74	86	133	101	GAGAG	5'	113
General cellular processes									
	HP0082	methyl-accepting chemotaxis transducer (TlpC)	3	13	4	16	GGGAG	5'	122
	HP0103	methyl-accepting chemotaxis protein (TlpB)	0	1	3	5	GGGAG; CAGGG	3'; 3'	6; 2
Chaperones									
	HP0010	molecular chaperone (GroEL)	250	209	281	282	CAGCG	3'	424
	HP0109	molecular chaperone (DnaK)	28	82	101	119	CAGUG; GGGAG	3'; 3'	287; 206
Protein and peptide secretion	HP0795	trigger factor (Tig)	7	5	4	1	GAGAG	3'	13
Trans-formation	HP0520	cag pathogenicity island protein (Cag1)	1	6	2	1	GAGAG	3'	38
Detoxification									
	HP0390	adhesin thiol peroxidase (Tpx)	15	18	17	9	GAGAG	3'	93
	HP1563	alkyl hydroperoxide reductase (AhpC)	262	343	275	219	CAGCG; GAGAG	5'; 3'	87; 23
Energy metabolism									
	HP0121	phosphoenol pyruvate synthase (PpsA)	3	7	5	7	GGGAG	5'	5
	HP0961	glycerol-3-	5	11	4	9	GAGAG;	3'; 5'	8; 76

		phosphate dehydrogenase, NAD(P)+ dependent					CAGCG		
	HP1099	2-keto-3-deoxy-6-phosphogluconate aldolase (Eda)	35	48	41	23	CCAUGAUCUUUUUG	3'	1
	HP1103	glucokinase (Glk)	16	19	22	33	CAGCG	3'	1
	HP1186	carbonic anhydrase	2	7	17	6	GGGAG; GGGAG	5'; 3'	142; 172
	HP1238	aliphatic amidase (AimE)	5	2	2	0	CAGCG	5'	149
	HP1325	fumarase (FumC)	6	5	8	7			
	HP1399	arginase (RocF)	1	2	0	2	GAGAG	3'	78
Purines, pyrimidines, nucleotides									
	HP0742	phosphoribosylpyrophosphate synthetase (PrsA)	18	10	9	10	GGGAG; GGGAG	5'; 3'	9; 18
	HP0825	putative thioredoxin reductase? (Note: not the same as TrxR (HP1164)	33	133	98	82	GGGAG	3'	203
Regulation									
	HP1287	transcriptional regulator (TenA)	0	2	2	7	CAGGG	5'	95
Replication									
	HP0275	ATP-dependent nuclease (AddB)	5	4	9	9	CAGUG	5'	23
	HP0501	DNA gyrase, sub B (GyrB)	1	4	1	6	GAGAG; GAGAG; CAGCG	3'; 3'; 3'	38; 40; 34
	HP0835	histone-like DNA-binding protein (Hup)	12	16	14	15	CAGGG	3'	31

Translation									
	HP0083	ribosomal protein S9 (Rps9)	7	5	7	5	GGGAG	3'	30
	HP0177	translation elongation factor EF-P (Efp)	15	16	21	15	CUGUG; GAGAG	3'; 3'	100; 398
	HP0794	ATP-dependent Clp protease proteolytic subunit (ClpP)	11	6	7	9	GAGAG	5'	1
	HP0960	glycyl-tRNA synthetase, alpha subunit (GlyQ)	1	4	2	2	GAGAG	5'	2
	HP1199	50S ribosomal protein L7/L12 (RpL7/Rpl12)	11	20	32	42	CUGUG	5'	37
	HP1200	ribosomal protein L10 (Rpl10)	1	3	0	2	CUGUG	3'	5
	HP1205	elongation factor Tu (Tuf)	42	86	116	141	GAGAG	3'	13
	HP1320	ribosomal protein S10 (Rps10)	1	5	2	11	GAGAG	5'	4
	HP1350	protease	6	5	1	7	GGGAG	5'	132
	HP1379	ATP-dependent protease (Lon)	2	2	5	8	GAGAG	5'	8
Transport									
	HP1172	glutamine ABC transporter (GlnH)	2	21	5	14	CAGGG	3'	25
	HP1562	iron(III) ABC transportersubstrate-binding protein (CeuE)	14	25	33	36	CAGCG	5'	150
Hypothetical									
	HP0129	hypothetical protein	26	45	40	40	GAGAG	3'	349

