Development of Molecular Tools to Evaluate Colonization Factors of *Helicobacter pylori*

by

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Abstract

Helicobacter pylori is a Gram-negative, spiral-shaped, microaerophilic bacterium that establishes life-long infections of the gastric mucosa causing gastritis, peptic ulcers, MALT lymphomas and increases the risk of gastric cancer. The remarkable ability of *H. pylori* to survive the acidic environment of the stomach and chronic inflammation is bestowed by a strong urease and a diverse antioxidant defense system. Unfortunately, the genetics of *Helicobacter* are not easily manipulated, therefore, advancements in its biology has been hindered by the lack of genetic tools. The aim of this study was to evaluate fundamental mechanisms involved in the colonization of *H. pylori* in the gastric mucosa. This theme was divided into two areas where we: (i) demonstrated that *H. pylori* senses pH, possibly through the methyl-accepting chemoreceptor protein TlpB, and responds using tactic behavior which is essential for mouse colonization and; (ii) delineated the role of the antioxidant AhpC during colonization. In the latter, we developed a novel antisense RNA interference strategy to target and knockdown AhpC protein levels between 30 and 74%. These knockdowns correlated with increased sensitivity to oxidative stress, however, did not alter the efficiency to colonize the mouse stomach. To further the application of antisense RNA, antisense constructs were also constructed to target two essential two-component regulators, HP0166 (ArsR) and HP1043 which are thought to be involved in acid resistance and the regulation of *tlpB,* respectively. Knockdowns were achieved with the former while several attempts to knockdown HP1043 were unsuccessful. This work demonstrates a fundamental process (pH taxis) probably involved during initial colonization of *H. pylori* to a new host and that the host oxidative defense system may not be a barrier for initial colonization. Additionally, the application of antisense RNA technology is a useful tool for the evaluation of gene function in *H. pylori* and may provide a way forward for the study and characterizing factors involved in colonization and pathogenesis.

List of Abbreviations and Symbols Used

8-oxoG	7,8-dihydro-8-oxo-2'-deoxyguanosine
Ahp	Alkyl hydroperoxide reductase
Amp	Ampicillin
AP	Apurinic or apyrimidic
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
BER	Base excision repair
BHI	Brain heart infusion
bp	Base pair
BSA	Bovine serum albumin
CFU	Colony forming unit
Carb	Carbenicillin
Cm	Chloramphenicol
CuZnSOD	Copper-zinc superoxide dismutase
Cys	Cysteine
ddH_2O	Distilled deionized water
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
dsRNA	Double stranded RNA
DTT	Dithiothreitol
e^-	Electron

EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked Immunoabsorbant assays
EMSA	Electrophoretic Mobility Shift Assay
FAD	Flavin adenine dinucleotide
[Fe-S]	Iron sulfur cluster
FeSOD	Iron superoxide dismutase
Fur	Ferric uptake regulator
GFP	Green fluorescent protein
H^+	Hydrogen ion
H_2O	Water
H_2O_2	Hydrogen peroxide
HAMP	Histidine kinases, adenylyl cyclases, methyl-binding proteins and Phosphatases
IgG	Immunoglobulin G
IL-N	Interleukin- [N = number; e.g. IL-2, IL-8 or IL-12]
iNOS	Inducible nitric oxide synthase
IPTG	Isopropyl-beta-D-thiogalactopyranoside
kb	Kilobase pair
kDa	Kilodalton
Km	Kanamycin
LB	Luria Bertani
LDS	Lithium dodecyl sulfate
Le^b	Lewis b
NAD	Nicotinamide adenine dinucleotide

NADP	Nicotinamide adenine dinucleotide phosphate
NCS	Newborn calf serum
MBC	Minimal Bactericidal Concentration
MetSO	Methionine sulfoxide
MCP	Methyl-accepting chemoreceptor protein
miRNA	Micro RNA
MnSOD	Manganese superoxide dismutase
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	Messenger RNA
Msr	Methionine sulfoxide reductase
Mtz	Metronidazole
MW	Molecular weight
NBT	Nitro blue tetrazolium
NEB	New England Biolabs
•NO	Nitric oxide
$•NO_2$	Nitrogen dioxide
nt	Nucleotide
O_2	Oxygen
$O_2•^-$	Superoxide
OD	Optical density
•OH	Hydroxyl radical
Ohr	Organic hydroperoxide resistance
$OONO•^-$	Peroxinitrite

PAS	Periodic clock protein, aryl hydrocarbon receptor and single-minded protein
PBS	Phostphate buffered saline
PCR	Polymerase chain reaction
pI	Isoelectric point
Prx	Peroxiredoxin
ROH	Organic alcohol
ROS	Reactive oxygen species
ROOH	Organic hydroperoxide
RNA	Ribonucleic acid
RNAi	RNA interference
RNS	Reactive nitrogen species
SAM	S-adenosyl-methionine
SDS	Sodium dodecyl sulfate
SDS PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA	Small interfereing RNA
sLe^a	Sialylated Lewis a
sLe^x	Sialylated Lewis b
SOD	Superoxide dismutase
S-OH	Sulfenic acid
TAE	Tris-Acetate EDTA
tBOOH	*tert*-butyl hydroperoxide
Taq	*Thermus aquaticus* DNA polymerase
TCA	Tricarboxylic acid cycle

TE	Tris-EDTA
Tet	Tetracyclin
Tris-HCl	2-Amino-2-(hydroxymethyl)-1,3-propanediol, hydrochloride
Trx	Thioredoxin
TrxR	Thioredoxin reductase
Tween 20	Polyoxyethylene sorbitan monolaurate
UV	Ultraviolet
[X]~P	Phosphorylated gene (e.g. CheY~P is phosphorylated CheY)
X-gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

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Chapter 1: Introduction

In 1982, small, spiral-shaped bacteria were successfully isolated and cultured from the stomach of a symptomatic patient by Barry Marshall and Robyn Warren in Perth, Australia. It was their belief that the unidentified bacilli were the causative agents for antral gastritis and gastric ulcers in humans (Marshall and Warren, 1984); symptoms originally attributed to a combination of environmental factors, alcoholism and stress. This organism was originally named *Campylobacter pyloridis* (corrected to *C. pylori*) based upon similar morphological features to other *Campylobacter* spp.; however, it was renamed *Helicobacter pylori* (*H. pylori*) as structural and genetic features warranted a distinct genus (Goodwin *et al.*, 1989; Romaniuk *et al.*, 1987).

H. pylori is a genetically diverse, Gram-negative, motile, spiral-shaped, microaerophilic bacterium that colonizes the gastric mucosa. It is catalase, oxidase and urease positive, contains 4-6 unipolar flagella and is approximately 2-4 μm in length. Circular coccoid bodies can be observed in prolonged culture or after stress conditions and is believed to be indicative of cellular death (Kusters *et al.*, 1997). Three genomes have been sequenced: 26695 isolated from a patient suffering from gastritis (Tomb *et al.*, 1997); J99 isolated from a patient with a duodenal ulcer (Alm *et al.*, 1999); and more recently HPAG1, a strain isolated from a patient with chronic atrophic gastritis (Oh *et al.*, 2006). Analysis of the genomes shows a G+C content between 35-40% and each to be approximately 1.6 Mb in size, nearly three times smaller than the genome for *Escherichia coli* (4.7-5.3 Mb; Blattner *et al.*, 1997). Some *H. pylori* isolates carry cryptic plasmids, but no virulence factors or antibiotic resistance determinants have been found to be harbored on the plasmids (Heuermann and Haas, 1995).

With a small genome, it may not be surprising that there are very few transcriptional regulators found in *H. pylori* compared to other pathogens. The regulators that are present seem to be involved in regulating a broad array of genes (Delany *et al.*, 2001; Dietz *et al.*, 2002; Merrell *et al.*, 2003b). Additionally, it is predicted that the genome contains many essential genes as an estimated 30-79% of the genome is required for viability (Chalker *et al.*, 2001; Jenks *et al.*, 2001; Salama *et al.*, 2004). To this end, it is remarkable that an organism with such a small genome has evolved the ability to survive the harsh environment of the stomach and establish life-long infections. Therefore, it is arguable that *H. pylori* is one of the most successful of human pathogens.

Nearly 25 years after the original discovery of *H. pylori,* a large research effort has been undertaken to progress our understanding and knowledge of the microbiology and pathogenicity of this organism. How these organisms establish life-long infections of the stomach is of fundamental interest and the subject of much investigation.

The studies found within this thesis address two areas of colonization: (i) during exposure to acid and; (ii) the role of the oxidative stress response of *H. pylori.* Following up on observations that *H. pylori* uses pH gradients found within the gastric mucin to orient in the gastric mucosa (Schreiber *et al.*, 2004), we hypothesized that *H. pylori* senses pH and responds using tactic systems. Defined isogenic mutants of the four MCPs (Tlps) will be made to determine their involvement in the tactic responses. Additionally, efforts will be made to study transcriptional proteins involved in responses to acid using novel technology that will be developed to study the oxidative stress response and colonization (see below).

The abundant antioxidant, AhpC, has been shown to be essential for viability under normal growth conditions (Baker *et al.*, 2001; Lundstrom and Bolin, 2000). Subsequent work, however, demonstrated that *ahpC* mutants can be achieved at 2% O_2 (Olczak *et al.*, 2002) and are unable to colonize mice and thus it was suggested that AhpC is a virulence factor (Olczak *et al.*, 2003). Since the *ahpC* mutants were enfeebled for growth at 2% O_2 (Olczak *et al.*, 2002), we wished to further determine the importance of AhpC in colonization. To address this, we seek to develop an antisense RNA interference strategy that will knock down protein levels of AhpC, yet still retain growth under normal culture conditions. This antisense technology will also be broadened and applied to the study of two essential transcriptional regulators though to be involved in acid resistance (HP0166; ArsR) (Pflock *et al.*, 2004; Pflock *et al.*, 2006a) and possibly regulation of proteins involved in pH sensing (HP1043) (Delany *et al.*, 2002). We hypothesize that protein knock downs of these two regulators will provide insights on the regulons involved in acid resistance and pH taxis which may further be involved during colonization.

Chapter 2: Literature Review

2.1 The Family Helicobacteraceae

Helicobacter belongs to the epsilon subclass of the proteobacteria along with *Wolinella succinogenes* and members of the genus *Campylobacter* which are both in the order Campylobacterales (Vandamme *et al.*, 1991). Many of the members are microaerophilic and inhabit the digestive tract of humans and/or animals but can either be pathogenic or commensal, depending on the host (Kusters *et al.*, 2006).

Even though spiral shapped bacteria were observed in the stomachs of dogs nearly 100 years ago, the impetus to study these further was lost until recently. Investigations of gastritis and stomach disorders in animals have led to the realization that few mammals escape infection by *Helicobacter*. Currently, 24 *Helicobacter* species have been described that infect various animals such as cats, dogs, birds and rodents (Dewhirst *et al.*, 1994; Fox *et al.*, 1988; Fox *et al.*, 1994; Paster *et al.*, 1991; Stanley *et al.*, 1993). Other *Helicobacter* species, such as *H. heilmannii*, have been observed with gastric biopsies, but have yet to be successfully cultured (Andersen *et al.*, 1996). Less than half of the *Helicobacter* spp. are gastric colonizers while the others are considered to be non-gastric and have been found to colonize other organs such as the intestine or liver (Solnick and Schauer, 2001).

The most prevalent human *Helicobacter* infection is caused by *H. pylori* whilst uncommon *Helicobacters* only make up 0.01% of infections in developed countries (Foschini *et al.*, 1999) and 6.2% of infections in undeveloped countries (Yali *et al.*, 1998). Many of the uncommon *Helicobacter* infections are believed to be transmitted via pets or contact with other animals (Dieterich *et al.*, 1998) and have been isolated from

stomachs and gastrointestinal tracts of humans (Solnick and Schauer, 2001; Solnick, 2003).

2.2 Clinical Significance, Diagnosis and Treatment

It has been estimated that nearly 50% of the world's population is infected with *H. pylori* (Dunn *et al.*, 1997). The prevalence of disease, however, varies between countries as lower incidences (20-50% of the population) are seen in developed countries and higher incidences (80-90%) in developing countries (Frenck and Clemens, 2003; Rothenbacher and Brenner, 2003). The route of transmission is thought to be fecal-oral or oral-oral and to occur during childhood (Malaty and Graham, 1994). Individuals infected with *H. pylori* will develop gastritis, but approximately 15% will require medical attention for more severe outcomes due to chronic gastritis, duodenal (95%) and gastric ulcers (70%) (McColl *et al.*, 1993; McColl, 1997; Veldhuyzen van Zanten *et al.*, 1997).

In 1994 the International Agency of Research on Cancer (IARC), part of the World Health Organization (WHO), classified *H. pylori* as a group 1 carcinogen (IARC, 1994) as colonization of *H. pylori* is estimated to increase the risk of developing gastric cancer by 10-fold (Kuipers, 1998). The lifetime risk of developing gastric cancer is thought to be 1-2% in Western countries and 60-80% in developing countries (Kuipers, 1999). Additionally, *H. pylori* is also implicated in mucosal-associated lymphoid tissue (MALT) lymphomas which can occur in 1% of *H. pylori*-positive patients (Parsonnet and Isaacson, 2004).

Diagnosis of *H. pylori* can be done invasively or non-invasively. Invasive techniques usually involve endscopy with collection of several antral biopsies. These biopsies are subjected to histological examination for the organism and/or neoplastic

lesions, primary isolation on selective media and rapid urease testing (Vaira *et al.*, 2002). Non-invasive techniques include serology by detecting *H. pylori* IgG antibodies in the blood with enzyme linked immunoabsorbant assays (ELISA), a fecal antigen test to look for *H. pylori* antigens shed in feces (Vaira *et al.*, 1999) or the ^{13}C-urea breath test that relies on the urease of *H. pylori* to evolve $^{13}CO_2$ in expired breath (Logan and Walker, 2001). Although invasive tests are more specific for detection of *H. pylori* (though normally require specific expertise), non-invasive tests are typically less expensive but also have their shortcomings. For example, false-positives can arise from serological tests as serum antibodies can exist for long durations of time and the ^{13}C-urea test requires expensive equipment (Vaira *et al.*, 2002). The fecal antigen test is more widely used in Europe than North America but may offer an alternative to endoscopes of children.

Eradication of *H. pylori* can improve and/or cure several gastroduodenal diseases (Candelli *et al.*, 2005). Initially, monotherapies with antibiotics were not successful in eradicating infections so triple therapies using two antibiotics, with the most widely used being amoxicillin, clarithromycin, metronidazole or tetracycline, with a proton pump inhibitor such as omperazole is commonly used in a 7-14 day regiment (Candelli *et al.*, 2005). Over recent years, however, *H. pylori* has shown an increasing resistance to metronidazole and clarithromycin (Chowdhury *et al.*, 2002; van der Wouden *et al.*, 2001) with the latter contributing to a decrease in eradication rates (Tankovic *et al.*, 2001). In case of failures of first-line treatments, employment of quadruple therapies has been suggested (Perri *et al.*, 2003) or the use of other antimicrobials such as bismuth salts, rifabutin or furazolidone can be used (Megraud and Lamouliatte, 2003).

2.3 Genetic Diversity

The genetics of *H. pylori* may be the most diverse of all bacterial species. Comparison of the two originally sequenced strains, 26695 and J99, showed that the nucleotide sequence of genes differed by approximately 7%, although most are found at the third codon position and do not affect the proteome (Alm *et al.*, 1999). To put the 7% difference into context, comparison of the nucleotide sequences *Salmonella typhimurium* and *Salmonella typhi* only show a 1-2% difference (Zahrt and Maloy, 1997). Moreover, between the two unrelated strains, 6% of the genes were determined to be strain-specific (Alm *et al.*, 1999). An expansion of this survey to 15 genomes determined that 12-18% of the genes were strain-specific (Salama *et al.*, 2000) with many of these strain-specific genes found in a highly variable plasticity zone (Alm *et al.*, 1999; Salama *et al.*, 2000). To date, no clinical relevance has been attributed to any of these strain-specific genes. In addition to the diversity between strains, isolates from a single patient are thought to change overtime. The sequenced *H. pylori* J99 strain was reisolated 6 years after the original isolation and through microarray analysis, it was determined that there was approximately 3% difference in genomic content (Israel *et al.*, 2001). Since transmission is thought to occur during childhood, it is unforeseeable the amount of changes that might occur to *H. pylori* until adulthood when treatment is sought due to the onset of symptoms. Perhaps, the most drastic changes occur during initial colonization as *H. pylori* adapts to its new host.

Diversity may be attributed to a few features of *H. pylori.* First, *H. pylori* lacks the classical DNA mismatch repair system, *mutHSL* that may contribute to a high mutation rate (Bjorkholm *et al.*, 2001). *H. pylori* is also naturally competent and can

readily take up foreign DNA from other *H. pylori* strains or perhaps from other species that temporarily colonize the stomach. It has been shown that *H. pylori* undergoes recombination more frequently than *E. coli* or *Neisseria gonorrhoeae* (Suerbaum *et al.*, 1998). Since mutation rates of *H. pylori* and the ability to take up DNA can be variable between strains (Ando *et al.*, 2000; Bjorkholm *et al.*, 2001), intragenomic recombination may also contribute to genome diversity. It was shown that *H. pylori* contains a broad distribution of identical DNA repeats throughout the chromosomal and that intragenomic recombination may lead to gene duplications or deletions (Aras *et al.*, 2003).

Spontaneous point mutations are also believed to contribute to the diversity of *H. pylori.* Mutations can lead to phase variation, by turning "on" or "off" the expression of certain genes (Cooke *et al.*, 2005). Moreover, point mutations that lead to alterations of surface proteins (antigenic variation) may prevent detection by the immune system, or alter affinity for host receptors (Cooke *et al.*, 2005). The result of this diversity may permit host-specific adaptations and immune evasion that may attribute to the life-long survival and chronicity of *H. pylori.*

2.4 Colonization, Persistence and Disease Factors

H. pylori establishes infections of the gastric mucosa long before the onset of disease. This niche is a harsh environment with high acidity and a constant turnover of the gastric mucus. After entrance into the gastric lumen, *H. pylori* must first survive the low pH by maintaining pH stasis (urease) (Sachs *et al.*, 2005). Second, *H. pylori* must escape the acidity of the stomach by traversing the thick mucosa. This is achieved by high motility governed by sheathed flagella that are also protected from acid (Eaton *et al.*, 1996). In order to reach the gastric mucosa, *H. pylori* must sense the pH gradient between

the gastric epithelium and lumen to properly orient itself in a less acidic environment (pH taxis) (Croxen *et al.*, 2006). Upon reaching the epithelial cells, *H. pylori* can evoke a strong inflammatory response (Rautelin *et al.*, 1994), thought to be for the acquisition of nutrients, and establishing life-long colonization.

2.4.1 Acid Acclimation: Urease and α-Carbonic Anhydrase

H. pylori is a neutralphile, and unlike other neutralphiles, is able to survive and grow in the acidic environment of the stomach (Berg *et al.*, 1997). *H. pylori* expresses a nickel-containing urease enzyme that hydrolyzes urea into carbon dioxide and ammonia (Mobley *et al.*, 1988). Originally, it was hypothesized that *H. pylori* underwent altruistic lysis and that the released urease would bind the surface of another bacterium and the activity would thus created a microenvironment of neutrality (Phadnis *et al.*, 1996). This hypothesis was later dismissed as the optimal urease activity would be abolished in the acidic environment and the fact that gastric levels of urea (1-3 mM) were insufficient in maintaining the neutral microenvironment (Sachs *et al.*, 2005). The prevailing view is presented below.

The urease genes are clustered in an operon with the *ureAB* genes encoding the two subunits of the urease enzyme and further downstream, the *ureIEFGH* genes which encode accessory proteins (Labigne *et al.*, 1991). Of the accessory genes, the UreI is important in transport of urea into the cell and is uniquely found in all gastric *Helicobacter* spp. (Scott *et al.*, 2000). First, *ureI* mutants were unable to survive in acid in the presence of urea (Weeks *et al.*, 2000). Secondly, eloquent experiments were performed in *Xenopus laevis* oocytes expressing *H. pylori* UreI that demonstrated that when the pH is less than 6, the UreI gate begins to open and permit facilitated diffusion

of urea. At neutral pH, the gate is closed and urea can only enter the cell via diffusion through the cytoplasmic membrane (Weeks *et al.*, 2000; Weeks and Sachs, 2001). Finally, *ureI* was able to complement a *yut* mutant (an unrelated single-component urea channel) of *Yersinia pseudotuberculosis* and only active at pH > 5.0 suggesting that urea uptake by UreI is independent of other factors (Sebbane *et al.*, 2002).