	HP0130	hypothetical protein	10	12	15	13	GAGAG; GAGAG; GGGAG; CUGUG; CUGUG; GAGAG	5'; 3'; 5'; 5'; 5'; 3'	58; 392; 191; 342; 228; 394
	HP0486	hypothetical protein	21	12	12	12	GAGAG	3'	1
	HP0654	hypothetical protein	0	0	1	1	GAGAG	5'	225
	HP0891	hypothetical protein	7	14	5	1	CAGUG	5'	178
	HP1098	hypothetical protein	14	29	32	27	CCAUGAUCUUUUUG	5'	56
	HP1173	hypothetical protein	9	16	15	14	CAGGG; CAGUG	5'; 3'	93; 70
	HP1414	hypothetical protein	0	0	1	2	GGGAG	3'	29
	HP1454	hypothetical protein	17	39	11	27	GAGAG; GAGAG; GAGAG; CUGUG	3'; 3'; 3'; 3'	327; 759; 791; 103
	HP1457	hypothetical protein	2	9	5	7	CUGUG; CAGUG; GGGAG	5'; 5'; 5'	74; 151; 12
	HP1588	hypothetical protein	7	16	11	6	GAGAG; GGGAG; CAGCG	5'; 3'; 5'	186; 74; 59
Other									
	HP0485	catalase-like protein	39	44	26	57	GGGAG	3'	60
	HP0596	tumor necrosis factor alpha-inducing protein	20	25	28	23	CUGUG; CUUAAAGAUUUAUG	5'; 5'	24; 36
	HP1104	cinnamyl-alcohol dehydrogenase (Cad)	60	60	53	63	CAGCG	5'	157

***** NSC = Normalized spectral count from proteomic approach

****** = bp from start or stop codon

SUPPLEMENTARY TABLE 3.2 (Table S3.2) Putative targets for indirect regulation by aconitase based on proteomics approach

Category	**Locus**	**Description**	**WT 2% NSC ***	**WT 12% NSC**	***acnB* 2% NSC**	***acnB* 12% NSC**
Amino acid biosynthesis						
	HP0002	riboflavin synthase beta chain (RibE)	8	8	2	6
	HP0098	threonine synthase (ThrC)	13	19	25	21
	HP0106	cystathionine gamma-synthase (MetB)	42	27	35	28
	HP0107	cysteine synthetase (CysK)	7	5	11	7
	HP0283	3-dehydroquinate synthase (AroB)	3	0	2	3
	HP0330	ketol-acid reductoisomerase (IlvC)	6	3	1	4
	HP0380	glutamate dehydrogenase (GdhA)	40	38	39	32
	HP0397	phosphoglycerate dehydrogenase (SerA)	2	1	4	3
	HP0570	leucyl aminopeptidase (PepA)	97	82	62	69
	HP0649	aspartate ammonia-lyase (AspA)	39	22	11	19
	HP0672	aspartate aminotransferase (AspB)	27	26	47	27
	HP0736	phosphoserine aminotransferase (SerC)	11	13	12	7
	HP0777	uridine 5'-monophosphate (UMP) kinase (PyrH)	4	5	4	7
	HP1013	dihydrodipicolinate synthetase (DapA)	16	24	29	25
	HP1038	3-dehydroquinate dehydratase (AroQ)	27	61	40	45
	HP1468	branched-chain-amino-acid aminotransferase (IlvE)	13	5	21	17
Biosynthesis of cofactors, prosthetic groups, and carriers						
	HP0407	biotin sulfoxide reductase (BisC)	25	12	13	12
	HP0604	uroporphyrinogen decarboxylase (HemE)	8	5	6	16
	HP0824	thioredoxin (Trx1)	53	53	36	57
	HP0928	GTP cyclohydrolase I (FolE)	1	0	1	1
	HP1058	3-methyl-2-oxobutanoate hydroxymethyltransferase (PanB)	4	5	5	0
	HP1164	thioredoxin reductase (TrxR)	6	2	17	15
	HP1355	nicotinate-nucleotide pyrophosphorylase (NadC)	2	2	3	2
	HP1582	pyridoxal phosphate biosynthetic protein J (PdxJ)	11	29	23	13

Cell envelope						
	HP0003	3-deoxy-d-manno-octulosonic acid 8-phosphate synthetase (KdsA)	24	40	35	25
	HP0229	outer membrane protein (Omp6)	1	0	1	3
	HP0232	secreted protein involved in flagellar motility	7	12	10	6
	HP0252	outer membrane protein (Omp7)	2	2	0	1
	HP0254	outer membrane protein (Omp8)	0	3	3	3
	HP0295	flagellin B homolog (Fla)	7	10	3	6
	HP0317	outer membrane protein (Omp9)	21	21	23	37
	HP0472	outer membrane protein (Omp11)	33	49	30	58
	HP0601	flagellin A (FlaA)	34	37	34	46
	HP0638	outer membrane protein (Omp13)	3	3	7	6
	HP0655	protective surface antigen D15	5	18	7	16
	HP0671	outer membrane protein (Omp14)	3	5	3	3
	HP0706	outer membrane protein (Omp15)	4	6	4	4
	HP0797	flagellar sheath adhesin (HpaA)	11	22	23	22
	HP0830	Glu-tRNA(Gln) amidotransferase, subunit A	4	9	7	1
	HP0857	phosphoheptose isomerase	3	7	2	0
	HP0870	flagellar hook (FlgE)	58	54	21	48
	HP0907	hook assembly protein, flagella (FlgD)	4	0	6	5
	HP0912	outer membrane protein (Omp20)	19	8	20	18
	HP1125	peptidoglycan-associated lipoprotein	19	35	35	29
	HP1177	outer membrane protein (Omp27)	3	8	17	24
	HP1275	phosphomannomutase (AlgC)	8	14	7	5
	HP1375	UDP-N-acetylglucosamine acyltransferase (LpxA)	1	2	2	2
	HP1456	membrane-associated lipoprotein (Lpp20)	32	51	22	25
	HP1469	outer membrane protein (Omp31)	6	14	12	12
Central intermediary metabolism						
	HP0068	urease accessory protein (UreG)	2	1	8	3
	HP0072	urease subunit beta (UreB)	306	341	493	202
	HP0073	urease subunit alpha (UreA)	329	566	443	225
	HP0075	urease protein (UreC)	5	9	9	3
	HP0147	cytochrome c oxidase, diheme subunit, membrane-bound (FixP)	3	3	8	1
	HP0620	inorganic pyrophosphatase (Ppa)	12	21	16	12
	HP0723	L-asparaginase II (AnsB)	49	47	25	56
	HP0900	hydrogenase expression/formation	5	2	13	14