The current proposed model for pH stasis is that upon urea uptake into the cytoplasm, urease (UreAB) hydrolyses the urea to $2NH_3$ and CO_2 (Mobley *et al.*, 1988). Both NH_3 and CO_2 readily cross the cytoplasmic membrane into the periplasmic space. In the periplasm, NH_3 can react and neutralize H^+ ions by forming NH_4^+. In addition, periplasmic α-carbonic anhydrase converts CO_2 to HCO_3^- + H^+. The later H^+ can then react with the second NH_3 to make another NH_4^+. HCO_3^- provides buffering at a range of pH 6.1 (Marcus *et al.*, 2005). The major flaw in this model is the fact that ammonia has a pKa of 9.25. Therefore, in the cytoplasm (pH 6 – 7), NH_3 would most likely exist as NH_4^+ and would not be able to cross the periplasmic membrane. Perhaps the major contributor to buffering the periplasm is attributed to CO_2 conversion to HCO_3^- by α-carbonic anhydrase.

Mutations in the urease genes render *H. pylori* unable to colonize mice (Skouloubris *et al.*, 1998; Tsuda *et al.*, 1994a; Tsuda *et al.*, 1994b). Contradictory to the previous results, Mine *et al.* isolated a urease-negative *H. pylori* and showed that it was able to colonize the stomach of the Mongolian gerbil model and thus suggest that urease is not required for colonization of the gastric mucosa (Mine *et al.*, 2005). Nevertheless, the unique feature of a powerful urease and the ability to transport urea at lower pH may

be one piece in explaining why *H. pylori* can survive acid and occupy the human stomach.

2.4.2 Motility

In addition to short term survival in an acidic environment, *H. pylori* must traverse the gastric mucus to colonize the epithelial cell line of the gastric mucosa where the pH is more favorable. Therefore, the ability of *H. pylori* to be motile and chemotactic is important for colonization. The genome of *H. pylori* indicated that there are approximately 50 genes involved in the structure and regulation of the flagella and appear to be unlinked (Alm *et al.*, 1999; Oh *et al.*, 2006; Tomb *et al.*, 1997). *H. pylori* has 4-6 unipolar flagella covered in a sheath that is thought to protect the flagella from acid (Jones *et al.*, 1997). The flagellar filament is made up of two subunits, *flaA* and *flaB* and is associated with the basal body and flagellar hook (Leying *et al.*, 1992; O'Toole *et al.*, 1994; Suerbaum *et al.*, 1993). Mutations in these flagellar subunits render *H. pylori* non-motile (weak motility in *flaB* mutants) and completely cleared from the stomachs four days after infection (Eaton *et al.*, 1996) underscoring the importance of motility for colonization. Moreover, a mutation in the flagellar motor (*motB*) that still produce flagella, were non-motile and showed a reduced ability to colonize the stomach (Ottemann and Lowenthal, 2002).

Despite the important role of motility during infection, *H. pylori* can become non-motile in vitro by switching off flagellar biosynthesis. This has been shown to be due to slipped strand mispairing in a c-tract upstream of *fliP,* the basal body of the flagella (Josenhans *et al.*, 2000). Although this has only been observed in vitro, it is possible that it may be beneficial in vivo when *H. pylori* is adherent to the gastric epithelium.

Although discussed in detail later in the text, chemotaxis is also an important factor that usually modulates motility. The sensing of pH gradients and positive taxis towards certain compounds (e.g. mucin or urea) may contribute to proper alignment in the gastric mucosa for protection from luminal acid and a successful colonization (Croxen *et al.*, 2006; Schreiber *et al.*, 2004).

2.4.3 Adhesins: BabA and SabA

H. pylori can be found swimming in the mucus layer or attached to the gastric epithelial lining (Hessey *et al.*, 1990). Adherence to host cells may protect *H. pylori* against shedding of the mucosal layer or stomach peristalsis and may allow the delivery of toxins to host cells. There are approximately 32 different *Helicobacter pylori* outer membrane proteins (HOPS), some of which have been identified as adhesins (Alm *et al.*, 2000; Ilver *et al.*, 1998). It has been noted that many of these HOPS are unique in structure and may be a reflection of the adaptation to the acidic nature of the stomach. Further adaptation may be attributed to both antigenic and/or phase variation that has recently been observed in several HOPS (Alm *et al.*, 2000).

The best studied adhesin is BabA, a 78 kDa protein that binds to the fucosylated Lewis b blood antigen (Le^b) that are found on the surface of gastric epithelial cells (Aspholm-Hurtig *et al.*, 2004; Boren *et al.*, 1993; Ilver *et al.*, 1998). Therefore, it is thought that BabA is involved in the initial colonization of the gastric mucosa (Ilver *et al.*, 1998; Linden *et al.*, 2002). The result of this adhesion is thought to enhance mucosal inflammation (Rad *et al.*, 2002) and facilitate denser colonization (Sheu *et al.*, 2006). Additionally, adhesion by BabA has been associated with increased risk of duodenal ulcers and gastric cancer (Gerhard *et al.*, 1999), however, among strains that expressed

detectable BabA, there was considerable variability that may contribute to different clinical outcomes (Hennig *et al.*, 2004).

SabA has been identified as a second major adhesin in *H. pylori* that recognizes sialylated Lewis a (sLe^a) and Lewis x (sLe^x) antigens (Mahdavi *et al.*, 2002) and gangliosides (Roche *et al.*, 2004). During chronic infections and prolonged inflammation, the dominant Le^b antigens on gastric epithelial cells are replaced by the sLe^a and sLe^x (Ota *et al.*, 1998). Therefore, SabA is hypothesized to enhance binding to inflamed tissue after changes in Lewis antigen presentation and contribute to chronicity of infection. In addition to a role in adhesion, SabA is also thought to activate neutrophils in releasing an oxidative bust (Petersson *et al.*, 2006; Unemo *et al.*, 2005). SabA is believed to be expressed in patients with gastric cancer than those with duodenal ulcers and it has been suggested that acidity decreases its expression (Merrell *et al.*, 2003a; Yamaoka *et al.*, 2006) and may determine niches by allowing detachment from inhabitable areas and movement to a less harsh environment, perhaps using pH taxis (Croxen *et al.*, 2006).

The current interest in the BabA and SabA adhesins revolves around the observations that they are able to undergo antigenic and phase variation (Mahdavi *et al.*, 2002; Solnick *et al.*, 2004). Both *babA* and *sabA* have repetitive CT-tracks upstream of the gene making them prone to slipped strand mispairing mutagenesis (Backstrom *et al.*, 2004; Mahdavi *et al.*, 2002). This can create a frame shift mutation resulting in proteins that are unable to adhere to their receptors and thus are given a "on" or "off" status. This phenomenon seems to becoming more apparent in many HOPS (Peck *et al.*, 1999; Yamaoka *et al.*, 2000) and efforts are ongoing to try and link "on" and "off" statuses between them (Yamaoka *et al.*, 2006).

In addition to phase variation, BabA has also been shown to undergo antigenic variation (Solnick *et al.*, 2004). After BabA-positive strains were passaged through rhesus monkeys, many isolates had lost *babA* as it was replaced by a duplicate *babB*, a gene of unknown function. It was further shown that *H. pylori* strains with a silent *babA* locus can undergo recombination with a *babB* locus to form an activated BabB/A chimera. BabB/A had the same adhesion affinity as wild type BabA, albeit decreased expression, and was subject to the "on/off" phase variation phenotype (Backstrom *et al.*, 2004). To add another degree of diversity, point mutations in *babA* genes have been shown to have different affinities to different Lewis b antigens, depending on the different terminal modifications (Aspholm-Hurtig *et al.*, 2004). It was demonstrated that certain classes of BabA recognized an array of modified Le^b antigens while others were exclusive to a specific type. Moreover, *babA* and *babB* profiles have been shown to be altered based on selective pressures from the host (Colbeck *et al.*, 2006).

The phase and antigenic variation of *H. pylori* HOPS may offer survival advantages of a subpopulation based on altered host receptor presentation or changes in the local environment. It is possible that switching to an "off" status may allow the bacteria to detach from the epithelium and then use its motility and chemotaxis to leave an undesirable environment and move to a less harsh or more nutritious niche. Although only a handful of HOPS have been studied, the possibility of microdiversity within any number of the >30 HOPS expressed by *H. pylori* provides the opportunity for optimal host-specific interactions with its host that may very well contribute to the chronicity of infection.

2.4.4 *cag* Pathogenicity Island (*cag*PAI) and CagA

Between 50-70% of all *H. pylori* isolates have a 40 kb insertion in the glutamate racemase gene in the chromosome called the *cag* Pathogenicity Island (*cag* PAI) (Akopyants *et al.*, 1998; Censini *et al.*, 1996; Cover *et al.*, 1995) and are designated cag^+. Eighteen of the 31 genes encoded on this island are essential for the formation of a type IV secretion apparatus (Fischer *et al.*, 2001b). Type IV secretion systems are transporters translocate substrates (often times protein) from the bacterium into the cytosol of the host cell (Backert and Meyer, 2006). The model type IV secretion system comes from *Agrobacterium tumefaciens* that delivers protein and T-DNA into plant cells to cause crown-gall tumors (Christie and Vogel, 2000). The *cag* PAI has been shown to transolcate the cytotoxin-associated gene (*cagA*) (Censini *et al.*, 1996) and more recently, peptidoglycan (Viala *et al.*, 2004) into host cells.

After a multitude of epidemiological studies, it is evident that cag^+ strains enhance the risk of peptic ulcers and gastric cancer, however, both clinical manifestations can occur with cag^- strains (Nomura *et al.*, 2002) suggesting the involvement of other factors. CagA is a large, 120-145 kDa immunodominant antigen (Covacci *et al.*, 1993) that is involved in apoptosis of T-cells, release of proinflammatory cytokines, increased cell motility and the hummingbird phenotype, characterized by elongated cellular morphology of gastric cells in vitro (Cox *et al.*, 2001; Paziak-Domanska *et al.*, 2000; Segal *et al.*, 1999; Wang *et al.*, 2001). Once CagA is injected into the host cell, it is targeted into the inner surface of the plasma membrane due to the Glu-Pro-Ile-Tyr-Ala (EPIYA) sites found at the carboxyl-terminus (Higashi *et al.*, 2005). Here, CagA recruits and interacts with the Src family kinases (SFK) and becomes phosphorylated on the

tyrosine residue of the EPIYA motif (Selbach *et al.*, 2002; Stein *et al.*, 2002) which can be present in multiple repeats (Higashi *et al.*, 2002a).

As an example of an affect of CagA, the phosphorylated CagA (CagA~P) can interact with the host cytoplasmic tyrosine phosphatase called SHP-2 at each of the two Src homology-2 (SH-2) domains found at the N and C-terminus (N-SH2 and C-SH2, respectively) (Higashi *et al.*, 2002b). Binding of CagA-P to the SH-2 domains induces a conformational change in SHP-2 allowing access to the catalytic domain and activating phosphatase activity (Higashi *et al.*, 2002a). SHP-2 is known to activate Erk MAP kinases (Neel *et al.*, 2003) and interactions with CagA~P result in a continued activation of Erk which is important in progression of mitosis (Higashi *et al.*, 2004) Therefore, CagA may be involved in deregulated growth of gastric epithelial cells, but the reasons are unclear. SHP-2 is also involved in regulation cellular processes that include cell proliferation, cell morphogenesis and motility (Neel *et al.*, 2003) which would result in the hummingbird phenotype; however, the signaling pathways are unknown.

CagA~P can also interact with the SH2 of Csk and stimulate kinase activity which phosphorylates the C-terminal inhibitory domain of SFK (Tsutsumi *et al.*, 2003). Therefore, it is thought that this serves as a negative feedback mechanism for CagA: as SFK phosphorylates CagA to CagA~P, CagA can also inhibit phosphorylation of itself by stimulating Csk to inhibit SFK. This may prevent excessive toxicity to the gastric epithelial cells and give a longer time to exert its effects.

It is now also becoming evident that CagA also has activity in host cells independent of tyrosine phosphorylation. Through interactions of other signal pathways CagA can also alter cell motility and morphological changes in vitro (Churin *et al.*, 2003;

Mimuro *et al.*, 2002). There have also been observations that CagA can disrupt the epithelial barrier between tight junctions of cells similar to VacA (see below) (Amieva *et al.*, 2003). This may allow for leakage and greater access to nutrients at the site of bacterial attachment, similar to that of VacA (see below).

While the above studies have expanded our knowledge on the biology of CagA it will be important to progress the in vitro observations to determining its role in *Helicobacter* colonization and progression of disease in the host.

2.4.5 Vacuolating Cytotoxin (VacA)

After observations that large intracellular vacuoles were forming in mammalian cells challenged with *H. pylori* culture filtrates the *vacA* gene was discovered (Cover and Blaser, 1992; Leunk *et al.*, 1988). Mature VacA is a large, 88 kDa protein that can be found on the surface of *H. pylori* as well as secreted as soluble proteins (Cover and Blaser, 1992; Ilver *et al.*, 2004). VacA is found in all *H. pylori* strains (Schmitt and Haas, 1994); however, it can be classed into different families based on diversities found in the 5' region (s-region; s1a, s1b, s1c and s2) and in the mid region (m-region; m1 and m2) (Atherton *et al.*, 1995). An increased risk of development of peptic ulcers and gastric cancers are associated with s1 types as compared to the inactive s2 types, additionally, m1 types increase the risk of gastric epithelial damage than m2 types (van Doorn *et al.*, 1998; van Doorn *et al.*, 1999).

VacA is secreted by a type V (autotransporter) secretion mechanism (Fischer *et al.*, 2001a) and is believed to be a ligand for the receptor protein tyrosine phosphatases, (RPTP)-α and RPTP-β (Fujikawa *et al.*, 2003). Secreted VacA forms an anion-selective membrane channel in the host cell plasma membrane and the channels are internalized by

endocytosis into a late endosome (Molinari *et al.*, 1997). These pores allow the release of bicarbonate and urea from the cell cytosol, thus altering the permeability of the late endosomal compartment and permit an influx of water which leads to vesicle swelling and ultimately vacuole formation. This eventually results in cellular death (Cover *et al.*, 2003; de Bernard *et al.*, 2004).

VacA is also believed to have other functions such as the loosening of cellular junctions by unknown actions (Papini *et al.*, 1998; Pelicic *et al.*, 1999), perhaps to supply nutrients for *H. pylori.* Additionally, VacA may contribute to immunomodulation as it has been reported that VacA prevents proliferation of T-cells by suppressing nuclear factor of activated T-cells (NFAT) thus inhibiting IL-2 secretion (required for T-cell viability and proliferation) (Boncristiano *et al.*, 2003; Sundrud *et al.*, 2004).

Although animal studies with *vacA* mutants showed that colonization is not affected (Eaton *et al.*, 1997; Salama *et al.*, 2001; Wirth *et al.*, 1998), long term animal studies have yet to be done and the role of VacA in persistence is unknown. Given the multifunction of VacA, in vivo characterizations will need to be done in order to determine the role of VacA in persistence and its role in evading the immune system.

2.4.6 Miscellaneous Disease Factors: OipA, DupA and IceA

Several other genes of increasing interest seem to be involved in severity of clinical outcomes such as the outer inflammatory protein (*oipA*; severe gastritis) (Yamaoka *et al.*, 2000), duodenal ulcer-promoting gene (*dupA*) (Lu *et al.*, 2005a) and induced by contact with epithelium genes (*iceA*; peptic ulcers & gastric cancer) (Peek *et al.*, 1998). Although noteworthy, little is known about the function of these genes.

2.4.7 Global Regulation: Fur, NikR and ArsRS

The small genome and lack of regulatory systems may be reflective of the fact that *H. pylori* can survive in a harsh environment where there is little competition from other microorganisms. It has, however, been shown that *H. pylori* can perceive and respond to environmental stimuli and alter its gene expression (Chuang *et al.*, 2005; Gancz *et al.*, 2006; Merrell *et al.*, 2003a; van Vliet *et al.*, 2004).

Analysis of the *H. pylori* genome shows that it encodes three sigma factors (RpoD, RpoN and FliA) (Tomb *et al.*, 1997), four histidine kinases and six response regulators, two of which are orphan regulators and essential for viability (HP1201 and HP1043) (Beier and Frank, 2000). The paired kinases and response regulators are: (i) CheA/CheY (described later, Foynes *et al.*, 2000); (ii) CrdRS (copper-resistance, Waidner *et al.*, 2005); (iii) HP0244/FlgR (flagella, Jimenez-Pearson *et al.*, 2005b) and; (iv) ArsRS (acid resistance, Beier and Frank, 2000). Additionally, *H. pylori* encodes few repressors including, but not limited to: (i) HspR and HrcA (heat shock repression, Spohn *et al.*, 2004); (ii) CsrA (carbon storage regulator, Barnard *et al.*, 2004); (iii) Fur (iron homeostasis, van Vliet *et al.*, 2002b) and; (iv) NikR (nickel homeostasis, van Vliet *et al.*, 2002a). The latter two and ArsRS have been of most recent interest and will be discussed further.

The classic ferric uptake regulator (Fur) of *E. coli* is bound by Fe^{2+} and controls expression of more than 90 genes involved in Fe^{2+}-utilization, uptake and storage (Andrews *et al.*, 2003). In situations of iron-depletion, where Fur is found unbound by Fe^{2+} (apo-Fur), repression is alleviated. A homolog of Fur is found in *H. pylori* that also regulates genes involved in Fe^{2+} uptake and storage (Delany *et al.*, 2001). Unlike Fur of

E. coli, *H. pylori* Fur directly regulates gene expression positively and negatively. Recently, immunoprecipitation assays have identified Fur to directly regulate 59 genes, 25 of which are positively regulated (Danielli *et al.*, 2006) which was in accordance with many genes identified by a transcriptome analysis (Ernst *et al.*, 2005a). Interestingly, *fur* has been shown to be down regulated during the acid response (Bijlsma *et al.*, 2002) which was later shown to be mediated through NikR (van Vliet *et al.*, 2004).

NikR is an ortholog of the Ni^{2+}-response regulator of *E. coli* that represses the Ni^{2+} uptake system when Ni^{2+} concentrations are high (De Pina *et al.*, 1999). It has only been recently shown that NikR from *H. pylori* actually binds Ni^{2+} (Abraham *et al.*, 2006). NikR has been shown to be both an activator and repressor of transcription in *H. pylori* in response to Ni^{2+} levels. Genes involved in Ni^{2+} uptake (*nixA*), a histidine rich storage protein (*hpn*) and genes involved in motility have been identified in a transcriptome analysis (Contreras *et al.*, 2003). It was originally thought that NikR upregulated expression of the nickel transporter, NixA, during increased Ni^{2+} concentrations (Contreras *et al.*, 2003), however, subsequent work demonstrated the opposite (Ernst *et al.*, 2005c; Wolfram *et al.*, 2006) exposing inconsistencies with microarray analysis. Furthermore, it has been shown that NikR is an activator of urease (van Vliet *et al.*, 2002a) and under acid conditions a repressor of Fur (van Vliet *et al.*, 2004) thought to be mediated by increased NikR levels by an unknown mechanism (Pflock *et al.*, 2006b).

Two-component systems act to sense environmental changes to elicit a response. They are typically comprised of a membrane bound sensor and a cytoplasmic response regulator. Generally, the sensor kinase specifically senses a stimulus and facilitates autophosphorylation of a conserved histidine residue. The phosphate is subsequently

transferred to a conserved aspartate residue on the receiver domain of a response regulator that mediates a conformational change and can bind DNA to facilitate transcription (Stock *et al.*, 2000). Of most interest in *H. pylori,* ArsRS has been shown to be involved in the acid response in sensing pH (Dietz *et al.*, 2002). It was previously shown that there are between 101 and 280 genes regulated by *H. pylori* in response to acid (Bury-Mone *et al.*, 2004; Merrell *et al.*, 2003a; Wen *et al.*, 2003). Attempts to study the ArsRS regulon have been made by transcriptome analysis and magnetocapture with recombinant ArsR (Dietz *et al.*, 2002; Forsyth *et al.*, 2002a). Included in this regulon are the negative regulation of *rocF* and proteins of unknown functions as well as positive regulation of urease and *amiE* and *amiF* thought to be involved in converting amides to ammonia (van Vliet *et al.*, 2003). Studies of ArsRS have been somewhat hindered because although the sensor kinase (ArsS) is non-essential, the response regulator (ArsR) is (Beier and Frank, 2000). Site-directed mutations in the aspartate residue, thereby abolishing the ability to be phosphorylated, can be achieved therefore it is possible that ArsR has two functions; (i) control of non-essential genes in response to acid and; (ii) control of an essential gene or genes in the dephosphorylated state (Schar *et al.*, 2005). Undoubtedly, the inability to create isogenic mutants of *arsS* has hampered the identification of other genes in the ArsRS regulon.

2.5 Oxidative Stress and Protection

Microaerophlic organisms, such as *H. pylori,* are capable of growth in O_2, but at tensions lower than atmosphere. Thus, microaerophlic organisms are especially sensitive to oxidative stress (Krieg and Hoffman, 1986), a term used to describe exposure to toxic reactive oxygen species (ROS). Additionally, bacteria are also sensitive to nitrostive

stress caused by reactive nitrogen species (RNS). Therefore, bacteria have evolved mechanisms to deal with oxidative stress by evading exposure or reducing the ROS and RNS into non-toxic products and *H. pylori* is no exception. In particular, *H. pylori* has a wide array of strong antioxidant machinery to combat oxidative burst, a hallmark of the inflammatory response, and must be efficient in doing so as inflammation is chronic. The roles and current knowledge of oxidative stress in *H. pylori* will be addressed below.

2.5.1 Sources of ROS

Bacteria are subjected to oxidative stress both endogenously and/or exogenously. Endogenous ROS are usually a byproduct of aerobic metabolism while exogenous sources can come from oxidative bursts created from phagocytes in an attempt to clear the infection.

2.5.1.1 Endogenous Sources

Several ROS are formed by autoxidation of dehydrogenases during improper electron transfer from flavins to iron-sulfur clusters or quinones (via the 1 e^- transfer to form semiquinones) to oxygen (Messner and Imlay, 2002). This results in the formation of superoxide ions ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) in the cytoplasm (Massey, 1994). Since $O_2^{\bullet -}$ can not readily cross the periplasmic membrane due to its charge, periplasmic $O_2^{\bullet -}$ can arise endogenously through menaquinone as the donor (Korshunov and Imlay, 2006), although the mechanism is currently speculative. *H. pylori* encodes a soluble quinone reductase, MdaB that catalyses the $2e^-$ transfer from NADPH to quinones (Wang and Maier, 2004). Isogenic mutants of *mdaB* were more susceptible to H_2O_2 and hydroperoxides suggesting a role in protection against oxidative stress. It is proposed that

MdaB may compete against $1e^-$ transfers to quinones in forming semiquinones that can further react with O_2 to form $O_2\bullet^-$ and H_2O_2.

Superoxide ions can oxidize iron-sulfur clusters of dehydratases, releasing iron and inhibiting the function of the enzymes (Gardner and Fridovich, 1991a, b). Many of these dehydratases (e.g. fumarase and aconitase) are found in the tricarboxylic acid cycle (TCA) which would become inactivated during oxidative stress. This is important in *H. pylori* as the TCA is an important source of energy and reducing equivalents (Pitson *et al.*, 1999). The release of free iron is important because it increases intracellular iron levels and can bind DNA to react with H_2O_2 for further damage.

Hydrogen peroxide can freely diffuse through membranes (Seaver and Imlay, 2001b) and is a strong oxidant that can oxidize sulfur containing amino acids creating sulfenic acids that can be further oxidized to an irreversible sulfonic acid or mediate carbonylation of proteins (Brot and Weissbach, 2000; Imlay, 2003). This oxidation usually results in loss of function of the protein. Perhaps the most important reaction involving H_2O_2 is with iron bound to DNA during Fenton chemistry and the formation of hydroxyl radicals (OH•) (Green and Paget, 2004):

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + [OH\bullet] + OH^-$$

It was recently determined that endogenous H_2O_2 concentrations are sufficient for cell toxicity (Park *et al.*, 2005).

Hydroxyl radicals are the most toxic of all ROS as it can extract electrons from sugar and base moieties of DNA causing lesions and possibly lethal mutations (Hagensee and Moses, 1989; Hutchinson, 1985). In addition, OH• can oxidize proteins and lipids as they can propagate through unsaturated fatty acids in the lipid membrane as lipid radicals

which can then become in close proximity for oxidation of other macromolecules (Buettner, 1993).

2.5.1.2 Exogenous Sources

As part of innate immunity, neutrophils create oxidative bursts in an attempt to eradicate the invading pathogen and results in inflammation. Oxidative bursts are mediated through NADPH oxidases inside of the neutrophils. Resting neutrophils have unassembled NADPH oxidases that require stimulation before they become activated. Upon activation, the phosphorylation of the $p47^{phox}$ triggers itself, $p67^{phox}$ and $p40^{phox}$ to translocate from the cytoplasm to the membrane to bind flavocytochrome b_{558} and another cytoplasmic protein, Rac2 (Allen, 2001). The formed holoenzyme transferred electrons from NADPH to O_2 to generates $O_2^{\bullet -}$ and H_2O_2 in the lumen of the phagosome which normally kills the organism.

H. pylori has been shown to evoke a strong inflammatory response in the gastric mucosa in vitro and increased levels of ROS have been measured in the gastric mucosa of chronically infected patients (Baik *et al.*, 1996). This differs from other organisms such as *Salmonella typhi*, *Brucella abortus* and *Yersini* spp. that have been shown to elicit little to no oxidative burst (Charnetzky and Shuford, 1985; Kossack *et al.*, 1981; Kreutzer *et al.*, 1979). *H. pylori* stimulates chronic inflammation by recruiting neutrophils into the gastric mucosa, and although the oxidative burst is sufficient to cause tissue damage, *H. pylori* is not cleared (Rautelin *et al.*, 1994). Recruitment of neutrophils is thought to be mediated by the neutrophil-activating protein (NapA) (Dundon *et al.*, 2002; Evans *et al.*, 1995) and activation may be through host interactions with SabA (Petersson *et al.*, 2006). Moreover, BabA has been shown to elicit an IL-8 response that also activates neutrophils

(Rad *et al.*, 2002). There was a lot of controversy over the involvement of CagA in the proinflammatory response as *cagA*-mutants still stimulated IL-8, however, specific mutants in the *cag*PAI did not (Fischer *et al.*, 2001b). It has been proposed that the *cag*PAI secretes *H. pylori* peptidoglycan which was shown to stimulate IL-8 in vitro (Viala *et al.*, 2004).

Recent evidence has shown that *H. pylori* is able to disrupt the NADPH oxidase targeting to the phagosome and instead is targeted to the plasma membrane (Allen *et al.*, 2005). This would result in the release of ROS into the extracellular space contributing to host tissue damage and evasion of phagocytic killing of *H. pylori.* This may be due to NapA as it was shown that it was able to modify the generation and magnitude of the oxidative burst, however, the mechanism remains unknown (Petersson *et al.*, 2006).

2.5.2 Endogenous and Exogenous Sources of RNS

RNS are oxidative nitrogen intermediates that can be derived from endogenous denitrification (nitrite is converted to •NO via nitrite reductase) or from an inducible nitric oxide synthase (iNOS; converts L-arginine and oxygen to citrulline and •NO) of phagocytic cells (Fang, 2004). These intermediates include nitric oxide (•NO), S-nitrosothiols, nitrogen dioxide ($\bullet NO_2$) and peroxynitrate ($OONO\bullet^-$). There are several outcomes for each intermediate; •NO and S-nitrosothiols have been shown to inhibit DNA replication by interacting with zinc of metalloproteins (Schapiro *et al.*, 2003), •NO can inhibit ribonucleotide reductase thereby limiting precursors for DNA synthesis and repair and prevent cellular respiration (Lepoivre *et al.*, 1991; Pacelli *et al.*, 1995). In the presence of oxygen and hydroxyl radicals, •NO can be converted into $\bullet NO_2$ and $OONO^-$ respectively, that cause similar damage as ROS (Burney *et al.*, 1999).

The •NO byproduct of the iNOS reaction is also involved in sustaining the Th1 immune response by stimulating secretion of cytokines. Therefore increased levels of iNOS and •NO is involved in the maintenance of inflammation (Coleman, 2001). *H. pylori* is able to stimulate iNOS production from gastric epithelial cells in vitro and in vivo (Li *et al.*, 2001). Moreover, high levels of nitrated proteins and higher levels of iNOS mRNA can be detected in patients suffering from various gastroduodenal diseases (Kaise *et al.*, 2003). Therefore, iNOS stimulation by *H. pylori* may be another method to promote chronic inflammation in the host.

An arginase, *rocF*, is a 37 kDa cell membrane-associated enzyme that forms a homooligmer of 100-300 kDa was identified in *H. pylori* to hydrolyze L-arginine to L-ornithine and urea (McGee *et al.*, 1999; McGee *et al.*, 2004; Mendz *et al.*, 1998). The role in protection in oxidative stressed became of interest when it was shown that RocF can compete for L-arginine with iNOS of phagocytic cells and limit the amount of NO• production (Gobert *et al.*, 2001). It has also been recently shown that if RocF becomes damaged through oxidative or nitrosative stress, that thioredoxin (Trx) may act as a chaperone for the proper renaturation of the protein so that it can aid in protection against further oxidative stress (McGee *et al.*, 2006).

2.5.3 Bacterial Antioxidant Defenses

Bacteria employ many defense mechanisms to deal with the burden of oxidative stress. Superoxide dismutases, catalases and peroxiredoxins are common mechanisms found in virtually all organisms to prevent against oxidative damage of DNA, protein and lipids (Storz and Imlay, 1999). Since the microaerophile *H. pylori* is especially

vulnerable to oxidative stress the three aforementioned detoxifying enzymes can be found as a line of defense against ROS and RNS.

2.5.3.1 Superoxide Dismutase

Superoxide dismutase (SOD) was first discovered in 1969 (McCord and Fridovich, 1969) and was subsequently found in all living organisms (Fridovich, 1995). SOD is a metalloenzyme and typically exists as a homodimer and catalyzes the disproprotionation of $O_2\bullet^-$ to form H_2O_2 and oxygen to prevent the formation of •OH:

$$2O_2\bullet^- + 2H^+ \rightarrow H_2O_2 + O_2$$

Unlike *E. coli*, *H. pylori* encodes only one SOD, an iron-containing SOD (FeSOD) called SodB (Pesci and Pickett, 1994; Spiegelhalder *et al.*, 1993). It was determined that SodB is 24 kDa and was localized on the cell surface, although no signal peptide was found (Spiegelhalder *et al.*, 1993). No follow up studies on the localization of SodB have been reported and this may need to be addressed as it is difficult to imagine that any organism would lack protection from endogenous $O_2\bullet^-$. In contrast, SodB of *E. coli* is found in the cytoplasm (Yost and Fridovich, 1973) along with another manganese-containing SOD (MnSOD; *sodA*) (Keele *et al.*, 1970). The third SOD of *E. coli*, SodC (copper-zinc; CuZnSOD), is found in the periplasm (Benov *et al.*, 1995). Due to the surface localization of *H. pylori* SodB, it is thought that its main function is to protect *H. pylori* against the oxidative burst ($O_2\bullet^-$) from infiltrating neutrophils during inflammation (Spiegelhalder *et al.*, 1993).

Isogenic *sodB* mutants were only viable at oxygen tensions of 2% and had a higher frequency of spontaneous mutations (Seyler *et al.*, 2001). Moreover, the mutants were unable to colonize mice suggesting the protective role against host oxidative

defenses. In addition, *sodB* mutants were found to have an accumulation of free Fe^{2+} inside of the cell than the wild type strain during the latter stages of growth which was also accompanied with an increased DNA damage (Wang *et al.*, 2005b). This is similar to what was seen in *E. coli* (Keyer and Imlay, 1996) and is reflective of the importance of SodB in detoxifying $O_2\bullet^-$. If $O_2\bullet^-$ is allowed to persist, they can disrupt the iron-sulfur clusters of hydrogenase, fumarate reductase, aconitase and ferredoxins (Olson *et al.*, 2000) thus elevating intracellular Fe^{2+} and providing more of an opportunity for Fenton chemistry.

2.5.3.2 Catalase

Found in almost all aerobic and anaerobic organisms, hydroperoxidase, or more commonly known as catalase, is a well studied enzyme that catalyzes the degradation of H_2O_2 into water and O_2 (Chelikani *et al.*, 2004):

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

Catalases can be broken into three classes; (i) monofunctional, heme-containing catalases; (ii) bifunctional, heme-containing catalase-peroxidase and; (iii) non-heme, manganese-containing catalases (Chelikani *et al.*, 2004). The latter two are the most common and are typically found in the cytoplasm and periplasm, respectively. Catalase-peroxidases have two activities; catalase (described above) and peroxidase:

$$2AH_{red} + H_2O_2 \rightarrow 2H_2O + 2A_{ox}$$

Peroxidase activity is also involved in the removal of H_2O_2, however, the substrate (AH_{red}) in vivo remains unknown (Carpena *et al.*, 2004).

Bacteria can encode several different catalases, for example *E. coli* encodes two catalases; a bifunctional catalase peroxidase (KatG) and a monofunctional catalase

(KatE). KatG is regulated in response to oxidative stress (Christman *et al.*, 1989) while KatE is regulated by the stationary phase sigma factor, RpoS (Loewen *et al.*, 1985). *H. pylori*; however, only encodes one catalase (KatA) which is a monofunctional catalase that forms a homotetrameric structure with each monomer having a molecular mass of 59 kDa (Hazell *et al.*, 1991; Loewen *et al.*, 2004). *H. pylori* also has a catalase-like protein (*hp0485*) in its genome that appears to lack a C-terminal domain essential for catalase activity (Alm *et al.*, 1999; Tomb *et al.*, 1997). No studies on this gene have been reported.

KatA has been shown to be localized in the cytoplasm and periplasm (Harris and Hazell, 2003). It makes up approximately 2-3% of the protein weight of *H. pylori* (Wang *et al.*, 2004) and has been shown to retain activity at pH $\geq$ 3.0 and at 56 °C (Bauerfeind *et al.*, 1997; Hazell *et al.*, 1991). However, catalase activity can be partially inactivated in the presence of organic hydroperoxides (Wang *et al.*, 2004). Mutants of *katA* were shown to be more sensitive to H_2O_2 than the wild type (Odenbreit *et al.*, 1996) and although catalase activity does not appear to be required for intial colonization of mice, it may be important in persistence since mutants showed a decrease in bacterial load during long-term infections (Harris *et al.*, 2003). This was also accompanied by a decrease in inflammation suggesting that the *katA*-mutant was not able to sustain a long-term infection owing to the clearing by the inflamed gastric mucosa. This is also supported by in vitro evidence that *katA*-mutants were unable to survive oxidative bursts from neutrophils (Ramarao *et al.*, 2000) or survive in macrophages (Basu *et al.*, 2004).

Immediately downstream of *katA* is an independently-transcribed *katA*-associated protein (*kapA*) (Harris *et al.*, 2002; Odenbreit *et al.*, 1996). Interest arose in KapA after a

yeast two-hybrid assay identified protein-protein interactions between KatA and KapA (Rain *et al.*, 2001). Mutants in *kapA* did not affect catalase activity; however, it was noted that it was more susceptible to H_2O_2 than the wild type strain (Harris *et al.*, 2002). In fact, *kapA* mutants had a similar phenotype in the mouse model as the *katA* mutant: decreased colonization during a persistent infection when compared to wild type (Harris *et al.*, 2003).

Putative roles of KapA surfaced when the cellular localization of KatA was evaluated (Harris and Hazell, 2003). The authors demonstrated that KatA was localized in the periplasm as well as the cytoplasm. However, KatA activity in the periplasm was dependent on the presence of KapA. In the *kapA* mutant, periplasmic KatA activity was decreased greater than 5-fold than in the wild type strain. Therefore it was speculated that KapA may act as a chaperone or as a transport accessory protein for the localization of KatA (Harris and Hazell, 2003).

2.5.3.3 Peroxiredoxins

Peroxiredoxins (Prxs), also known as thiol peroxidases or alkyl hydroperoxide reductases (Ahp), are ubiquitous, cytosolic, non-heme peroxidases that rely on a specific active cysteine to catalyze the reduction of H_2O_2, organic hydroperoxides (ROOH) and peroxinitrites to their corresponding alcohols (ROH) (Bryk *et al.*, 2000; Jacobson *et al.*, 1989; Poole, 2005):

$$ROOH + 2e^- \rightarrow ROH + H_2O$$

Prxs can be classed into three groups based upon the recycling mechanism of the sulfenic acid intermediate (S-OH); (i) typical 2-Cys; (ii) atypical 2-Cys and; (iii) 1-Cys (Poole, 2005). The formation of the S-OH is the same with all families; the active

peroxidatic cysteine is oxidized to S-OH by the peroxide substrate (Ellis and Poole, 1997a, b). The formation of the disulfide bond by the resolving cystiene is the distinguishing characteristic for each family (Poole, 2005). Three Prxs are encoded by *H. pylori*; thiol-specific peroxidase (Tpx), bacterioferritin co-migratory protein (Bcp) and an alkyl hydroperoxide reductase (AhpC) (Alm *et al.*, 1999; Tomb *et al.*, 1997). Bcp is a 1-Cys Prx while Tpx and AhpC is an atypical and typical 2-Cys Prx, respectively. Reduction of Bcp is through the Trx / thioredoxin reductase (TrxR) system (discussed below) (Wang *et al.*, 2005c) but the reduction of 1-Cys Prxs (e.g. Bcp) in *E. coli*, though thought to be mediated through glutathione / glutaredoxin / glutathione reductase, remains unclear (Rouhier *et al.*, 2006). Tpx has been shown to be reduced by the Trx / TrxR system in *E. coli* (Baker and Poole, 2003) but reduction mechanism has not been reported for *H. pylori.*

The reduction of *H. pylori* AhpC differs from that of *Salmonella* and *E. coli.* Their reduction is mediated through a 52 kDa flavoprotein subunit (AhpF) of the AhpCF complex (Tartaglia *et al.*, 1990). It has a bound flavin adenine dinucleotide (FAD) and passes $2e^-$ from NAD(P)H to the reducing disulfide bonds of Prxs (Poole, 1996; Poole and Ellis, 1996). *H. pylori* AhpC was shown to be reduced by the Trx / TrxR system and not by AhpF as depicted in Figure 1 (Baker *et al.*, 2001). The Trx / TrxR system uses redox active disulfide bonds for the transfer of electrons and helps maintain a reducing environment of the cytoplasm (Carmel-Harel and Storz, 2000). TrxR is 35 kDa, FAD-bound protein that contains one redox active disulfide bond and forms homodimers (Moore *et al.*, 1964; Zanetti and Williams, 1967). TrxR reduces a small, 12 kDa Trx (also forms a homodimer) by passing electrons from NADPH to FAD then to the redox active

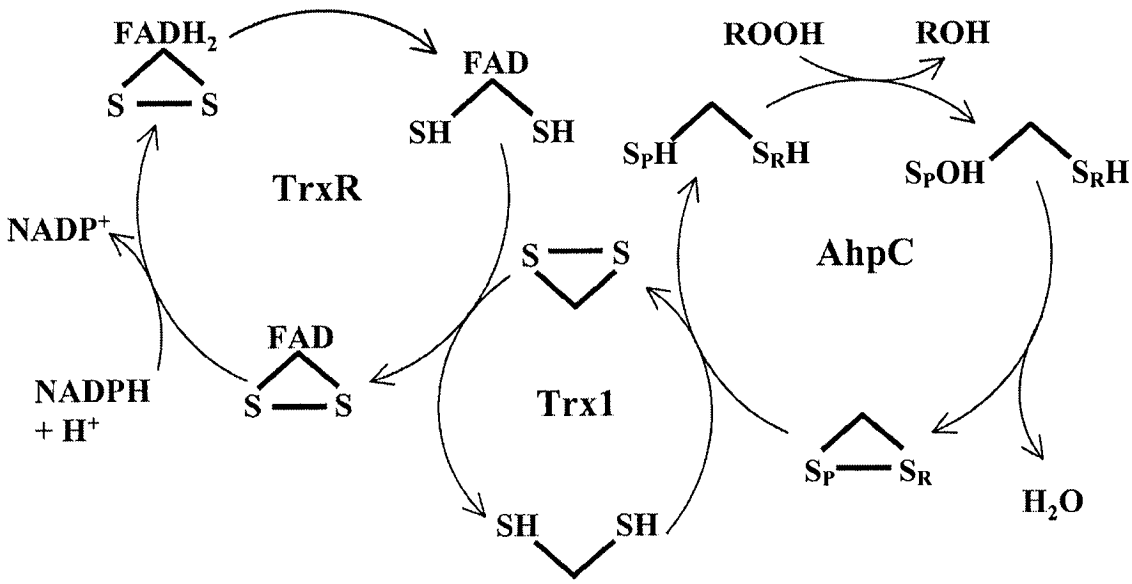


Figure 1. Trx Based Reduction of *H. pylori* AhpC. Two electrons are transferred from $NADPH_2$ through the FAD cofactor of TrxR to reduce Trx1 (Trx in the text). Trx1 then reduces the disulfide bond in between two AhpC subunits (S_P – S_R) to the peroxidatic cysteine (S_PH) and resolving cysteine (S_RH). The S_PH is oxidized to a sulfenic intermediate (S_POH) by the organic peroxide (ROOH) thereby reducing it to its corresponding alcohol (ROH). The recycling of S_POH occurs by resolution by S_RH from the other AhpC subunit, releasing water and reforming the disulfide bond (S_P – S_R). Figure adapted from Baker *et al.*, (2001).

disulfide bond of TrxR. The newly formed dithiols go through a dithiol-disulfide bond exchange with the redox active disulfide bond of Trx (Holmgren, 1985). Reduced Trx can further reduce a variety of disulfide bonds in various proteins, including Prxs.