		protein (HypB)				
	HP1014	7-alpha-hydroxysteroid dehydrogenase (HdHA)	5	4	4	4
	HP1111	pyruvate ferredoxin oxidoreductase, beta subunit	6	11	14	12
General cellular processes						
	HP0392	histidine kinase (CheA)	10	8	17	22
	HP0599	hemolysin secretion protein precursor (HylB)	1	7	4	8
	HP1067	chemotaxis protein (CheY)	8	12	16	20
Cell division						
	HP1069	cell division protein (FtsH)	5	7	5	4
Cell killing						
	HP0887	vacuolating cytotoxin	15	23	4	11
Chaperones						
	HP0011	co-chaperonin (GroES)	30	70	55	84
	HP0110	co-chaperone and heat-shock protein (GrpE)	3	13	14	7
Protein and peptide secretion						
	HP0786	preprotein translocase subunit (SecA)	4	9	19	23
Transformation						
	HP0543	cag pathogenicity island protein (Cag22)	1	2	1	1
	HP0547	cag pathogenicity island protein (Cag26)	18	39	35	26
Detoxification						
	HP0243	neutrophil activating protein (NapA)	98	156	71	134
	HP0389	iron-dependent superoxide dismutase (SodB)	86	146	121	75
	HP0875	catalase (KatA)	545	560	581	453
Energy metabolism						
	HP0026	citrate synthase (GltA)	112	98	46	91
	HP0027	isocitrate dehydrogenase	39	43	59	78
	HP0056	delta-1-pyrroline-5-carboxylate dehydrogenase	3	5	10	7
	HP0154	enolase (Eno)	28	10	23	7
	HP0176	fructose-bisphosphate aldolase (Tsr)	104	166	140	160
	HP0191	iron-sulfur subunit of fumarate reductase (FrdB)	8	7	7	6

	HP0192	fumarate reductase flavoprotein subunit	22	31	11	34
	HP0194	triosephosphate isomerase (Tpi)	8	5	7	5
	HP0588	delta subunit of 2-oxoglutarate:acceptor oxidoreductase	4	3	3	4
	HP0589	alpha subunit of 2-oxoglutarate:acceptor oxidoreductase	59	53	86	49
	HP0590	beta subunit of 2-oxoglutarate:acceptor oxidoreductase	13	19	28	15
	HP0591	gamma subunit of 2-oxoglutarate:acceptor oxidoreductase	3	4	3	3
	HP0631	quinone-reactive Ni/Fe hydrogenase, small subunit (HydA)	2	4	2	3
	HP0974	phosphoglycerate mutase (Pgm)	3	4	7	4
	HP1100	phosphogluconate dehydratase	18	11	30	5
	HP1108	pyruvate flavodoxin oxidoreductase subunit gamma (PorC, PorG)	16	7	8	13
	HP1110	pyruvate flavodoxin oxidoreductase subunit alpha (PorA)	33	18	39	27
	HP1132	F-type H+-transporting ATPase subunit beta (AtpD)	60	66	68	84
	HP1133	ATP synthase F1, subunit gamma (AtpG)	18	6	14	10
	HP1134	F-type H+-transporting ATPase subunit alpha (AtpA)	47	21	43	32
	HP1161	flavodoxin (FldA)	58	93	97	92
	HP1325	fumarase (FumC)	6	5	8	7
	HP1345	phosphoglycerate kinase	6	10	8	3
	HP1346	glyceraldehyde-3-phosphate dehydrogenase (Gap)	4	4	5	4
	HP1385	fructose-1,6-bisphosphatase	11	14	9	20
	HP1386	ribulose-phosphate 3-epimerase	3	2	1	0
	HP1398	alanine dehydrogenase (Ald)	33	31	30	25
	HP1461	cytochrome c551 peroxidase	20	9	5	5
	HP1495	transaldolase	3	11	13	7
Fatty acid and phospholipid metabolism						
	HP0201	fatty acid/phospholipid synthesis protein (PlsX)	3	3	1	5
	HP0372	deoxycytidine triphosphate deaminase (Dcd)	3	6	4	6
	HP0195	enoyl-(acyl-carrier-protein) reductase (NADH) (FabI)	2	8	2	3
	HP0371	biotin carboxyl carrier protein (FabE)	8	12	9	3