Tpx was first described as an H_2O_2 scavengase and transcribed divergently from the *sodB* gene (Wan *et al.*, 1997). Isogenic *tpx* mutants were shown to be more sensitive to H_2O_2 and cumene hydroperoxide but not to nitrosative stress (Comtois *et al.*, 2003; Olczak *et al.*, 2003). Moreover, the importance of Tpx in establishing infection was shown as *tpx* mutants were cleared from mice after three weeks (Olczak *et al.*, 2003). Mutants of Bcp, on the other hand, were not susceptible to H_2O_2 and only slightly more susceptible to paraquat (superoxide generator) and organic hydroperoxides than the wild type strain (Comtois *et al.*, 2003; Wang *et al.*, 2005c). In addition, *bcp*-mutants failed to establish colonization in the mouse model (Wang *et al.*, 2005c). Though, in agreement with what was determined in *E. coli*, Bcp was shown to have a preference for the reduction of lipid hydroperoxides (LOOH) and is therefore thought to be more specialized in function than Tpx or AhpC (see below) (Wang *et al.*, 2005c). This is important for *H. pylori* as they contain polyunsaturated fatty acids as part of their phospholipids, albeit in small amounts (Geis *et al.*, 1990). During lipid oxidation, peroxides can facilitate $1e^-$ reductions of other lipids and quickly propagate to form peroxyl radicals (Girotti, 1998). Much like $O_2^{\bullet -}$, peroxyl radicals can also release Fe^{2+} from iron-sulfur clusters that could possibly lead to Fenton chemistry.

AhpC, is a homolog of human Prxs (Chuang *et al.*, 2006), has a molecular mass of 26 kDa (Lundstrom and Bolin, 2000) and makes up approximately 2% of the protein weight of *H. pylori* (Wang *et al.*, 2005c). The crystal structure of AhpC indicates that it

forms a symmetrical pentamer of homodimers [$(\alpha_2)_5$ decamer] which is reflective of other 2-Cys Prxs (Papinutto *et al.*, 2005). AhpC was originally thought to be essential for viability as several attempts to make an isogenic mutants were unsuccessful (Baker *et al.*, 2001; Lundstrom and Bolin, 2000). Mutants of *ahpC* were, however, obtained under low oxygen tension (2% O_2) (Olczak *et al.*, 2002) and were shown to be more sensitive to oxygen stress and unable to colonize the stomachs of mice suggesting its importance in establishing infection (Olczak *et al.*, 2003).

AhpC has been shown to reduce and detoxify H_2O_2, organic hydroperoxides, lipid peroxides and peroxynitrite (Baker *et al.*, 2001; Bryk *et al.*, 2000). In contrast, AhpC, and not catalase of *E. coli* is thought to be a major scavenger of endogenous H_2O_2 (Seaver and Imlay, 2001a) and that Tpx provides *E. coli* with protection from organic hydroperoxides (Cha *et al.*, 2004). However, with a strong catalase, the function of *H. pylori* AhpC is probably more protective against organic hydroperoxides incurred from inflammation and Tpx may merely provide redundancy (discussed later). It was determined that the *ahpC* mutant had a three-fold increase in the amount of lipid peroxides than wild type strain (Wang *et al.*, 2004). With the ability to reduce lipid peroxides it also seems that both AhpC and Bcp may perform similar roles in offering protection against lipid peroxides (Wang *et al.*, 2004; Wang *et al.*, 2006b). Additionally, accumulation of Fe^{2+} was detected in the *ahpC* mutant, much like with the *sodB* mutant that also. Although accumulation of Fe^{2+} in *sodB* mutants of *E. coli* had been described before, this was the first report for *ahpC* mutants (Wang *et al.*, 2005b). This further demonstrates the importance of AhpC in detoxifying ROS as the authors made a

correlation between the higher Fe^{2+} levels and increased DNA damage, presumably due to Fenton chemistry.

A novel function of AhpC has recently been described (Chuang *et al.*, 2006). They reported that during long-term exposure to oxidative stress, AhpC may act like a chaperone. They propose that under short-term oxidative stress, low molecular weight AhpC complexes (75-125 kDa) readily reduce ROS. However, during prolonged oxidative stress, AhpC forms high molecular weight complexes (>669 kDa) and acts as a chaperone to prevent misfolding or the unfolding of other proteins. They showed that during long exposures of AhpC to H_2O_2, the cysteine residues in the high molecular weight complexes were oxidized to sulfinic ($-SO_2H$) or sulfonic ($-SO_3H$) acids and could not be reduced by dithiothreitol (DTT). Additionally, this complex promoted the renaturation of denatured insulin and protected it against further reduction by DTT (Chuang *et al.*, 2006). Although AhpC may provide chaperone capabilities during prolonged oxidative stress, the Prx activity is probably inactivated due to the formation of $-SO_2H$ and $-SO_3H$ at the reactive cysteines. However, it is possible that Tpx may still provide protection against organic hydroperoxides as both Tpx and AhpC appear to have redundant enzymatic activities. However speculative, enzymatic function of Tpx in *H. pylori* has not been reported but may serve to support the hypothesis.

2.5.3.4 Non-Enzymatic Antioxidants

Some bacteria utilize non-enzymatic means for protection against oxidative stress. Though examples are numbered, a couple are; (i) phenolic glycolipids of *Mycobacteria* which are thought to scavenge •OH and $O_2^{\bullet -}$ (Chan *et al.*, 1989); (ii) an exopolysaccharide called alginate that is believed to scavenge oxidants as well as the

production of a pigment, pyocyanin, that can protect against exogenous •NO by *Pseudomonas aeruginosa* (Simpson *et al.*, 1989; Warren *et al.*, 1990). To date, none of these mechanisms have been reported in *H. pylori.*

NapA shares sequence similarity to Dps proteins from *E. coli.* Dps is involved in forming nucleoprotein complexes and sequestering free iron, probably to prevent the Fenton reaction and protects DNA from •OH (Park *et al.*, 2005; Wolf *et al.*, 1999). More recently, a NapA homolog in *H. hepaticus* that shares 59% identity to *H. pylori* NapA was shown to bind DNA and protect DNA against oxidative stress (Hong *et al.*, 2006). Originally, it was shown that NapA could not bind *H. pylori* DNA (Cooksley *et al.*, 2003); however, it was later determined that it could bind DNA when bound by Fe^{2+} (Wang *et al.*, 2006c). Since *napA* mutants in *H. pylori* were more susceptible to normal oxygen and organic hydroperoxides than wild type (Olczak *et al.*, 2002; Wang *et al.*, 2006c), this may be one example of a non-enzymatic mechanism of oxidative protection in *H. pylori.*

2.5.4 Oxidative Damage Repair

Despite the detoxifying enzymes that are employed to help protect bacteria against oxidative stress, damage can still occur. Damage to DNA and proteins must be fixed or deleterious effects may ensue. Bacteria encode several systems involved in the repairing of DNA lesions and oxidized proteins.

2.5.4.1 DNA Repair: Base Pair Excision

DNA lesions can occur by •OH formation during the Fenton reaction and nearly 100 different kinds of base and sugar damages have been identified (Gros *et al.*, 2002).

Such damaged bases include 7,8-dihydro-8-oxo-2'-deoxyguanosine (8-oxoG), formamidopyrimidine and thymine glycols (Devasagayam *et al.*, 1991; Schuchmann *et al.*, 1984). 8-oxoG incorporations into DNA can result in miscoding and mutations during DNA replication while formamidopyrimidine and thymine glycols are thought to block DNA and RNA polymerases (Fink *et al.*, 1997; Laval *et al.*, 1998).

Most oxidized bases are substrates for the base excision repair system. Small, monomeric, proteins called N-glycosylases recognize oxidized bases and are involved in their removal. In *E. coli*, the N-glycosylase, Fpg (also called MutM), recognizes a broad range of oxidized purines, including 8-oxoG, and several oxided pyrimidines (Tchou *et al.*, 1991). Nth (endonuclease III) is another well studied N-glycosylase that recognizes a broad spectrum of oxidized pyrimidines (Gros *et al.*, 2002; Radman, 1976). N-glycosylases catalyze the hydrolysis of the N-glycosidic bond between the target base and the deoxyribose. This releases the base and leaves an apurinic or apyrimidic (AP) site flanked by a 5' and 3' phosphate (Graves *et al.*, 1992). Subsequently, a 3'-OH primer is created by the 5'-phosphatase activity of endonuclease IV (EndoIV) (Izumi *et al.*, 1992) allowing for filling in of the gap by DNA polymerase III and DNA ligase (Dianov *et al.*, 1992). It should be noted that many different N-glycosylases exist with different specificities and slightly different mechanisms.

Repair of DNA in *H. pylori* may be more limited than *E. coli*. *H. pylori* has a paucity of N-glycosylases involved in base pair excisions of oxidized bases such as Fpg (MutM) and lacks MutH and MutL from the *mutHSL* mismatch repair system (Tomb *et al.*, 1997; Wang *et al.*, 2005a). Two genes, *hp0585* and *hp0602*, were annotated as homologs of Nth (EndoIII). Mutational analysis demonstrated that HP0602 was a 3-

methyladenine DNA glycosylase that was unable to excise oxidized bases (O'Rourke *et al.*, 2000). The function of HP0585 turned out to be similar to that of Nth of *E. coli* as *nth* mutants were unable to repair oxidized pyrimidines (O'Rourke *et al.*, 2003). Moreover, *H. pylori nth* mutants were hypersusceptible to H_2O_2 stress which is not seen in *E. coli nth* mutants. Finally, *H. pylori nth* mutants displayed reduced colonization of mice over time suggesting that throughout the progression of disease and prolonged exposure to inflammation (oxidative bursts). *H. pylori nth* mutants were unable to repair DNA damage.

More recently, more N-glycosylases and an AP endonuclease were identified in *H. pylori* (Huang *et al.*, 2006; Mathieu *et al.*, 2006). They demonstrated that MutY and Ung were N-glycosylases and that allelic replacements rendered *H. pylori* more susceptible to spontaneous mutations 26 and 4-fold respectively than wild type (Huang *et al.*, 2006). It was also shown that *mutY* mutants were only 30% efficient in the mouse colonization model (Eutsey *et al.*, 2006). Additionally, an AP endonuclease, XthA, was also identified and *xthA* mutants were 4-fold more susceptible to spontaneous mutations (Huang *et al.*, 2006). Although the mutants were not tested in vivo or against oxidative stress, it is reasonable to hypothesize that a similar observation would be made as what was reported with *H. pylori nth* or *mutY* mutants (Eutsey *et al.*, 2006; O'Rourke *et al.*, 2003).

2.5.4.2 Protein Repair: Msr

Methionine residues in proteins are subjected to oxidation by ROS to form methionine sulfoxides (MetSO) that can result in a loss of biological function. MetSO can exist as two different diastereoisomers Met(S)SO and Met(R)SO. Two enzymes,

methionine sulfoxide reductase A and B (MsrA and MsrB) were identified and shown to reduce MetSO back to methionine with specificity to the –S and –R isomers respectively (Grimaud *et al.*, 2001; Sharov *et al.*, 1999).

Unlike some bacteria that express multiple *msr* genes (Singh and Moskovitz, 2003), *H. pylori* encodes a single, 42 kDa Msr protein that has both MsrA and MsrB translationally fused together (Alamuri and Maier, 2004). This has also been observed in *Neisseria* spp. and *Haemophilus influenzae* (Ezraty *et al.*, 2005). Interestingly, *H. pylori* Msr does not exhibit MsrA activity, only MsrB activity and was reduced by the Trx / TrxR system (Alamuri and Maier, 2006) similar to *E. coli* (Mossner *et al.*, 1999). Mutants in *msr* contained more oxidized proteins, were more susceptible to oxidative stress and were unable to colonize the stomachs of mice when compared to the wild type strain (Alamuri and Maier, 2004).

Recently cross-linking and immunoprecipitation studies identified several proteins that interacted with Msr (Alamuri and Maier, 2006). Most interestingly was a DNA repair enzyme, SSR, KatA and the heat shock protein GroEL (molecular chaperone). The latter two only displayed interactions with Msr only after challenge with peroxide. Msr-GroEL interactions had been demonstrated in *E. coli* (Khor *et al.*, 2004) but not with catalase. Additionally, a higher Msr-KatA-GroEL complex was also identified suggesting that a larger complex may be utilized to assure the proper reduction and folding of oxidized KatA. Further evidence to support the repair of KatA was demonstrated by the addition of purified Msr to extracts of *msr* mutants which restored KatA activity from 50% to 85% of the wild type (Alamuri and Maier, 2006).

2.5.5 Regulation of Oxidative Stress Genes

Analaysis of the *H. pylori* genomes indicates few transcriptional regulators (Alm *et al.*, 1999; Oh *et al.*, 2006; Tomb *et al.*, 1997). To this end, homologs of the H_2O_2 response regulators OxyR of *E. coli* (Christman *et al.*, 1989) and PerR of *B. subtilis* (Bsat *et al.*, 1998) that regulate catalase, AhpCF and Fur are not found in *H. pylori.* Additionally, the superoxide / nitric oxide induced SoxRS of *E. coli* that regulate MnSOD and DNA repair enzymes (Amabile-Cuevas and Demple, 1991; Ding and Demple, 2000) or the organic hydroperoxde resistance (OhrR) of *Xanthomonas* spp. (Mongkolsuk *et al.*, 1998) are also not found in *H. pylori.*

Despite the lack of known oxidative regulators, *H. pylori* has displayed some regulation of its antioxidants systems. Many genome-wide microarray analysis have implicated up and down regulation of antioxidant genes in response to acid, metals or host-attachment (Bury-Mone *et al.*, 2004; Danielli *et al.*, 2006; Ernst *et al.*, 2005a; Kim *et al.*, 2004; Merrell *et al.*, 2003a; Merrell *et al.*, 2003b; Pflock *et al.*, 2006a), although little follow up or direct evidence has been reported. A mutant in the carbon storage regulator (CsrA), a repressor in *E. coli* of stationary genes and carbon storage, showed an increased sensitivity to oxidative stress (Barnard *et al.*, 2004). The authors attempted to correlate this sensitivity to the expression of known proteins involved in protection of oxidative stress. They found that in the wild type, levels of *ahpC*, *katA*, *napA, sodB* were increased during exposure to oxidative stress. In the *csrA* mutant levels of all genes tested were unaltered except for *napA* which was shown to be decreased in response to stress. In a separate study, Msr was shown to be increased 3-fold in response to hydroperoxides and nitric oxide and 3.5-fold in response to low Fe^{2+} which appear not to be related to Fur or

NikR (Alamuri and Maier, 2006). A further study using proteomics to look at regulated genes of oxidative stress demonstrated, perhaps surprisingly, that *ahpC* levels decreased during prolonged exposure to oxygen (Chuang *et al.*, 2005).

It was noted during the first characterization of *katA* that there was a Fur-box upstream of the coding sequence (Odenbreit *et al.*, 1996). Further work showed that there were actually two Fur-boxes upstream of *katA* and that Fur was able to bind (Delany *et al.*, 2001). However, they could not demonstrate Fur-specific regulation at the mRNA level in *fur* mutants. A further group did, however, show that KatA activity was decreased in *fur* mutants suggesting at least in part, an indirect role of Fur in the regulation of KatA (Harris *et al.*, 2002). This could be also due to alterations of heme synthesis and its unavailability for KatA activity, however, this has not been explored.

Fur may also play a role in the regulation of *napA* and *sodB*. NapA was shown to have multiple Fur-boxes upstream of the coding sequence and it was shown to be repressed by low Fe^{2+} (Cooksley *et al.*, 2003). This repression did not occur in *fur* mutants; however, the DNA binding of Fur to the *napA* promoter was not shown. Similarly, *sodB* was also shown to be repressed by apo-Fur (low iron) with direct evidence using electrophoresis gel mobility shift assays and DNA footprinting (Ernst *et al.*, 2005b). This report was the first demonstration of oxidative regulation in *H. pylori.*

Perhaps of most interest in recent reports is the compensatory mechanism of antioxidant proteins in *H. pylori.* This phenomenon has been demonstrated in *L. pneumophila* (Leblanc *et al.*, 2006) and *Burkholderia pseudomallei* (Loprasert *et al.*, 2003). Initial observations were made in *ahpC* mutants showing that there was a 5-fold increase in *napA* expression (Olczak *et al.*, 2002). This was followed by the observation

that *mdaB* was increased in an *ahpC napA* double mutant (Wang and Maier, 2004). Subsequent work demonstrated that mutants in *ahpC*, *katA*, *sodB* and *tpx* had an increase in *napA* and *mdaB* between 2-5 fold (Olczak *et al.*, 2005). This phenotype was further enhanced in the presence of double mutants in which levels of *napA* and *mdaB* were increased between 6-10 fold. Additionally, *katA* expression was shown to be increased 4-fold in *ahpC sodB* and *ahpC napA* double mutants. Therefore, there might be a novel mechanism allows *H. pylori* to recognize that it is an enfeebled state for protection against ROS and attempts to compensate by increasing other antioxidants.

2.6 Taxis

Motile bacteria seek favorable niches by quick responses to attractants and repellants in the environment. Attractants and repellants are sensed by transmembrane chemoreceptor proteins that send a signal through chemotaxis proteins (*che* genes) to ultimately determine the direction of motility. Bacteria swim by rotating flagella that are attached to an individual motor at a rate of 18,000 rpm that can switch between counter-clockwise or clockwise rotations (Berg, 2003). By default, flagella rotate in a counter-clockwise direction that form a bundle that cooperatively act to propel the bacteria which is called "smooth swimming" or "running". If a flagellar motor is triggered to turn the flagella clockwise, the flagella become uncoordinated with the other flagellum and the bacteria will "tumble". Cells typically perform a "random walk" which consists of running followed by a brief tumble followed by a run in a new direction (Berg, 2003). When an increase in attractants or decreases in repellants are sensed, tumbling is reduced thereby allowing the bacteria to run towards the favorable area. To date, the best characterized taxis systems are found in *E. coli* and *Salmonella enterica* serovar

typhimurium for Gram-negative bacteria, and *Bacillus subtilis* (*B. subtilis*) for Gram-positives.

Since *E. coli* is the prototypical system for our understanding in taxis, it will be described below and integrated to what little is known about taxis in *H. pylori.*

2.6.1 Chemotaxis

Chemotaxis is used by bacteria to seek optimal conditions by sensing gradients of attractant or repellent chemicals in the environment. The sensing of these gradients provides the organism with sufficient information to quickly alter its behavior and move to a more favorable niche. *E. coli* has been shown to sense attractants (e.g. amino acids, sugars) and repellents (acid, indols, Ni^{2+}) (Mowbray and Sandgren, 1998). *H. pylori* has also been shown to sense attractants (amino acids, bicarbonate, mucin, urea) and repellents (bile, certain amino acids, pH and in one report, urea) (Cerda *et al.*, 2003; Croxen *et al.*, 2006; Mizote *et al.*, 1997; Nakamura *et al.*, 1998; Worku *et al.*, 2004; Yoshiyama *et al.*, 1999) whilst others have been unable to reproduce the work (Andermann *et al.*, 2002). These attractants and repellents are sensed in bacteria by methyl-accepting chemoreceptor proteins (MCP; Figure 2A).

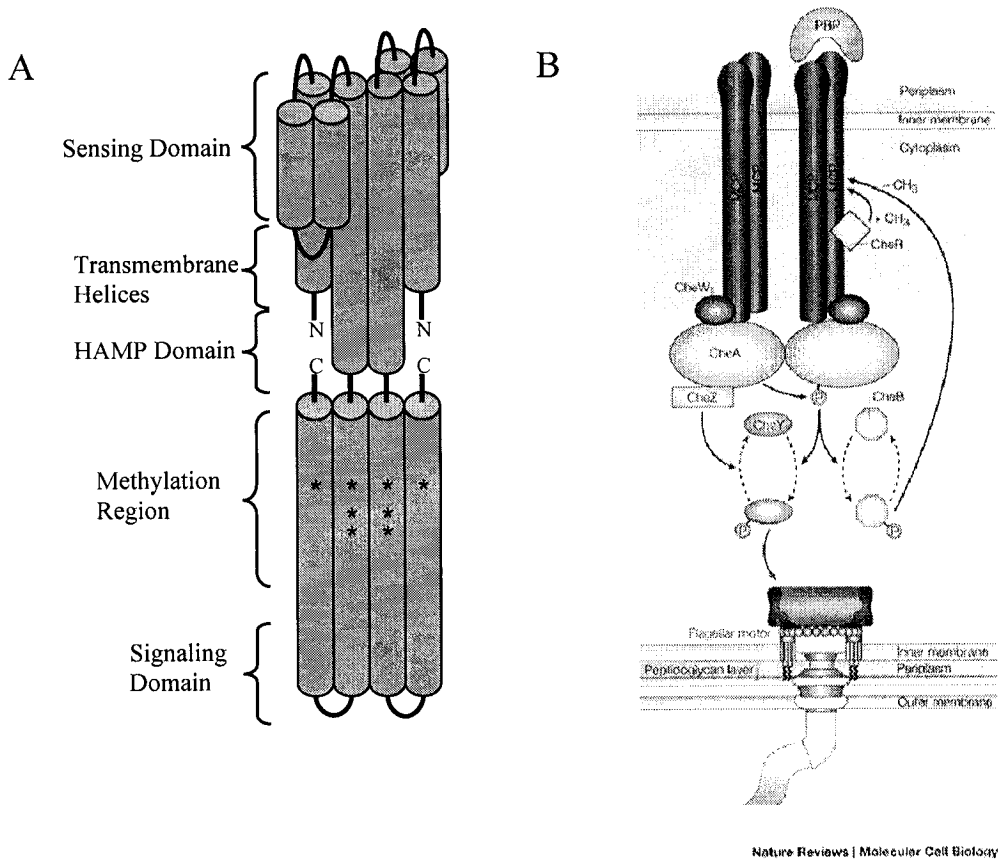


Figure 2. Schematic of MCP and Chemotaxis Systems in *E. coli*. (A) The typical structure of an MCP. Depicted is the sensing domain, transmembrane helicies, HAMP domains, methylation region and signaling domain. Asterisks represent methylation residues in the methylation region. Figure adapted from Szurmant and Ordal, (2004). (B) Relay of sensory information in *E. coli*. Two MCPs, one interacting with a periplasmic binding protein (PBP) show the scaffolding made with CheW and CheA. Repellent signals are sensed by MCPs and are sent through CheW to stimulate kinase activity of CheA. CheA facilitates phosphorylation of CheY that readily diffuses to the flagellar motor to promote clockwise rotations (tumble) away from the substrate. CheZ, also associated with CheY, enhances dephosphorylation of CheY~P thereby terminating the signal. Additionally, CheA phosphorylates CheB which competes with CheR for the methylation / demethylation of glutamate residues in adaptation process. Methylation alters the conformation of MCPs so that it will only trigger further chemotactic signals to CheA in response to a higher concentration than it previously sensed. Attractant signals prevent CheA kinase activity thereby decreasing CheY~P which promotes counterclockwise flagellar rotation (runs) towards the substrate. Figure adapted from Wadhams and Armitage, (2004).

2.6.1.1 Receptors

E. coli encodes four MCPs called Tar, Tap, Tsr and Trg (Mowbray and Sandgren, 1998). Tar and Tsr are highly abundant in the cell (ca 1,600 and 900 copies/cell respectively) and recognize aspartate and serine respectively, while Tap and Trg are lowly expressed (ca 150 copies/cell) and sense dipeptides and galactose respectively (Hazelbauer and Harayama, 1983). Some ligands, such as galactose and dipeptides, in addition to other small ligands must first be bound to proteins before they are recognized by MCPs (Falke *et al.*, 1997). *H. pylori* contains three classical MCPs referred to as TlpA (HP0099), TlpB (HP0103), TlpC (HP0082) and a fourth soluble MCP ortholog, TlpD (HP0599) (Tomb *et al.*, 1997). TlpA may be involved in the sensing of arginine and bicarbonate since expression of *tlpA* in *E. coli* conferred this phenotype and a *tlpA* mutant in *H. pylori* was not chemotactic to the substrates (Cerda *et al.*, 2003). TlpB was shown to have a possible role in the sensing of pH and allowing *H. pylori* to display pH taxis (Croxen *et al.*, 2006) and will be discussed in more depth in the thesis. The substrates of TlpC and TlpD remain unknown.

MCPs are transmembrane proteins that are clustered at one or both polls of the bacteria (Maddock and Shapiro, 1993) and that they form as mixed trimer of dimers (Studdert and Parkinson, 2004, 2005). They are composed of a variable N-terminal periplasmic (sensing) domain that are specialized to interact with specific ligands and a well conserved C-terminal, cytoplasmic domain (Le Moual and Koshland, 1996). The cytoplasmic domain can be broken into subdomains; a) Histidine kinases, adenylyl cyclases, methyl-binding proteins and phosphatases (HAMP) domain; b) two methylated helices and; c) signaling domain. HAMP domains, found in most sensor kinases of *E.*

coli, are postulated to transmit signals from external input domains to the cytosolic, output domains (Aravind and Ponting, 1999) but, the actual mechanism is unknown. The two methylated helices contain at least four glutamate residues that are subject to methylation and demethylation by CheR and CheB respectively (Terwilliger *et al.*, 1983; Terwilliger and Koshland, 1984) and are involved in the adaptation response (discussed later). Finally, the signaling domain is believed to provide a scaffold for binding and interactions between the CheA and CheW chemotaxis proteins (Le Moual and Koshland, 1996).

2.6.1.2 Sensing and Signaling

A schematic of chemotaxis can bee seen in Figure 2B. Sensing by the MCPs is thought to occur at one of the monomers of the homodimers. As a ligand binds one of the monomers, a conformational change ensues in that monomer which is thought to act in a piston-like mechanism. This is believed to send the proper signal from the periplasmic domain to the cytoplasmic domains (Ottemann *et al.*, 1998; Ottemann *et al.*, 1999). The signaling domain of MCPs form a scaffold for binding of CheW and CheA which are now believed to interact with all of the MCPs to form tight clustered network of sensors (Homma *et al.*, 2004). CheW is thought to regulate CheA activity through stabilization of electrostatic interactions depending on signals/conformation from/of the MCPs (Baker *et al.*, 2006). CheA, a histidine kinase, is part of a two-component system that, depending on its interactions with CheW, can autophosphorylate histidine to quickly transfer the phosphate group to the aspartate residue of the response regulator protein CheY (CheY~P) (Borkovich *et al.*, 1989). CheY~P can freely dissociate from CheA and act as an allosteric regulator of the flagellar motors, FliM, to promote a clockwise rotation of

the flagella (tumble) (Alon *et al.*, 1998). Therefore, an attractant will cause CheW to destabilize CheA and inhibit the CheA kinase activity so that there will be a lower pool of CheY~P and the cell will continue to run towards the attractant. Conversely, a repellent will favor interactions between CheW and CheA permitting CheA activity to increase CheY~P levels so that the cell will tumble.

H. pylori has orthologs of the following chemotaxis proteins; CheY1 (HP1067); CheAY2 (HP0392; a bifunctional protein with CheY fused to CheA at the C-terminal) and CheW (HP0391). Mutants in all three proteins, although motile as judged by microscopy, were unable to swarm in a soft-agar chemotaxis assay nor able to colonize pigs or mice (Foynes *et al.*, 2000; Pittman *et al.*, 2001). However contrasting results were found by another group that reported that each mutant displayed the same phenotypes except they were able to colonize the stomachs of Mongolian gerbils (Andermann *et al.*, 2002; Terry *et al.*, 2005). Even within the same group, one paper reports that their *cheY1* mutant colonizes mice (Terry *et al.*, 2005) while a subsequent paper published one month later indicates that it cannot infect gerbil or mouse stomachs (McGee *et al.*, 2005).

The phosphate flow of *H. pylori* has briefly been studied between CheAY2 and CheY1 (Jimenez-Pearson *et al.*, 2005a). Their findings suggest that both CheAY2 and CheY1 are phosphorylated by the CheA kinase activity of CheAY2 with a higher affinity towards CheY1. These results are consistent with the findings in *E. coli*. Although direct interactions between the Tlps, CheW and CheAY2 have not been reported, the transduction of phosphates appears to be analogous to other bacterial systems.

2.6.1.3 Adaptation

E. coli has the ability to adapt to persistent stimulation and return to a null state (i.e. to the same sensory state as if it were not stimulated) even though the stimulus is still present. This allows the bacteria to sense changes in the level of stimulation instead of responding to an absolute stimulus (Lai *et al.*, 2006b). Adaptation is accomplished by the methylation of glutamate residues in the methylated helices of MCPs and is modulated by CheR, a methyltransferase (Djordjevic *et al.*, 1998) and CheB, a methylesterase that is activated by phosphorylation by CheA (West *et al.*, 1995). Unstimulated cells have an equal rate of methylation and demethylation that keeps an intermediate kinase activity of CheA (null state). When an attractant is sensed by a MCP, CheA activity is inhibited and, as a result, the CheY~P and CheB~P pools are decreased. This, and a conformational change in the MCP, favors methylation of CheR over demethylation (Barnakov *et al.*, 2002). Increased methylation of the MCPs create a compensatory change in the MCP-CheW-CheA complex returning CheA activity back to null state (Lai and Hazelbauer, 2005; Lai *et al.*, 2006a). The MCPs are now adapted to the current state of the environment and can respond to new changes in attractant concentrations.

H. pylori lacks homologs of CheR and CheB (Tomb *et al.*, 1997). However, three CheV paralogs called CheV1, CheV2 and CheV3 (HP0019, HP0616 and HP0393 respectively) have been annotated. CheV proteins have been identified in the adaptation process for the chemotaxis system of *B. subtilis* (Karatan *et al.*, 2001). Of the three CheVs in *H. pylori, cheV1* mutants could not swarm in a chemotactic assay while *cheV2*, *cheV3* and double *cheV2 cheV3* mutants had no change in phenotype compared to wild type (Pittman *et al.*, 2001). It was later demonstrated that the three CheV paralogs could

dephosphorylate CheAY2 by accepting the phosphate on their CheY-like acceptor domain (Jimenez-Pearson *et al.*, 2005a). It is tempting to speculate that the three CheV act to compete for phosphates with CheY1 thereby being involved in the termination of the signals. Since the affinity for CheAY2 kinase activity in vitro is low, this may not be the case. This however should not rule out additional accessory proteins or modification of the CheV proteins that may facilitate an enhanced affinity for phosphorylation by CheAY2. In any event, the role of CheV, particularly CheV1 will require further investigation.

2.6.1.4 Removal of Signal

CheY possesses phosphatase activity which can be enhanced by the binding of CheZ (Blat and Eisenbach, 1994). CheZ binds to a truncated version of CheA called CheA short ($CheA_S$) that is expressed from an alternate translational initiation site (Cantwell *et al.*, 2003; Kofoid and Parkinson, 1991). $CheA_S$ dimerizes with CheA and is part of the MCP-CheW-CheA core machinery. CheA preferentially binds CheY and phosphorylates it as CheZ immediately binds CheY~P to enhances dephosphorylation (Cantwell *et al.*, 2003). Therefore, an increased and sustained CheA activity must occur to out compete CheZ and exceed a threshold of CheY~P to trigger tumbling. This may also aid to reduce a tumbling event due to fluctuations in signals (Bourret and Stock, 2002).

Since *H. pylori* lacks CheZ, it was proposed that the CheY2 domain of CheAY2 acts as a phosphate sink which can drain phosphate groups for CheY1 to terminate the signal. This is analogous to findings in *Sinorhizobium meliloti* which also contains a CheAY1 and CheY2 (note different annotation of genes) (Sourjik and Schmitt, 1996,

1998). Returning to *H. pylori* annotations, it is thought that CheY1 becomes phosphorylated by CheAY2 but CheAY2 can remove the phosphate group from CheY1~P and place it the CheY2 domain of CheAY2. This would reduce the pool of CheY1~P that is able to act at the flagellar motor. Since CheA is associates with MCPs and CheW (Le Moual and Koshland, 1996), CheAY2~P would be unlikely to diffuse to a flagellar motor and alter the rotation of the flagella unless they are colocalized with the flagellar apparatus as found in swarming *Caulobacter crescentus* (Alley *et al.*, 1992). This may be possible since the motility of *cheY1* mutants were still able to tumble (though excessively) and *cheAY2* mutants could only swim in straight lines (no tumbles) suggesting that CheY1 is not the main regulator of the flagellar motor (Foynes *et al.*, 2000).

More recently, it was reported that mutations in *hp0170* could suppress the *cheW* mutant phenotype to permit swarming in a soft-agar chemotactic assay (Terry *et al.*, 2006). Bioinformatic analysis using secondary structures indicate that HP0170 may be a homolog of CheZ from *E. coli*. Although not tested, they proposed that HP0170 is part of signal termination by enhancing the autodephosphorylation CheY1~P. Fundamentally, unless the function of *cheW* is compensated by another protein, then a *cheW* mutant should not be able to stimulate kinase activity from CheAY2 to phosphorylate CheY1. Therefore, the absence of HP0170 should not suppress the *cheW*. They argue, however, that it is possible that the CheW domain found in all of the CheV paralogs may restore CheW function as demonstrated in *B. subtilis* (Rosario *et al.*, 1994), although the CheVs of *H. pylori* were shown not to complement *cheW* in *H. pylori* (Pittman *et al.*, 2001). Despite no mention of this in Terry *et al.*, (2006), their proposal remains that the CheVs

may restore some CheW activity such that the kinase activity of CheAY2 can phosphorylate CheY1 in small amounts (Terry *et al.*, 2006). In the absence of HP0170, sufficient CheY1~P may accumulate to act on the flagellar switch. Therefore, to critically assess the role of HP0170 in the chemotaxis of *H. pylori*, it will be important that further studies are performed to demonstrate dephosphorylation activity and critical assessment of the CheV to support this hypothesis.

2.6.2 Energy Taxis (Aerotaxis)

Energy taxis is used by bacteria to seek a gradient for optimal cellular energy and is thought to be by monitoring the redox state of electron transport or the proton motive force across the membrane (Alexandre *et al.*, 2004). Studies on energy taxis have begun due to the discovery of a novel chemoreceptor, Aer, which is the major oxygen-sensing transducer in *E. coli* in a process called aerotaxis (Bibikov *et al.*, 1997). Aerotaxis allows movement of the bacteria towards optimal concentrations of oxygen and other electron receptors (Rebbapragada *et al.*, 1997). This allows the bacteria to find the best environment for optimal energy.

Aer is a low abundance MCP that has the highly conserved C-terminal signaling domain that couples signaling activity to CheW and CheA. Unlike the other MCPs, Aer is not subject to sensory adaptation of CheR and CheB (Bibikov *et al.*, 2004) and is anchored on the inner side of the cytoplasmic membrane where a conserved PAS (periodic clock protein, aryl hydrocarbon receptor and single-minded protein) domain is thought to sense oxygen stimulus (Amin *et al.*, 2006; Repik *et al.*, 2000). PAS is a common domain found in proteins that sense redox potential, energy levels and light (Taylor and Zhulin, 1999). Environmental oxygen is thought to be monitored by the

reduction or oxidation status of a flavin adenine dinucleotide (FAD) by the electron transport system (Bibikov *et al.*, 2000). It is proposed that the redox status of FAD facilitates a conformational change in the PAS and HAMP domains so that signaling can be passed along to CheW and CheA and subsequently control the rotational direction of the flagella (Alexandre *et al.*, 2004). The signaling of Aer is not well understood but is being elucidated (del Carmen Buron-Barral *et al.*, 2006; Gosink *et al.*, 2006).

In addition to Aer, the highly abundant Tsr has also been implicated in energy taxis and is thought to sense proton motive force (Greer-Phillips *et al.*, 2003; Rebbapragada *et al.*, 1997). Other than phenotypic observations, there is currently no direct evidence to support the findings that Tsr is a proton motive force sensor (Alexandre *et al.*, 2004). Two hypotheses are; (i) Tsr can sense pH and may be able to sense ΔpH of the proton motive force (Taylor *et al.*, 1999) and; (ii) changes in proton motive force may alter mobility of Tsr anchoring in the membrane by affecting the charges involved in stabilization (Alexandre and Zhulin, 2001).

B. subtilis can also sense oxygen through a soluble protein called HemAT (Hou *et al.*, 2000). The signaling domain is similar to that of other MCPs which associates with CheW and CheA. The sensory domain has globin folds that contain a heme group that can bind internal O_2 (Hou *et al.*, 2001; Zhang and Phillips, 2003). When the heme group binds O_2, and only O_2, a conformational change occurs in HemAT to send a signal from the sensory domain to the signaling domain (Zhang *et al.*, 2005). The mechanism of selective O_2 sensing is unknown.

Although aerotaxis has been described in *C. jejuni* (Hazeleger *et al.*, 1998) with proposed Aer-like proteins designated CetAB (Hendrixson *et al.*, 2001), no reports of aerotaxis or Aer orthologs in *H. pylori* have been made.

2.7 Antisense RNA

Antisense was first recognized in 1983 when it was discovered that RNA complementary to the Shine-Dalgarno and ATG start site can function as a repressor of the target gene (Simons and Kleckner, 1983). Now it is becoming increasingly apparent that natural, small RNAs (antisense RNAs) are a common strategy of bacteria to control gene expression (Gottesman, 2004). Antisense RNAs (also known as small RNAs or noncoding RNAs), are untranslated, highly structured RNAs that act via complementation to the target RNA. Antisense RNA can be encoded in *cis* on the opposite strand of the target sense RNA, or in *trans* with only partial regions of complementarity, but may have more than one target. Antisense RNAs are usually between 35 and 150 nucleotides (nt) in length and are believed to be stabilized by GC-rich stem-loop structures (Hjalt and Wagner, 1995). They function post-transcriptionally with their target mRNAs and can result in; (i) translational repression; (ii) translational activation and; (iii) target mRNA degradation (Gottesman, 2004).

Recently, approximately 50 new antisense RNAs were discovered in a genome-wide scan of *E. coli* (Argaman *et al.*, 2001; Gottesman *et al.*, 2001; Wassarman *et al.*, 2001). Although many are yet to be characterized, the sheer number underscores the importance of antisense RNA in gene regulation. Below is an example of one of recently discovered, and subsequently better studied, antisense RNAs, RyhB.

2.7.1 Natural Antisense RNA: RyhB

Although Fur functions as a repressor of transcription, there are a number of genes that are positively activated by Fur such as *acnA*, *fumA* (both iron-binding genes of the tricarboxylic acid cycle), *sdhCDAB* (succindate dehydrogenase operon) and *sodB* (iron superoxide dismutase; FeSOD) as well as various others (Dubrac and Touati, 2000; Hantke, 2001; Masse *et al.*, 2005). This positive regulation is through the repression of a small, 90 bp antisense RNA called RyhB. RyhB, when expressed (i.e. during iron depletion), targets the aforementioned positively regulated genes for mRNA degradation (Masse and Gottesman, 2002). The degradation of *sodB* has been the best characterized gene targeted for degradation by RyhB.

The RyhB RNA has a predicted secondary structure that contains three stem-loops (SL-) called; SL-1, SL-2 and SL-3 (Figure 3A). SL-2 contains the complementary sequences to *sod*B mRNA (Masse and Gottesman, 2002). The *sodB* mRNA contains two stem loops; SL-A and SL-B (encompasses the ribosomal binding site) that are separated by an AU rich linker region (Figure 3A) (Geissmann and Touati, 2004). Hfq (host factor 1), a small, basic protein that forms a hexameric-shaped structure, (Sauter *et al.*, 2003) binds many small RNAs and is thought to protect RyhB from degradation and facilitate RyhB-*sodB* interactions (Masse and Gottesman, 2002; Masse *et al.*, 2003). Hfq associates with RNase E and can then form ribonucleoprotein complexes with antisense RNAs (in this situation, RyhB) (Morita *et al.*, 2005). The interaction with RyhB is believed to recruit the Hfq/RNase E to the target *sodB* mRNA. Here, Hfq can bind to the AU linker region of *sodB* and open SL-B to make a larger loop thereby making the ribosomal binding site more accessible to interactions with SL-2 of RyhB (Figure 3A and 3B)

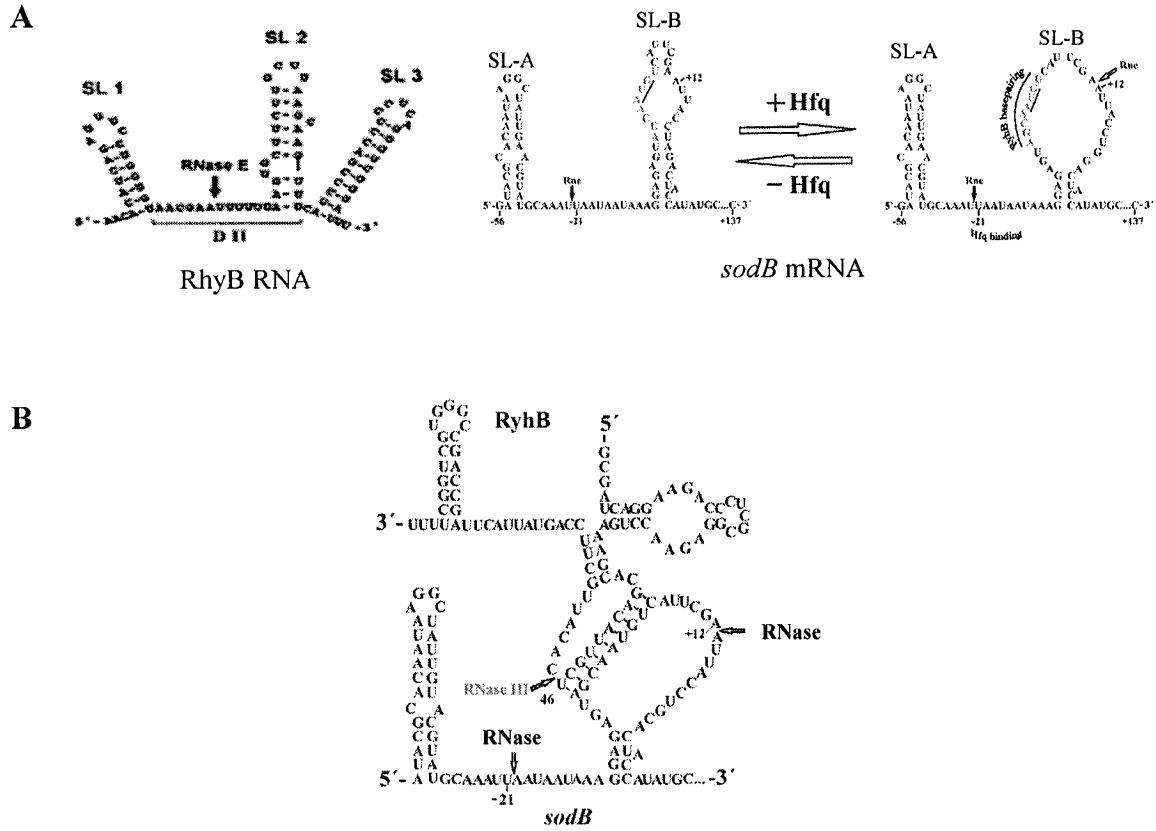


Figure 3. Proposed Function of RyhB RNA in *E. coli*. (A) Predicted secondary structures of RyhB RNA (adapted from Moll *et al.*, (2003)) and *sodB* mRNA (adapted from Afonyushkin *et al.*, (2005)). The three stem loop (SL-) structures are depicted for RhyB with the complementary sequence to the Shine-Dalgarno of *sodB* found in SL-2. The two SL structures of *sodB* mRNA (SL-A and SL-B) are shown separated by an AU rich linker region that is the binding sequence for Hfq. Dynamic changes in *sodB* mRNA upon Hfq binding alter SL-B to open up and expose the Shine-Dalgarno sequences to RyhB. (B) Upon recruitment of the ribonucleoprotein complex (RhyB-Hfq-RNase E; not depicted) RhyB basepairing (via SL-2) to the SL-B of *sodB* mRNA sequesters the Shine-Dalgarno from ribosomes thereby inhibiting translation initiation. Additionally, the RhyB-*sodB* interaction is subject to degradation by RNase III and RNase E as sites are depicted by arrows. Figure adapted from Afonyushkin *et al.*, (2005).