	HP0558	3-oxoacyl-ACP synthase	27	34	57	22
	HP0690	acetyl coenzyme A acetyltransferase (thiolase) (FadA)	57	61	58	54
	HP1045	acetyl-CoA synthetase (AcoE)	1	3	3	2
	HP1376	(3R)-hydroxymyristoyl-(acyl carrier protein) dehydratase (FabZ)	8	15	13	6
Purines, pyrimidines, nucleotides						
	HP0198	nucleoside diphosphate kinase (Ndk)	4	7	9	4
	HP0646	UDP-glucose pyrophosphorylase	1	1	2	7
	HP0757	beta-alanine synthetase homolog	2	4	3	4
	HP0829	inosine 5'-monophosphate dehydrogenase	23	14	7	9
	HP0865	deoxyuridine 5'-triphosphate nucleotidohydrolase	10	13	9	16
	HP1178	purine-nucleoside phosphorylase (DeoD)	20	25	11	14
Regulation						
	HP0088	RNA polymerase sigma-70 factor (RpoD)	8	8	11	12
	HP0224	peptide methionine sulfoxide reductase (Msr)	11	16	29	18
Replication						
	HP0054	adenine/cytosine DNA methyltransferase	1	2	12	13
	HP0323	membrane bound endonuclease (Nuc)	14	12	10	20
	HP0500	DNA polymerase III subunit beta (DnaN)	18	17	19	26
	HP0615	DNA ligase (Lig)	4	1	1	1
Transcription						
	HP1198	DNA-directed RNA polymerase, beta subunit (RpoB)	50	93	84	97
	HP1213	polynucleotide phosphorylase (Pnp)	1	0	2	7
Translation						
	HP0264	ATP-dependent protease binding subunit/heat shock protein (ClpB)	20	43	58	48
	HP0296	ribosomal protein L21 (Rpl21)	2	2	2	4
	HP0297	ribosomal protein L27 (Rpl27)	6	5	3	16
	HP0402	phenylalanyl-tRNA synthetase, beta subunit (PheT)	1	7	2	0
	HP0470	oligoendopeptidase F (PepF)	8	3	1	7
	HP0515	ATP-dependent HslUV protease (HslV)	12	14	11	14

	HP0617	aspartyl-tRNA synthetase	4	2	1	4
	HP0657	processing protease	4	3	2	7
	HP0944	putative translation inhibitor protein	11	14	7	15
	HP1019	serine protease (HtrA)	45	18	37	31
	HP1040	ribosomal protein S15 (Rps15)	2	1	5	6
	HP1048	translation initiation factor IF-2 (InfB)	17	13	19	18
	HP1147	ribosomal protein L19 (Rpl19)	2	0	1	3
	HP1195	translation elongation factor EF-G (FusA)	34	51	36	46
	HP1196	ribosomal protein S7 (Rps7)	15	20	17	24
	HP1197	ribosomal protein S12 (Rps12)	1	0	2	3
	HP1201	ribosomal protein L1 (Rpl1)	4	3	3	2
	HP1202	50S ribosomal protein L11 (RplK)	9	26	38	35
	HP1241	alanyl-tRNA synthetase (AlaS)	11	7	11	12
	HP1256	ribosome releasing factor (Frr)	17	28	16	12
	HP1296	ribosomal protein S13 (Rps13)	9	7	11	22
	HP1299	methionine amino peptidase (Map)	2	0	7	6
	HP1301	ribosomal protein L15 (Rpl15)	2	9	4	16
	HP1303	ribosomal protein L18 (Rpl18)	31	61	36	46
	HP1304	ribosomal protein L6 (Rpl6)	9	15	11	4
	HP1305	ribosomal protein S8 (Rps8)	3	5	1	3
	HP1307	ribosomal protein L5 (Rpl5)	3	6	9	3
	HP1310	ribosomal protein S17 (Rps17)	4	0	1	2
	HP1312	ribosomal protein L16 (Rpl16)	1	1	1	3
	HP1314	ribosomal protein L22 (Rpl22)	3	3	2	5
	HP1315	ribosomal protein S19 (Rps19)	1	0	4	2
	HP1316	ribosomal protein L2 (Rpl2)	1	6	2	3
	HP1317	ribosomal protein L23 (Rpl23)	3	4	3	5
	HP1319	ribosomal protein L3 (Rpl3)	2	1	5	5
	HP1422	isoleucyl-tRNA synthetase (IleS)	5	6	13	19
	HP1441	peptidyl-prolyl cis-trans isomerase B (Ppi)	13	9	8	18
	HP1547	leucyl-tRNA synthetase (LeuS)	5	2	4	4
	HP1555	elongation factor Ts (Tsf)	17	10	19	14
Transport						
	HP0298	dipeptide ABC transporter substrate-binding protein (DppA)	27	21	24	16
	HP0653	nonheme iron-containing ferritin (Pfr)	55	58	60	55
	HP1339	biopolymer transport protein (ExbB)	1	2	0	2
	HP1561	iron(III) ABC transportersubstrate-binding protein (CeuE)	6	14	12	15
	HP1576	ABC transporter, ATP-binding protein	20	13	8	7