(Masse *et al.*, 2003). Recent reports, however, suggest that the RhyB-*sodB* interaction alone is sufficient for gene repression in the absence of degradation machinery (Morita *et al.*, 2006).

Under high iron conditions, *sodB* is also targeted for degradation by RNase E, albeit at a slower rate. It is thought that ribosomal binding during translation protects the *sodB* mRNA from quick degradation (Afonyushkin *et al.*, 2005). When RyhB is expressed (low iron conditions), and the ribosomal binding site is more accessible to RyhB, it is thought that the RyhB-*sodB* interactions prevent ribosomes from binding to *sodB* thus exposing the downstream RNase E cleavage (Afonyushkin *et al.*, 2005). After *sodB* cleavage by RNase E, the RyhB-*sodB* interaction is cleaved by RNase III and the remaining fragments of RyhB and *sodB* are degraded by exo- and endonucleases (Afonyushkin *et al.*, 2005).

With the recognition of many antisense RNAs it is becoming more evident that these RNAs are important in gene regulation in bacteria.

2.7.2 Antisense RNA as a Molecular Tool

Genetic analysis of some microorganisms is hampered by a lack of functional molecular tools. In eukaryotic systems it has become the norm to utilize antisense RNA driven methods to selectively target and knockdown proteins (Sen and Blau, 2006). The evolution and improved design of these strategies have been of major focus as they may be potential therapies for disease (Lu *et al.*, 2005b). Although more primitive, antisense-RNA has been increasingly adapted to bacterial systems. In the future it may become a useful method for studying intermediary levels of target genes and provide a way forward in characterizing function of essential genes.

2.7.2.1 Antisense RNA in Eukaryotes (RNAi)

Antisense oligonucleotides were used in eukaryotic cells for many years. However, improved antisense methodology in eukaryotic systems began due to the discovery of microRNA (miRNA). miRNAs are ~22 bp, noncoding, endogenous RNAs that regulate gene expression (Lagos-Quintana *et al.*, 2001). Not the actual miRNAs per se that led to improved antisense methods, but the dissection and exploitation of the host machinery that processes miRNAs have given a way forward for what is now called RNA interference (RNAi). Double stranded RNA (dsRNA) is processed in eukaryotic cells by an RNaseIII enzyme called Dicer which cuts it into 21-23 bp dsRNAs (Bernstein *et al.*, 2001; Zamore *et al.*, 2000). These 21-23 bp dsRNAs are called small interfering RNAs (siRNA) and one of the single stranded siRNAs are complexed in a RNA-induced silencing complex (RISC), while the other is degraded (Hammond *et al.*, 2001). The single stranded siRNA acts as a guide to recruit RISC to the target mRNA. If there is perfect basepairing between the siRNA and target transcript, the transcript is degraded by an RNase H-like enzyme of the RISC complex named Argonaute2 (Hammond *et al.*, 2001; Liu *et al.*, 2004). Otherwise, partial baseparing only seems to result in gene silencing and not mRNA degradation (Zamore and Haley, 2005). The processing of miRNAs is used by eukaryotic cells to regulate gene expression.

Scientists have exploited this method to exogenously add their own siRNA to target their favorite gene. Two methods are generally employed; (i) transfection of predesigned (22 bp) siRNAs or; (ii) expression of longer siRNA from a plasmid or from a virus (Li *et al.*, 2006). The former is much more expensive than the latter. Though common methodology, RNAi has its problems, particularly for further progression of in

vivo studies. Firstly, exogenous siRNA would need to compete with host RISC machinery and thus saturation may prevent essential regulation of other host genes. Toxicity has been observed with higher concentrations of siRNA. Secondly, siRNAs are short and it has been shown to have non-specific effects on other genes. Finally, dsRNA in general evokes interferon stimulation, a cytokine that modulates host immune responses (e.g. against RNA viruses) (Racz and Hamar, 2006; Snove and Rossi, 2006). An additional caveat lies in the fact that the processing of longer siRNAs as it is an ATP-dependent process (Zamore *et al.*, 2000). Although it may be overshadowed by other forms of toxicity, increased energy consumption may also have deleterious effects on the host cell as it may lack energy to provide essential functions. Nonetheless, these concerns are of importance as there is a large drive to circumvent or minimize the issues (Snove and Rossi, 2006).

2.7.2.2 Antisense RNA in Prokaryotes

It has been known for some time that prokaryotic cells produce natural antisense RNAs to regulate gene expression and plasmid copy numbers. With the advancement of RNAi technology in eukaryotic cells, various reports show that antisense RNA can be a useful method of knocking down protein levels in bacterial backgrounds that lack other basic genetic tools. Successful antisense strategies have been developed primarily in Gram-positive backgrounds (Kernodle *et al.*, 1997; McGrath *et al.*, 2001; Sun *et al.*, 2005; Zheng *et al.*, 2005), but can also be used in Gram-negatives (Engdahl *et al.*, 1997). Although the mechanism of action is unknown, three possibilities can be hypothesized; (i) that binding of antisense to the Shine-Dalgarno and translational start site (ATG) prevents ribosomal loading; (ii) antisense binding downstream sterically impedes

progression of mRNA through the ribosome and/or; (iii) the formation of dsRNA is subjected to degradation by RNases. In any event, the end result is knockdown levels of protein and thus permits study of intermediate levels of genes.

Successful knockdown of a homolog of the cellular division protein, FtsZ in *Borrelia burgdorferi* has recently been achieved with antisense (Dubytska *et al.*, 2006), the first time a function could be assigned to the homolog. Virulence factors have also been targeted by antisense as it has been shown that knockdowns of the α-toxin of *Staphylococcus aureus* attenuated the lethality phenotype associated with the toxin (Ji *et al.*, 1999; Kernodle *et al.*, 1997) suggesting usefulness for in vivo studies. Additionally, the mechanism of action of antimicrobials have been tested against known drug targets that were knocked down due to antisense (Ji *et al.*, 2004) and the discovery of natural antimicrobials against *S. aureus* have been identified using antisense against the target gene (Young *et al.*, 2006).

Antisense has also been adapted for genome-wide searches. A shot-gun approach was used to create an antisense library (Ji *et al.*, 2001). The library was used to identifiy 150 essential genes in *S. aureus*, 40% of which were known essential genes, 30% had putative functions and the remaining 30% were unknown. This work identified clones based on decreased viability under strong inducing conditions. Therefore, the application of genome-wide searches using antisense can be broadened for searches of other phenotypes. During the same period, a second genome-wide search of *S. aureus* using antisense RNA was done by another group, again, identifying essential genes (Forsyth *et al.*, 2002b).

There have been a few reported problems with antisense RNA in bacteria. An antisense against *ahpC* in *Mycobacterium bovis* was shown to be lost when tested in vivo (Wilson *et al.*, 1998). However, this was not a problem with in vivo studies using antisense against the α-toxin of *S. aureus* (Ji *et al.*, 1999). This may be due to the fact that AhpC provides function for survival of the organism and selective pressure within the animal model resulted in revertants (restored wild type AhpC levels) whereas the α-toxin of *S. aureus* is non-essential and only contributes to virulence. Secondly, antisense directed towards early genes in bacteriophage life cycles have been successful, however when directed towards late genes antisense was ineffective in preventing phage replication (McGrath *et al.*, 2001; Sturino and Klaenhammer, 2002; Walker and Klaenhammer, 2000). This may be reflective of the importance of expression of early genes in phage life cycles and that expression of later genes may not be as critical in phage maturation.

As seen with the increasing number of reports, antisense RNA interference has given an invaluable tool and a way forward to characterize the function of essential genes and virulence factors, particularly in genetic backgrounds that, in the past, have been shown to be difficult to manipulate.

Chapter 3: Materials and Methods

3.1 Bacterial Strains, Media and Plasmids

Bacterial strains and plasmids used in this study can be found in Table 1. *Helicobacter pylori* (*H. pylori*) was grown at 37 °C in a humid microaerophilic environment (83% N_2, 10% CO_2, 7% O_2) on brucella agar (43 g per liter) supplemented with 7.5% Newborn Calf Serum (NCS), 10 μg/ml vancomycin, 5 μg/ml trimethoprim and 4 μg/ml amphotericin B. Soft agar brucella plates were made with brucella media (2.8 g per liter) and 0.4% agar (4 g per liter) with the same supplements as the aforementioned brucella agar plates. Acidified brucella broth + NCS had the pH adjusted by using concentrated HCl. After the appropriate pH was obtained the broth was filter sterilized. Broth cultures were prepared as follows. A loop full of *H. pylori* growth was scraped from a brucella agar plate and resuspended in 500 μl of sterile 1x phosphate buffered saline (0.2 g KCl, 0.2 g KH_2PO_4, 8.0 g NaCl, 2.16 g $NaH_2PO_4 \cdot 7H_2O$, pH 7.4 in 1 L; PBS). Approximately 250 μl of the *H. pylori* suspension was added to 30 ml of brucella broth (28 g per liter) supplemented with 10% NCS and 10 μg/ml vancomycin or in brain heart infusion (BHI; 37 g per liter) supplemented with 5% NCS and 10 μg/ml vancoymcin. The cultures were gently agitated on a microtitre dish shaker (Labnet International Inc.) at 175 rpm in the 37 °C microaerophilic incubator. Where necessary the following antibiotic concentrations were used: 20 μg/ml chloramphenicol (Cm20), 20 μg/ml kanamycin (Km20) and 18 μg/ml metronidazole (Mtz18). *H. pylori* was routinely checked for morphology/motility (microscopy), urease activity (small amount of phenol red + urea and ddH_2O; yellow to pink color change), catalase activity (bubbling with the addition of H_2O_2) and oxidase activity (N,N,N',N'-tetramethyl-p-phenylenediamine +

ddH_2O; color change of light brown to a deep blue/purple). Frozen stocks of *H. pylori* were prepared in brucella broth containing 20% glycerol and stored at -70 °C.

Similarly, *Helicobacter hepaticus* (*H. hepaticus*) was grown on and in the same media as *H. pylori* except at an oxygen tension of 2%. Additionally, *Campylobacter jejuni* (*C. jejuni*) was grown on and in the same media as *H. pylori* except with supplementation of 5% NCS and no addition of Dents antibiotics.

Escherichia coli (*E. coli*) was grown on Luria Bertani (LB) media (per liter of ddH_20; 10 g NaCl, 10 g tryptone peptone, 5 g yeast extract and for agar plates 15 g of agar) at 37 °C under normal aerobic conditions. Broth cultures were typically grown at 150 rpm. Where necessary the following antibiotic concentrations were used: 100 μg/ml ampicillin (Amp100), 100 μg/ml carbenicillin (Carb100), 20 μg/ml chloramphenicol (Cm20), and 25 μg/ml kanamycin (Km25) unless otherwise indicated. Frozen stocks were prepared in LB containing 10% dimethylsulfoxide (DMSO) and stored at -70 °C.

Proteus mirabilis (*P. mirabilis*) was grown under the same conditions as *E. coli*. Broth cultures were grown in LB while low swarming plates (LSW^-) were prepared (per litre of ddH_2O; 10 g tryptone, 5 g yeast extract, 0.4 g NaCl, 5 ml glycerol, 20 g of agar) for isolation of individual (non-swarming) colonies (Belas, 1994).

Table 1. Strains and Plasmids Used in This Study

Strain	Characteristics	Reference;Source
Escherchia coli		
DH5α	F^-, φ80d*lacZ*ΔM15, Δ(*lacZYA-argF*)U169, *deoR*, *recA*1, *endA*1, *hsdR*17(rk^-, mk^+), *phoA*, *supE*44, λ^-, *thi*-1, *gyrA*96, *relA*1	Stratagene
JM109	F′ *traD36 proA*$^+$*B*$^+$ *lacI*^{q} *Δ(lacZ)M15/ Δ(lac-proAB) glnV44 e14*$^-$ *gyrA96 recA1 relA1 endA1 thi hsdR17*	NEB
BL21 CodonPlus®(DE3)-RIL	F– *ompT hsdS*(r_B– m_B–) *dcm*+ Tet^r *gal l* (DE3) *endA* Hte *[argU ileY leuW* Cm^r]	Stratagene
Proteus mirabilis		
PMS2	wild type clinical isolate	Toptchieva *et al.*, 2003
Helicobacter pylori		
1061	wild type strain	Goodwin *et al.*, 1998
88-3887	wild type strain, motile, pig-passaged, mouse-colonizing strain, variant of KE26695	Josenhans *et al.*, 2000
G27	wild type strain	Censini *et al.*, 1996; Dr. Nina Salama
G27 *hp0405*::*cat*	*hp0405* replaced with chloramphenicol cassette (*cat*), Cm^R	This Study
G27 *hp0405*::P150T7	*hp0405* replaced with chloramphenicol cassette (*cat*) and 100bp antisense-*ahpC* + T7 terminator, Cm^R	This Study
G27 *rdxA*::*aphA-3*	*rdxA* locus replaced with kanamycin cassette (*aphA-3*); Km^R	This Study
G27 *rdxA*::GFP3K	*rdxA* gene replaced with a promoterless *gfpmut3*; Km^R	This Study
KE26695	wild type strain, TIGR strain, genome sequenced	Tomb *et al.*, 1997
SS1	wild type strain, mouse-adapted	Lee *et al.*, 1997; Dr. Adrian Lee

SS1 Δ*tlpA*	*tlpA* gene replaced with kanamycin cassette (*aphA-3*); Km^R	Croxen *et al.*, 2006
SS1 Δ*tlpB*	*tlpB* gene replaced with kanamycin cassette (*aphA-3*); Km^R	Croxen *et al.*, 2006
SS1 Δ*tlpB/tlpB*$^+$	Δ*tlpB* with wild type *tlpB* complemented in the *rdxA* gene; Cm^R, Km^R	Croxen *et al.*, 2006
SS1 Δ*tlpC*	*tlpC* gene replaced with kanamycin cassette (*aphA-3*); Km^R	Croxen *et al.*, 2006
SS1 Δ*tlpD*	*tlpD* gene replaced with chloramphenicol cassette (*cat*); Cm^R	Croxen *et al.*, 2006
SS1 *rdxA::aphA-3*	*rdxA* gene replaced with a kanamycin cassette (*aphA-3*); Km^R	This Study
SS1 *rdxA*::AS0166K	*rdxA* gene replaced with antisense-*hp0166* (P_{ureA} promoter, *fd* terminator); Km^R	This Study
SS1 *rdxA*::AS1043K	*rdxA* gene replaced with antisense-*hp1043* (P_{ureA} promoter, *fd* terminator); Km^R	This Study
SS1 *rdxA*::AS1043-2K	*rdxA* gene replaced with antisense-*hp1043* (P_{hp1043} promoter, *fd* terminator); Km^R	This Study
SS1 *rdx*::FAS1043K	*rdxA* gene replaced with antisense-*hp1043* (P_{ureA} promoter, full length, *fd* terminator); Km^R	This Study
SS1 *rdxA*::FAS1043-2K	*rdxA* gene replaced with antisense-*hp1043* (P_{hp1043} promoter, full length, *fd* terminator); Km^R	This Study
X47-2AL	wild type strain, cat isolate, mouse-adapted, herein referred to as X47	Handt *et al.*, 1995; Dr. Douglas Berg
X47 *rdxA::aphA-3*	*rdxA* gene replaced with kanamycin cassette (*aphA-3*); Km^R	This Study

X47 *rdxA*::AS1563K	*rdxA* gene replaced with antisense-*ahpC* (P_{ahpC}, full length, *fd* terminator); Km^R	This Study
X47 *rdxA*::AS1563-2K	*rdxA* gene replaced with antisense-*ahpC* ($P_{ureA,}$ full length, *fd* terminator); Km^R	This Study
Helicobacter hepaticus		
3B1	wild type strain	Fox *et al.*, 1994; Dr. James Fox
Campylobacter jejuni		
H480	wild type clinical isolate	Hoffman and Goodman, 1982
Plasmids		
pBlueScript SK+	cloning vector; also referred to as pBSK; Amp^R	Stratagene
pASAHPC100	100 bp antisense-*ahpC* cloned in pBSK; Amp^R	This Study
pASAHPC250	250 bp antisense-*ahpC* cloned in pBSK; Amp^R	This Study
pASAHPC	full length antisense-*ahpC* cloned in pBSK; Amp^R	This Study
pBScat	*Campylobacter coli* chloramphenicol cassette (*cat*) cloned in pBSK; Amp^R Cm^R	Hoffman Lab
pBC	cloning vector; Cm^R	Stratagene
pBC::P_{ureA}	P_{ureA} cloned in pBC; Cm^R	This Study
pHel2	*E.coli* / *H. pylori* shuttle vector; source of *fd*-bacteriophage terminator; Cm^R	Heuermann and Haas, 1998; Dr. Rainer Haas
pFD	*fd*-bacteriophage terminator cloned in pBC; Cm^R	This Study
pET-16b	10x His-tagged N-terminal overexpression vector; $Carb^R$	Novagen
pET16::*hp0166*	*H. pylori hp0166* (*arsR*) cloned in-frame with 10X N-terminal His-tag of pET-16b; $Carb^R$	This Study
pET16::*hp1043*	*H. pylori hp1043* cloned in-frame with 10X N-terminal His-tag of pET-16b; $Carb^R$	This Study
pET-29b	6x His-tagged C-terminal overexpression vector; Km^R	Novagen
pET-29b::*ahpC*	*H. pylori ahpC* (*hp1563*) cloned in-frame with 6x C-terminal His-tag of pET-29b; Km^R	This Study

pEU39cm	*H. pylori hp0405* cloned in pUC19; Amp^R, Cm^R	Olson *et al.*, 2000; Dr. Robert Maier
pEU39cm::P150T7	100bp antisense-*ahpC* + T7 terminator cloned into pEU39cm; Amp^R, Cm^R	This Study
pDH37	*E. coli / H. pylori* shuttle vector (same as pHel3); Km^R	Heuermann and Haas, 1998; Dr. Rainer Haas
pHASAHPC100	100 bp antisense-*ahpC* cloned in pDH37; Km^R	This Study
pHASAHPC250	250 bp antisense-*ahpC* cloned in pDH37; Km^R	This Study
pHASAHPC	full length antisense-*ahpC* cloned in pDH37; Km^R	This Study
pRDX	suicide vector to replace *rdxA* of *H. pylori* (Mtz^R), pBS backbone; Amp^R	This Study
pRDX-C	chloramphenicol resistant variant of pRDX; Amp^R, Cm^R	This Study
pRDX-C::*tlpB*	wild type *H. pylori tlpB* cloned in pRDX-C; Amp^R, Cm^R	This Study
pHP1	*E. coli / H.pylori* shuttle vector; source of *aphA-3* (kanamycin) cassette; Km^R	Kleanthous *et al.*, 1991; Dr. Robert Maier
pRDX+	improved pRDX (fully replaces *rdxA*; larger flanking sequence), pBC backbone; Cm^R	This Study
pRDX-K+	kanamycin resistant variant of pRDX+; Cm^R Km^R	This Study
pGFPmut3	*gfpmut3* cloned in pKEN2; Amp^R	Cormack *et al.*, 1996; Dr. Raphael Valdivia
pGFP3K	promoterless *gfpmut3* cloned in pRDX-K+; Km^R	This Study
pAS1563K	antisense-*ahpC* (P_{ahpC}, full length, *fd* terminator) cloned in pRDX-K+; Km^R	This Study
pAS1563-2K	antisense-*ahpC* (P_{ureA}, full length, *fd* terminator) cloned in pRDX-K+; Km^R	This Study
pAS0166K	antisense-*hp0166* (P_{ureA}, *fd* terminator) cloned in pRDX-K+; Km^R	This Study

pAS1043K	antisense-*hp1043* (P_{ureA}, *fd* terminator) cloned in pRDX-K+; Km^R	This Study
pFAS1043K	antisense-*hp1043* (P_{ureA}, full length, *fd* terminator) cloned in pRDX-K+; Km^R	This Study
pAS1043-2K	antisense-*hp1043* (P_{ureA}, *fd* terminator) cloned in pRDX-K+; Km^R	This Study
pFAS1043-2K	antisense-*hp1043* (P_{ureA}, full length, *fd* terminator) cloned in pRDX-K+; Km^R	This Study
pHel3	*E. coli / H. pylori* shuttle vector; Km^R	Heuermann and Haas, 1998; Dr. Rainer Haas
pHel3::FAS1043	antisense-*hp1043* (P_{ureA}, full length, *fd* terminator) cloned in pHel3; Km^R	This Study
pHel3::FAS1043-2K	antisense-*hp1043* (P_{hp1043}, full length, *fd* terminator) cloned in pHel3; Km^R	This Study
pUC19	cloning vector; Amp^R	NEB
pUC*tlpB*	*H. pylori tlpB* cloned under P_{lac} of pUC19, Amp^R	This Study

3.2 Molecular Techniques

3.2.1 Oligonucleotides

Oligonucleotides used in this study can be found in Table 2.

3.2.2 Restriction Digestion

Restriction digestions were performed in a final volume of 20 µl following manufacturer's instructions (New England Biolabs; NEB). In general 100-500 ng of DNA was cut with the appropriate 10x reaction buffer diluted to 1:10, 1x Bovine Serum Albumin (BSA) and 0.5 µl of the enzyme(s). Incubation was allowed to occur for 37 °C unless otherwise indicated by the manufacturer. When necessary, filling in 5' overhangs, or recessing 3' overhangs was performed by adding 0.5 µl 10 mM dNTPs and 0.5 µl of T4 DNA Polymerase and allowed to incubate at 12 °C for 15 minutes. Reactions were stopped with the addition of DNA loading buffer and run on a 1% agarose gel (see agarose gel electrophoresis).

Partial digests were performed with a buffer that contained less than 50% activity. If this was possible, a 1:2 dilution of the buffer with the least activity was used. Additionally, the restriction enzyme was diluted 1:2, 1:5 and 1:10 before added to the digestion mix. The reaction was incubated at 37 °C for approximately 10 minutes before being subjected to agarose gel electrophoresis.

Table 2. Oligonucleotides Used in This Study.

Name	Sequence (5' -> 3')	Notes
aahpC100BPE	CAGGAATTCCATGGTGAAGTTTGCCCAGCA	EcoRI
aahpC250BPE	CAGGAATTCGAAGAAGCGATCGCTTTGAG	EcoRI
aahpCB670R	GATACTCTGCAACGCCTTGGT	
aahpCBFPstI	ATGCCTGCAGGCTGATTGAGTGGAAAGCAT A	PstI
aahpCFBHI	CAGGGATCCAATAACGATGAAACAAGA	BamHI
ahpCA2F	TTCCCGGGATGTTAGTTACAAAACTTGC	SmaI
ahpCA2R	GAGAATTCAGAAAATTCCATTAAGCTT	EcoRI
ahpCAF	TTCCCGGGAATAACGATGAAACAAGA	SmaI
ahpCAR	ACGAATTCATCGTAACTCCTTAAGTG	EcoRI
ahpCFBamHI	GCGGATCCGATGTTAGTTACAAAACTTGCCC CA	BamHI
ahpCRXhoI	CCGCTCGAGAAGCTTAATGGAAT	XhoI
anti0166FPstI	ATGCCTGCAGACATTAACAAAGTTAATCGTT	PstI
anti0166RBamHI	GTAGGATCCATAATCTAGTGCTTTAATC	BamHI
anti1043FPstI	ATGCCTGCAGAATTACTCTTAAATGCAGG	PstI
anti1043RBamHI	GTAGGATCCTTAATGCTACGATAAGGTT	BamHI
aphA3F	CGATACTATGTTATACGCCAA	
aphA3R	GACATCTAAATCTAGGTACTA	
catF	GATATAGATTGAAAAGTGGAT	
catR	TTATCAGTGCGACAAACTGGG	
HP0166FNdeI	GAAGTCCCATATGATGATAGAAGTTTTAATG ATAGAAGATG	NdeI
HP0166RBamHI	CAGGGATCCTCAGTATTCTAATTTATAACCA ATCC	BamHI
HP1043FNdeI	GAAGTCCCATATGATGCGCGTTCTACTGATT G	NdeI
HP1043RBamHI	CAGGGATCCTTACTCTTCACACGCCGGTT	BamHI
pCMR	CATACTATATGTGCAGGGCG	
Php1043FSpeI	TAGCACTAGTCTTAATCGTAATCAAGCGG	SpeI
Php1043RBamHI	GTCGGATCCGAGTAATTCAGGCACTGAT	BamHI
pKMR	ATCGGTACCAAGTAATCGCATC	
PureAFSpeI	CGTAACTAGTAGTCGTGGCCACCATTATCA	SpeI
PureARBamHI	GTAGGATCCATCAAGGTTGGATGTAATTG	BamHI
RDXAI+SacI	GTAGAGCTCGCATTCGTGGGATGAGCTA	SacI
RDXAI+XbaI	GCATCTAGACTTGCAAGAATGGCGCTCG	XbaI
RDXAII+KpnI	CTGGGTACCGCTCAATCTGACAACCCAC	KpnI
RDXAII+XhoI	CGACTCGAGGTGGCAGAAGCGAGTCA	XhoI

rdxAIIKpnI	ATCGGTACCAAGTAATCGCATC	KpnI
rdxAIIXhoI	TTGCTCGAGTGCTTGGCG	XhoI
rdxAISacI	CACGAGCTCTGGTAATTGTTTCGTTAGGG	SacI
rdxAIXbaI	CACTCTAGACTTTATAAGACTCCGGATAG	XbaI
tlpA2Km-P1	AACAAGCTCGCTAAAGGCTG	
tlpA2Km-P2	TTGGCGTATAACATAGTATCGTCACAATCAA CGCCACGCAC	
tlpA2Km-P3	TAGTACCTAGATTTAGATGTCATCGGTTACT GAGGGCAATC	
tlpA2Km-P4	AAAGCTCGTTTTCTCTCGCC	
tlpAF	GAGCGCGAATAAGAGTTTGC	
tlpAR	ATGATCCCATTTAGCGCGTC	
tlpB2Km-P1	TGGTTACAGACGCTGATAGG	
tlpB2Km-P2	TTGGCGTATAACATAGTATCGAACACGACCA GCATGATACG	
tlpB2Km-P3	TAGTACCTAGATTTAGATGTCATGTGAGTGG AACGACCATG	
tlpB2Km-P4	CAAGCGGATGATCATCTC	
tlpBCompFBamHI	GCAGGATCCTCAAAGGATGGGAGGACTT	BamHI
tlpBCompREcoRI	GCAGAATTCACGGAAAGAATGGTGTCTTC	EcoRI
tlpBF	CGCCTACTCGCATTATGATG	
tlpBR	CATCCTTGCTCTCTTTGACC	
tlpC2Km-P1	ATCGCTAAAGTGTGGCTCAC	
tlpC2Km-P2	TTGGCGTATAACATAGTATCGTAACAACCGC ACACACCATC	
tlpC2Km-P3	TAGTACCTAGATTTAGATGTCGAAGATGTGA GCAGGAAG	
tlpC2Km-P4	TGAACGACGATATGGGTG	
tlpCF	TTTAGGCGTGCTGGTGTTAG	
tlpCR	TCCTTTCTTGTTTGTGGGCG	
tlpD2Cm-P1	GTGTGCATCCATGAATAAGA	
tlpD2Cm-P2	*CCCAGTTTGTCGCACTGATAA*GGAAGATAGTG CTAAACATG	
tlpD2Cm-P3	*ATCCACTTTTCAATCTATATC*TGTTCAAGCTTG CAAGATTCC	
tlpD2Cm-P4	CTTTAATCATTACACACCGC	
tlpDF	GCCTAAACGGCAATTCTTATG	
tlpDR	AACCAACGGAGCAATGAAAT	

Underlined indicate restriction endonuclease sites. Double underlined represents complementary sequences to either the *cat* (catF or catR) or *aphA3* (aphA3F or aphA3R) cassette primers. rdxAIIXhoI and rdxAIIKpnI designed from Smeets *et al.*, 2000.

3.2.3 Agarose Gel Electrophoresis

DNA larger than 200 bp was generally viewed on a 1% agarose gel (w/v) made up in 1x TAE (made in 50x stock; 242 g Tris Base, 57.1 ml glacial acetic acid and 100 ml of 0.5 M EDTA, pH 8.0, in a final volume of 1 L) and ethidium bromide (10 µl of 0.1 mg/ml stock in 25 ml of agarose). Smaller DNAs were viewed on a 2% agarose gel. All samples were mixed with 10x DNA loading buffer (200 mM Tris-HCl, pH 7.5, 200 µM EDTA, pH 8.0, 20% glycerol and 0.25% [w/v] bromophenol blue) and run in 1x TAE at 100 V on a Gel X_L^{Plus} Mini Gel Electrophoresis System (Labnet International, Inc.). Gels were visualized on a FOTO/Prep transilluminator (Fotodyne) and when necessary, bands were excised with sterile scalpels.

3.2.4 DNA Purification

DNA was purified from enzymatic reactions and agarose gel excisions using Qiagen Gel Extraction spin columns (Qiagen) according to the manufacturer's instructions. The DNA was typically eluted with a final volume of 30 µl. All DNA was frozen and stored at -20 °C.

3.2.5 DNA Ligation

DNA ligations were performed in a final volume of 20 µl using T4 DNA ligase (NEB). Sticky end ligations were set up with 1x T4 ligase buffer, equal amounts of DNA and 0.5 µl of T4 DNA ligase. The reactions were allowed to incubate for 1-2 hours at room temperature (22 °C). Blunt-end ligations were set up similarly except the use of 0.75x T4 ligase buffer, a 10:1 insert to vector ratio and an incubation of 2-4 hours at room temperature. The T4 DNA ligase was heat inactivated at 70 °C for 20 minutes.

3.2.6 Polymerase Chain Reaction (PCR)

PCR was set up using either Qiagen HotStarTaq (Qiagen) or Roche Expand High Fidelity Polymerase (Roche Applied Science) and run in a PTC-100™ Programmable Thermal Controller (MJ Research, Inc.) or GeneAmp^{R} PCR System 9700 (Applied Biosystems). Taq based PCR was set up in a 10 µl reactions containing 1x PCR buffer, 2.5 mM $MgCl_2$, 200 µM of each dNTP, 0.5 µM forward and reverse primers, ~50 ng template and 0.05 µl HotStarTaq and ddH_2O. Generally, PCR reactions started with a 15 minute denaturation at 95 °C followed by 35 cycles of amplification (95 °C denaturation for 30 seconds, 30 seconds at a determined annealing temperature [usually 48-56 °C] and 72 °C elongation at 1 minute per kb of DNA) followed by an elongation of 7 minutes at 72 °C and a final hold at room temperature (22 °C).

Roche polymerase based reactions were set up in 25 µl containing 1x PCR buffer, 1.5 mM $MgSO_4$, 200 µM of each dNTP, 0.4 µM forward and reverse primers, 20-50 ng of template and ddH_2O. PCR conditions were the same as above except the use of a 2 minute denaturation (where 0.25 µl Roche High Fidelity Polymerase was added), and a final hold at 4 °C. Amplified DNA was viewed with agarose gel electrophoresis.

For whole cell PCR, extracts were prepared depending on the organism. For *E. coli,* a single colony was picked up with a sterile pipette tip and resuspended to 200 µl ddH_2O. One microlitre of this suspension was used as template for PCR reactions. For *H. pylori,* a small amount of cells were picked with a sterile pipette tip and resuspended in 20 µl lysis buffer (50 mM NaOH, 0.025% SDS). This suspension was heated at 95 °C for 15 minutes and the volume brought up to 200 µl with ddH_2O and finally centrifuged at

16,000 x *g* for 5 minutes. One microlitre of the supernatant was used as a template for PCR reactions.

3.3 DNA Isolation

3.3.1 Genomic / Chromosomal DNA

Chromosomal DNA of *H. pylori* was isolated by taking two large loopfuls of overnight growth and added to 440 µl TE (Tris-HCl and EDTA, pH 8.0). After centrifugation at 3,300 x *g* for 4 minutes, the pellet was homogenously resuspended in TE before the addition of 50 µl Proteinase K (10mg/ ml Proteinase K made up in 50 mM Tris-HCl pH 8.0, 1 mM $CaCl_2$) and 10 µl 10% SDS. The sample was incubated for 1 hour at 37 °C until complete lysis. An equal volume of a 25:24:1 mixture of phenol/ chloroform / isoamyl alcohol was added and allowed to rock for 20 minutes at room temperature followed by centrifugation at 16,000 x *g* for 8 minutes. Approximately 450 µl of the aqueous phase was removed and added to an additional 50 µl of TE and another 500 µl of phenol/chloroform/isoamyl alcohol and the process was repeated 3-5 more times until the interface was clear. DNA was precipitated by adding 50 µl of 3.0 M sodium acetate, pH 5.0 and 1.5 ml of 95% ethanol and placed at -70 °C for 1-2 hours. After centrifugation at 16,000 x *g* for 10 minutes, the DNA pellet was washed 2 x with 750 µl 70% ethanol with 5 minute, 16,000 x *g* centrifugations in between. Once all of the ethanol was removed, the pellet was allowed to dry for approximately 10 minutes at 37 °C so that any residual ethanol would evaporate. The dried pellet was resuspended in 200 µl of 8 mM NaOH and stored at 4 °C. When used in PCR reactions, the chromosomal DNA was diluted fifty-fold.

3.3.2 Plasmid DNA

Plasmid DNA was generally isolated with QIAprep Mini Prep Kit (Qiagen) according to manufacture's instructions or by alkaline lysis. For alkaline lysis, a 50 ml culture of *E. coli* was grown overnight at 37 °C in LB broth with aeration. The bacteria was pelleted by centrifugation at 5,000 x *g* for 5 minutes. After decanting the supernatant, the pellet was resuspended in 2 ml of solution A (25 mM Tris-HCl pH 8.0, 50 mM glucose an 10 mM EDTA) and lysed with 4 ml of freshly made solution B (0.2 M NaOH, 1% SDS). The mixture was rocked gently until full lysis and then left on ice for 5 minutes. Then 3 ml of 3.0 M sodium acetate, pH 5.0, was added until precipitation occurred and was centrifuged at 5,000 x *g* for 15 minutes. The supernatant was saved and added to 5 ml of ice-cold isopropanol and mixed to precipitate the nucleic acids. This mixture was centrifuged for 10 minutes at 5,000 x *g* and the pellet washed with 70% ethanol. After a final centrifugation (5,000 x *g* for 5 minutes), the pellet was dried briefly at 37 °C to remove any residual ethanol and then resuspended in 1 ml of TE (100 mM Tris-HCl pH 8.0 and 10 mM EDTA pH 8.0). The solubilized nucleic acids were subjected to RNase treatment by addition of 2 µl of 10 mg/ml RNase (Sigma) and allowed to incubate for 1 hour at 37 °C. The RNase was removed by a phenol-choloroform extraction as follows; the reaction was split into two 500 µl aliquots and had 500 µl phenol added. Following centrifugation at 16,000 x *g* for 10 minutes the aqueous phase was removed and brought back up to a volume of 500 µl with TE and the process was repeated two more times except with 250 µl phenol and 250 µl chloroform or 500 µl chloroform added. The final aqueous phase was removed and plasmid DNA was precipitated by the addition of 50 µl 3.0 M sodium acetate and 1 ml 95% ethanol. After

precipitation and centrifugation at 16,000 x *g* for 10 minutes, residual salts were removed with 2 x washes with 70% ethanol and the final plasmid pellet was dried at 37 °C for 10 minutes before being resuspended in 50 µl sterile ddH_2O.

3.4 DNA Sequencing

All DNA sequencing was performed on a Beckman CEQ8000 capillary sequencer by DalGEN (Dalhousie University, Halifax, Nova Scotia).

3.5 Optical Density Determination

Bacterial optical densities were determined in either a Heλios γ (Thermo Electron Corporation) or SpectraMax M2 (Molecular Devices) spectrophotometers. Cell suspensions were diluted 1:10 in PBS to a final volume of 1 ml. The mixed suspension was added to plastic cuvettes (1 ml) (or microtitre plates (200 µl) in the case of the SpectraMax M2) and the optical density was read at a wavelength of 600 nm after being blanked with 1 ml of PBS.

3.6 Protein Extract Preparation and SDS-PAGE

Bacterial cultures were normalized to an OD_{600} of 0.5 and centrifuged for 4 minutes at 3,300 x *g*. After removal of the supernatant, the pellet was washed twice with 1x phosphate buffered saline (PBS, pH 7.4). The final pellet was then lysed with 100 µl loading buffer (25 µl 4X NuPAGE LDS (Invitrogen), 10 µl ß-mercaptoethanol and 65 µl ddH_2O). The lysed cells were boiled for 10 minutes and then centrifuged at 16,000 x *g* for 5 minutes to pellet cellular debris. Twelve and a half microliters of each sample was run on a 4-12% NuPAGE Gel (Invitrogen) at 150 V for approximately 1.5 hours in 1x MOPS SDS-PAGE buffer (Invitrogen) in an XCell *SureLock*™ Mini-Cell electrophoresis system

(Invitrogen). The gel was then stained with Coomassie blue (0.25% Coomassie Brilliant Blue R-250, 10% glacial acetic acid, 45% ethanol, 45% ddH_2O) and destained with destain buffer (10% ethanol, 40% glacial acetic acid, 50% ddH_2O). Alternatively, the gel was used for immunoblotting in lieu of Coomassie staining (see below).

3.7 Immunoblots

Proteins run on a SDS-PAGE were transferred to BioTrace®NT Pure/Nitrocellulose Blotting Membrane (Pall Corporation) using a The Panther™ Semidry Electroblotter HEP-1 (Owl Separation Systems). A piece of Whatman paper was folded in half and soaked in 1x Towbin buffer (192 mM glycine, 24 mM Tris, 20% ethanol, stored at 4 °C) and placed on the semidry transfer apparatus. The SDS-PAGE gel was then soaked in the 1x Towbin and placed on the Whatman paper, followed by Towbin-soaked nitrocellulose membrane and finally a second Towbin-soaked piece of folded Whatman paper. The transfer was run for 1 hour at 120 mA. After the transfer the nitrocellulose was quickly stained with 1x Ponceau S (made as 10x stock; 2% (w/v) Ponceau Red, 30% trichloroacetic acid, 30% sulfosalycilic acid) for 5 minutes and destained with ddH_2O to insure proper transfer and to assess equal protein loading. The nitrocellulose was then blocked for non-specific binding with 10 ml blocking buffer (0.5% skimmed milk in PBS + 0.1% Tween20 heat inactivated for 1 hour at 65 °C) for 1 hour at room temperature. Following the removal of the blocking buffer, polyclonal primary antiserum (either anti-HP0166 or anti-HP1043; see later) was diluted 1:1,000 in 10 ml of the blocking buffer and incubated with the nitrocellulose for 2 hours at room temperature. The primary antibody was removed with 3 x 15 minute washes with PBS + 0.1% Tween20. A secondary anti-mouse antibody conjugated with alkaline phosphatase

(Calbiochem) was diluted 1:5,000 in 10 ml of PBS + 0.1% Tween20 and incubated with the nitrocellulose for 30 minutes. The secondary antibody was washed with 3 x 15 minute washes with PBS + 0.1% Tween20. The blot was developed by briefly rinsing any residual Tween20 with ddH_2O and then incubated with 10 ml AP buffer (100 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$, pH 9.0) containing 24 µl of nitro blue tetrazolium (NBT; 75 mg/ml in 70% dimethylformamide [DMF]) and 40 µl of 5-bromo-4-chloro-3-indolyl phosphate (BCIP; 40 mg/ml in DMF). Development was stopped with several rinses with ddH_2O.