		(Abc)				
Hypothetical						
	HP0105	hypothetical protein	9	8	13	4
	HP0170	hypothetical protein	17	15	18	23
	HP0204	hypothetical protein	11	11	9	16
	HP0211	hypothetical protein	1	7	5	4
	HP0218	hypothetical protein	18	34	23	12
	HP0231	hypothetical protein	63	98	97	102
	HP0268	hypothetical protein	1	0	5	3
	HP0305	hypothetical protein	68	99	73	55
	HP0318	hypothetical protein	9	13	9	8
	HP0564	hypothetical protein	3	5	2	5
	HP0721	hypothetical protein	37	46	46	39
	HP0773	hypothetical protein	9	2	14	13
	HP0783	hypothetical protein	2	2	5	4
	HP0902	hypothetical protein	20	8	16	22
	HP0953	hypothetical protein	4	2	1	5
	HP0958	hypothetical protein	5	4	7	8
	HP0973	hypothetical protein	28	38	37	19
	HP1029	hypothetical protein	2	5	4	0
	HP1037	hypothetical protein	25	22	63	34
	HP1065	hypothetical protein	1	2	1	1
	HP1285	hypothetical protein	17	36	27	36
	HP1286	hypothetical protein	17	19	14	17
	HP1388	hypothetical protein	23	18	26	18
	HP1551	hypothetical protein	1	2	1	4
Other						
	HP0315	virulence associated protein D (VapD)	11	6	8	8
	HP0322	poly E-rich protein	5	5	7	4
	HP0480	GTP-binding protein, FusA-homolog	9	8	7	19
	HP0569	GTP-binding protein (Gtp1)	2	3	2	4
	HP0606	membrane fusion protein (MtrC)	3	3	6	5
	HP0630	NADPH-specific quinone reductase (MdaB)	5	20	9	14
	HP0658	Glu-tRNA(Gln) amidotransferase, subunit B	5	6	5	3
	HP0827	ss-DNA binding protein 12RNP2 precursor	4	1	5	10
	HP0930	stationary-phase survival protein	5	4	3	7
	HP1034	ATP-binding protein (YlxH)	9	6	11	6
	HP1430	conserved hypothetical ATP-binding protein	10	18	14	22

	HP1476	3-octaprenyl-4-hydroxybenzoate carboxy-lyase	11	17	5	12
	HP1496	general stress protein (Ctc)	6	5	2	1

***** NSC = Normalized spectral count from proteomic approach

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CHAPTER 4

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Helicobacter pylori is a Gram-negative, gastric pathogen that infects about half of the world's population and is the etiological agent for gastritis, peptic ulcer disease, and gastric cancer (1). This bacterium is remarkable in that it is able to successfully colonize the unique niche of the stomach, despite being a neutralophile. Not only does *H. pylori* have to travel through the acidic environment of the lumen to reach the gastric mucosa where it colonizes, but it also has to combat toxic reactive oxygen species that accumulate as part of the host inflammatory response. To circumvent damage by the acidic environment of the stomach, *H. pylori* uses the enzyme urease to hydrolyze urea into ammonia and carbon dioxide. The ammonia produced acts as a buffering agent to neutralize the surrounding microenvironment. *H. pylori* is also well equipped with a variety of enzymes that directly detoxify reactive oxygen species and repair damaged macromolecules. Interestingly, *H. pylori* possesses relatively few regulatory systems (as compared to some bacteria, for example, *Escherichia coli*) and lacks homologues of the stress-responsive transcriptional regulators, RpoS (σ^{38}) and RpoH (σ^{32}). It is therefore of interest to study how this bacterium regulates the response to environmental stresses, including acidity and oxidative stress. Aconitase is a bifunctional protein that participates in the tricarboxylic acid cycle and acts as a posttranscriptional regulator (2). I propose that *H. pylori* uses aconitase to posttranscriptionally regulate protein expression on a global scale.

I began this work by exploring the putative role for aconitase in posttranscriptionally regulating expression of peptidoglycan deacetylase (PgdA). PgdA is a peptidoglycan-modifying

enzyme that has been shown to be important for virulence in *H. pylori* (3). Expression of PgdA contributes to host immune system evasion and resistance to lysozyme degradation (3, 4). Initially, I examined the *pgdA* transcript for putative aconitase-binding patterns. I found that the *pgdA* 3' UTR contained two putative aconitase-binding stem-loop structures. I performed electrophoretic mobility shift assays and found that apo-aconitase binds to the *pgdA* 3' UTR. RNA footprinting revealed that some of the bases in the *pgdA* 3' UTR were protected from ribonuclease degradation as a result of aconitase-binding. I also found that expression of PgdA, especially in cells grown under 12% O_2, is significantly decreased in *acnB* as compared to the wild-type. Furthermore, *acnB* cells are more sensitive to lysozyme degradation and are attenuated for mouse colonization.

My research investigating the role of aconitase in mediating PgdA expression prompted me to conduct further experiments to determine if aconitase posttranscriptionally regulates expression of other proteins in *H. pylori*, particularly those related to oxidative stress. I subjected wild-type and *acnB* cells to 2% and 12% O_2 (control vs. oxidative stress) growth conditions and compared the protein expression differences between them. From the proteomic data, I identified numerous differentially expressed proteins in the *acnB* strain as compared to the wild-type strain. Many of these are likely to be indirect effects of the *acnB* deletion; however, some are putative targets for aconitase-mediated regulation. Proteins involved in oxidative stress, urease and hydrogenase proteins, and flagellar-related proteins were differentially expressed in the *acnB* strain as compared to the wild-type.

AhpC, a detoxification protein involved in the oxidative stress defense, as well as the UreAB subunits of urease were downregulated in *acnB* as compared to the wild-type. Additionally, from qPCR analysis, I found that the *acnB* strain has nearly 2-fold increased *hpn*

transcript than the wild-type. I used the Pattern Locator program (5) to search for established aconitase-binding patterns among the DNA sequences of putative targets of interest. Both the *ahpC* and *hpn* transcripts contain aconitase-binding patterns. Also, I found that the *acnB* strain is more sensitive to oxidative stress and has decreased urease and hydrogenase activities. From these data, I hypothesize that aconitase is directly influencing expression of AhpC and Hpn leading to the phenotypes I observed in the *acnB* strain.

Furthermore, the *acnB* strain is non-motile and this may be in part due to aconitase posttranscriptionally regulating expression of FlgR, the response regulator, which contains aconitase-binding patterns in its 3' UTR. The two component system FlgRS is necessary for activation of the RpoN regulon, which contains the flagellar genes *flaB* and *flgE*. From the proteomic study, both FlaB and FlgE were found to be differentially expressed in *acnB* as compared to the wild-type. This may be partly due to an aconitase-*flgR* interaction. It is necessary to confirm binding of aconitase to the *ahpC*, *hpn*, and *flgR* transcripts using electrophoretic mobility shift assays.

Numerous other proteins, for example, those involved in amino acid biosynthesis, transport, DNA replication, transcription, and translation were also identified to be differentially expressed in *acnB* as compared to the wild-type strain but not investigated any further. It would be interesting to determine if aconitase plays a role in mediating expression of any of those proteins. Analyses of putative target transcripts, qPCR, and Western blotting as well as establishing other phenotypes for the *acnB* strain are some of the preliminary experiments that can be performed to begin investigating other targets for aconitase-mediated regulation.

References

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