3.8 BCA Protein Assay

Protein concentrations were determined using a bicinchoninic acid (BCA) protein assay (Pierce) according to the manufacturer's instructions measured at a wavelength of 562 nm in a Heλios γ (Thermo Electron Corporation) spectrophotometer and compared to a BSA standard curve, plotted in Microsoft Excel (Microsoft Office Suite, 2003) with absorbance versus concentration. Standard curves having trendline slope with an R^2 greater than 0.995 were used to calculate concentrations of unknown samples.

3.9 Bradford Protein Determination

A standard curve was prepared by diluting a 2 mg/ml stock of BSA to 1, 2, 5, 10, 20 and 25 µg/ml in 1 ml of ddH_2O in triplicate. Protein concentrations were determined by taking 800 µl of the diluted protein and adding 200 µl of the Bradford reagent (Bio-Rad) and allowed to sit at room temperature for 15 minutes. Absorbances at 595 nm were read in a Heλios γ (Thermo Electron Corporation) spectrophotometer. Averaged absorbance values from the BSA standards were plotted in Excel (Microsoft Office Suite,

2003) versus the known concentrations and a standard curve showing a trendline with an R^2 of greater than 0.995 was used. Protein concentrations of the unknown samples were calculated using the equation of the standard curve.

3.10 Two Dimensional Gel Electrophoresis

Bacterial cells were pelleted and washed with sterile PBS by centrifugation (3,300 x *g* for 4 minutes). The washed pellet was resuspended in 0.5 ml of lysis buffer (110 mM Tris pH 7.4, 1.1 mM EDTA, 8.8M urea, 55 mM DTT, 11% glycerol and 5.5% Nonidet® P40) and incubated on ice for 10 minutes. Cellular debris was removed by centrifugation at 16,000 x *g* for 5 minutes and protein concentrations were determined using the Bradford assay. After protein determination, the protein was adjusted to 2 mg/ml made up in 10 µl 100X bromophenol blue, 5 µl 40% Bio-lyte® ampholytes (Bio-Rad), 100 µl ddH_2O and the aforementioned lysis buffer to a final volume of 1 ml.

One hundred microliters of the protein sample (0.2 mg) was added to 85 µl of IEF buffer (lysis buffer containing 0.01% bromophenol blue and 0.2% Bio-lyte® ampholytes). An 11 cm IPG strip (pH 3-10; Bio-Rad) was rehydrated with the aforementioned protein mix and 1.1 ml of mineral oil in a Protean IEF Cell (Bio-Rad) for 16 hours at 50 V and 20 °C. After the completion of the rehydration, two electrode wicks (Bio-Rad) were saturated with ddH_2O and placed between the electrode and the IPG strips and focusing started with the following parameters: start voltage = 0 V; end voltage = 8,000 V, volt-hours: 30,000 V-h, ramp = rapid and a temperature of 20 °C.

After focusing, the oil was removed and the strips were equilibrated in 3 ml of ReadyPrep™ Equilibration Buffer I (Bio-Rad) for 10 minutes. After removal of Equilibration Buffer I, the strips were then equilibrated with 3 ml ReadyPrep™

Equilibration Buffer II (Bio-Rad). Following removal of Equilibration Buffer II the strips were briefly soaked in 1x XT MOPS Electrophoresis Buffer (Bio-Rad) and placed on top of a one well, 8-16% Tris-HCl Criterion™ Precast Gel (Bio-Rad). Melted agarose (Bio-Rad) was added into the well to push the IPG strip down and allowed to solidify. The gel was run in a Criterion Cell (Bio-Rad) at 60 mA per gel for approximately two hours using 1x XT MOPS Electrophoresis Buffer. Gels were visualized with silver staining.

3.11 Silver Staining

Gels were incubated with 100 ml of fixing solution (30% ethanol, 10% glacial acetic acid and 60% ddH_2O) for 30 minutes followed by incubation with 30% ethanol for 15 minutes. Gels were then rinsed with 2 x 200 ml of ddH_2O for 5 minutes before being sensitized by a one minute incubation in 0.02% sodium thiosulfate solution. The sodium thiosulfate was removed by 2 x 200 ml washes with ddH_2O and followed by incubation with 100 ml of a freshly prepared 0.1% $AgNO_3$ solution for 30 minutes. The gels were developed by adding freshly prepared development solution (3% sodium carbonate and 0.025% formaldehyde) and stopped with a 1.4% EDTA solution for 10 minutes. Residual EDTA was removed with 3 x washes with 200 ml ddH_2O for 10 minutes.

3.12 Mass Spectometry

All mass spectrometry was performed on a a Finnigan LTQ-FT mass spectrometer system with a Protana nanospray ion source interfaced to an id Phenomenex Jupiter C18 reversed-phase capillary column by W.M. Keck Biomedical Mass Spectrometry Laboratory (University of Virginia; Charlottesville, Virginia, U.S.A.).

3.13 *E. coli* Competent Cells

3.13.1 $RbCl_2$ Competent Cells

An overnight culture of *E. coli* was grown in 5 ml LB broth at 37 °C. A 1:1,000 dilution of the overnight culture was made in 500 ml fresh LB and allowed to shake at 37 °C until the OD_{600} was between 0.4-0.5. The cells were pelleted with centrifugation at 5,000 x *g* for 5 minutes and resuspended in 200 ml TFB I (30 mM potassium acetate, 100 mM $RbCl_2$, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 15% glycerol) and allowed to sit on ice for 10 minutes. The cells were pelleted again with centrifugation and after decanting TFB I, the pellet was resuspended in TFB II (10 mM 3-(N-morpholino)propanesulfonic acid [MOPS], 75 mM $CaCl_2$, 10 mM $RbCl_2$, 15% glycerol, pH 6.3 adjusted with 1 M KOH). The suspension was aliquoted in 200 µl volumes and stored at -70 °C until needed.

3.13.2 $CaCl_2$ Competent Cells

Calcium chloride competent *E. coli* was prepared similarly to $RbCl_2$ except 50 ml of fresh LB was inoculated with the overnight growth. Following the harvest of the cells, the pellet was washed with 20 ml sterile 0.1 M $CaCl_2$ and incubated on ice for 20 minutes. After a second centrifugation, the pellet was resuspended in 4 ml of 0.1 M $CaCl_2$ and stored at 4 °C for approximately a week.

3.14 Transformation of *E. coli*

One hundred microlitres of either $RbCl_2$ or $CaCl_2$ competent cells were kept on ice after the addition of 10 µl of a ligation reaction or 0.5 µl plasmid DNA. The cells were heat shocked at 37 °C for 60 seconds before being placed back in ice for 5 minutes. After the addition of 900 µl of LB, the cells were placed at 37 °C and allowed to shake

with aeration for 30-45 minutes. When transformations were with ligation reactions, the cells were pelleted and resuspended in a final volume of 50 µl of fresh LB before being plated on an LB agar plate with the appropriate antibiotic selection. Otherwise, 20 µl of the 1 ml culture was plated for plasmid transformations. When necessary, 20 µl of 0.8 M isopropyl-beta-D-thiogalactopyranoside (IPTG) and 40 µl of 20 mg/ml 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal) was spread on the top of the plate for blue/white selection. The LB plates were incubated at 37 °C or 25 °C for 1 or 2 days, respectively.

3.15 Introduction of DNA in *H. pylori*

3.15.1 Preparation of Methylated DNA

Methylation of DNA for transformation of *H. pylori* was performed as described in Donahue *et al.* (2000). Briefly, A 25 ml broth culture of *H. pylori* was grown overnight to an OD_{600} ~0.7, harvested by centrifugation (5,000 x *g* for 4 minutes) and resuspended in 5 ml CFE Extraction Buffer (20 mM Tris-acetate pH 7.9, 50 mM potassium acetate, 5 mM EDTA, 1 mM dithiothreitol (DTT) and ½ tablet of Complete Mini Protease Inhibitor Cocktail (Roche Applied Science). The bacterial suspension was sonicated with 6 x 10 second pulses with 1 minute incubation on ice in between each pulse. The sonicated extract was centrifuged at 16,000 x *g* for 10 minutes to remove cellular debris and the supernatant was removed and labeled as the cell free extract (CFE). After protein concentration determination (BCA; Pierce) CFE were aliquoted so that they contained 300-400 µg of protein and stored at -70 °C.

Methylation of DNA was performed by incubating 10µg of DNA with 300-400 µg CFE in CFE extraction buffer supplemented with 200 µM S-adenosyl-methionine

(SAM) and incubated at 37 °C for 1 hour. It should be noted that DNA intended on being electroporated or transformed into a specific *H. pylori* strain was treated with CFE from that particular strain (e.g. DNA to be transformed into *H. pylori* SS1 would be treated with CFE from *H. pylori* SS1). Following treatment, a small sample was run on a 1% agarose gel to ensure no DNase activity from the CFE. The remaining treated DNA was purified using Qiagen DNA columns as described above.

3.15.2 Electroporation of *H. pylori*

Two milliliters of overnight broth cultures of *H. pylori* (OD_{600} ~0.6) were harvested by centrifugation (3,300 x *g*, 4 minutes) and were washed 3 x with 1 ml cold Hp-EB buffer (9% sucrose, 15% glycerol). After the final wash, the pellet was resuspended in 100 µl Hp-EB buffer and 500 ng - 1 µg of DNA. The *H. pylori* / DNA mix was shocked at 1.85 kV (usually 4-5 ms) with a Bio-Rad MicroPulser™ in a 0.2 ml electroporation cuvette (MolBio Products). The shocked cells were immediately spotted on a brucella agar plate without any selection and incubated overnight at 37 °C in the microaerophlic incubator. The following day, the spotted *H. pylori* was spread across a brucella agar plate supplemented with the proper selection. Individual colonies typically appeared after 3-5 days of incubation in the microaerophilic incubator.

3.15.3 Natural Transformation of *H. pylori*

A loopful of an overnight *H. pylori* growth from a brucella agar plate was spotted as a dime-sized spot on a fresh brucella plate without selection and allowed to incubate for 5 hours in a 37 °C microaerophlic incubator. Following incubation, 250-500 ng of DNA was mixed with the spot and spread-out slight (approximately the size of a quarter)

and allowed to incubate overnight in the microaerophilic incubator. Alternatively, a 3 ml aliquot *H. pylori* from a 24 hour broth culture for was harvested by centrifugation (5,000 x *g*, 4 minutes) was resuspended in 50 µl of sterile PBS. Approximately 250-500 ng of DNA was added to the suspension and spotted on a brucella agar plate without selection and placed in the microaerophilic incubator.

The next morning, the spot was spread across a brucella agar plate supplemented with the appropriate antibiotic selection and placed back in the incubator. Transformants typically appeared after 3-5 days of incubation.

3.16 pH Taxis Assay

pH taxis assays were set up as a wet-mount on a microscope slide that was sealed around the edges with high-vacuum grease (Dow Corning). Overnight broth cultures of *H. pylori* (OD_{600} ~0.6.-0.7), *H. hepaticus* and *C. jejuni* were diluted to an OD_{600} of 0.1 and a 10 µl aliquot of the bacterial suspension was placed between the grease and then covered with a 22 x 50 mm cover slip. Similarly, overnight cultures of *P. mirabilis* were diluted 1:1000 in fresh LB and allowed to grow for an additional 2 hours before being placed on the slide. The slide was visualized with the 40x objective of an inverted phase-contrast microscope (Olympus IX71) and video and images were recorded with an Evolution QEi (Media Cybernetics, Inc.) camera using the ImagePro Plus software (Media Cybernetics). Five microlitres of HCl (pH 0.1 to 3.0 or medium control) or 0.1 M NaOH or KOH was added to the open edge of the cover slip and allowed to diffuse with the bacterial suspension. After a few seconds, tactic behavior was recorded. Behavioral motility of individual bacterial cells (5 each) was tracked with the ImagePro Plus software (Media Cybernetics). The assay was performed in triplicate.

3.17 Construction of *tlp* Mutants

Isogenic mutants in *tlpA* (*hp0099*), *tlpB* (*hp0103*), *tlpC* (*hp0082*) and *tlpD* (*hp0599*) were created using a PCR-based mutagenesis method (Chalker *et al.*, 2001). Briefly, using *tlpD* as an example, approximately 500 bp of upstream and downstream flanking regions were PCR amplified from *H. pylori* SS1 chromosomal DNA using Roche High Fidelity Polymerase using primer pairs tlpD2Cm-P1/tlpD2Cm-P2 (upstream P1/P2 amplicon) and tlpD2Cm-P3/tlpD2Cm-P4 (downstream P3/P4 amplicon). The primers tlpD2Cm-P2 and tlpD2Cm-P3 contain an extended 5' region that is complementary to the primers that amplify the chloramphenicol (*cat*) cassette from *Campylobacter coli* (catF and catR). Approximately 50 ng of each flanking region (P1/P2 and P3/P4) was mixed with 10 ng of the *cat* cassette and diluted 1:5 before PCR amplification using Roche High Fidelity Polymerase and the tlpD2Cm-P1/tlpD2Cm-P4 primers. Initially, 4 pre-cycles were set with a 94 °C denaturation for 1 minute, a 5 minute annealing at 50 °C and a 5 minute elongation at 72 °C before a round of 40 cycles (94 °C for 30 seconds, 55 °C for 30 seconds and 72 °C for 3 minutes) and a final elongation at 72 °C for 10 minutes. Amplicons were gel purified, filled in ends with T4 Polymerase and cloned into EcoRV of pBlueScript KS+ for DNA sequencing. The amplicons were also used for natural transformation of *H. pylori* SS1 and selected on brucella agar supplemented with chloramphenicol (yielding *H. pylori* SS1 Δ*tlpD*). Mutants were verified using several different PCR reactions; a) tlpD2Cm-P1/tlpD2Cm-P4 to show a smaller amplicon in the mutant than the wild type; b) tlpD2Cm-P1/catR to show the upstream flanking region and *cat* cassette in the mutant but not wild type and; c)

tlpDF/tlpDR internal primers to show the absence in the mutants, but presence in the wild type.

Mutants of *tlpA*, *tlpB* and *tlpC* were made in a similar manner except the –P2 and –P3 primers contained complementary sequences to primers that amplify the kanamycin (*aphA-3*) cassette from *Campylobacter coli* (aphA3F and aphA3R) and thus resulted in kanamycin resistant mutants. It also should be noted that the *H. pylori* SS1 Δ*tlpB* and *H. pylori* SS1 Δ*tlpC* mutants were created by Gary Sisson and Dr. Roberto Melano (Dalhousie University) and that the pBlueScript::*tlpC* suicide vector was used to create the *tlpC* mutant as PCR amplicon alone did not result in any colonies.

3.18 Construction of pRDX-C and Complementation of *tlpB*

The pRDX-C vector was constructed with a pBlueScript SK+ backbone similarly as described by Smeets *et al.* (1998). The 5' and 3' regions of *rdxA* were PCR amplified with primers rdxAISacI/rdxAIXbaI and rdxAIIXhoI/rdxAIIKpnI, respectively, using Roche High Fidelity Polymerase from *H. pylori* KE26695 chromosomal DNA. The 5' amplicon was cloned into the SacI/XbaI sites of pBlueScript SK+ yielding pBS::RDXAI, transformed into $RbCl_2$ competent *E. coli* JM109 cells and selected on LB ampicillin plates. Following plasmid isolation and confirmation of proper insertion with restriction digestion, the 3' amplicon was cloned into the XhoI/KpnI site of pBS::RDXAI yielding pRDX. pRDX was transformed into $RbCl_2$ competent *E. coli* DH5α and selected on LB ampicillin plates. The resulting plasmid was confirmed by DNA sequencing.

A chloramphenicol (*cat*) cassette from *C. coli* was PCR amplified with primers catF/catR using Roche High Fidelity Polymerase and gel purified. The cassette had the ends filled in with T4 Polymerase before being blunt-end cloned into the EcoRV site of

pRDX yielding pRDX-C. pRDX-C was transformed into $RbCl_2$ competent *E. coli* DH5α and selected on LB plates supplemented with both ampicillin and chloramphenicol. Several colonies were picked to obtain one with the *cat* cassette in the forward orientation (i.e. same direction as *rdxA*) which was confirmed by restriction digestion with HindIII. The orientation was further confirmed with DNA sequencing.

Wild type *tlpB* containing approximately 300 bp upstream and 150 bp of downstream sequences was PCR amplified from *H. pylori* SS1 chromosomal DNA using primers tlpBCompFBamHI and tlpBCompREcoRI using Roche High Fidelity Polymerase. Following gel isolation, the 2.1 kb amplicon was digested with BamHI and EcoRI and cloned into the similarly digested pRDX-C yielding pRDX-C::*tlpB*. The resulting plasmid was transformed into $RbCl_2$ competent *E. coli* JM109 and selected on LB agar supplemented with ampicillin and chloramphenicol. The proper construct was confirmed with restriction digestion and DNA sequencing.

pRDX-C::*tlpB* was digested with SacI and KpnI and the ~3.55 kb fragment (*rdxA* flanking wild type *tlpB* and the *cat* cassette) was isolated and methylated with CFE from *H. pylori* SS1. After gel purification, the fragment was electroporated into *H. pylori* SS1 Δ*tlpB* and selected on brucella agar supplemented with kanamycin and chloramphenicol yielding *H. pylori* SS1 Δ*tlpB/tlpB*$^+$. The resulting colony was verified using PCR; a) tlpBF/tlpBR internal primers to show presence in wild type and in the complement (Δ*tlpB/tlpB*$^+$) but not in the Δ*tlpB* mutant and; b) tlpBCompFBamHI/tlpBCompREcoRI to show a 2.1 kb band in the wild type (wild type *tlpB*), a 1.5 kb band in the Δ*tlpB* mutant (flanking of *tlpB* and *aphA-3* cassette) and both the 2.1 kb and 1.5 kb bands in the Δ*tlpB/tlpB*$^+$. Additionally the Δ*tlpB/tlpB*$^+$ was confirmed to be properly inserted in the

rdxA gene with PCR using primer pairs rdxAISacI/tlpBCompREcoRI and rdxAISacI/pCMR and rdxAISacI/rdxAIIKpnI.

3.19 Soft Agar Chemotaxis Assay

Chemotactic behavior of *H. pylori* was performed in semisolid brucella agar (0.35% agar) containing 10% NCS. The plates were inoculated with a 1 µl volume of a 0.1 OD_{600} suspension of bacteria and placed in a 37 °C microaerophilic incubator. The migration (diameter of halo) was measured every 24 hours, starting at 48 hours (insufficient migration after 24 hours), for 5 days.

3.20 Aerotaxis Assay

Aerotaxis assays were performed with the help of Gary Sisson. Overnight *H. pylori* SS1, *H. pylori* SS1 Δ*tlpA*, *H. pylori* SS1 Δ*tlpB*, *H. pylori* SS1 Δ*tlpC* and *H. pylori* SS1 Δ*tlpD* broth cultures (OD_{600} ~0.6-0.7) were normalized to an OD_{600} of 1.0 in 100 µl of brucella broth. Each 100 µl suspension was mixed with 3 ml of 0.25% soft agar in a 13 ml Falcon tube and placed at 37 °C in a 5% CO_2 incubator and a microaerophilic incubator. The migration of a tight band was measured after several days of incubation. *C. jejuni* H840 was used as a positive control.

3.21 Mouse Infections

Mouse handling was performed by Donna Hutchison and Gary Sisson (Dalhousie University) or Elizabeth Wiznerowicz (University of Virginia). Broth cultures of *H. pylori* were grown for 15 hours to an OD_{600} of 0.6-0.7. Approximately 10^8 cells were centrifuged at 400 x *g* for 10 minutes and resuspended in 200 µl PBS before being injected orally using a syringe and a soft-tipped needle into five CJB/6J (Jackson

Laboratories; maintained in the University Of Virginia School Of Medicine Animal Quarters), or where indicated, five CJB/6J IL-12-deficient ($p40^{-/-}$) (bred in-house; Carleton Animal Care Facility, Dalhousie University), three times during a five day period. As a mock control, select mice were also orally injected with 200 µl sterile PBS. After three weeks, mice were sacrificed (CO_2 asphyxiation for CJB/6J IL-12-deficient mice (Dalhousie University) or halothane for CJB/6J mice (University of Virginia)) and the stomachs of the mice were isolated, weighed, homogenized and the contents were 10-fold serially diluted and plated on *H. pylori* selective media. Colonies were scored after 3-4 days of incubation in the microaerophilic incubator and verified as *H. pylori* using microscopy, urease test, catalase test and oxidase test (see §3.1).

3.22 Introduction of *H. pylori tlpB* into *Proteus mirabilis*

The *tlpB* gene was PCR amplified from *H. pylori* SS1 chromosomal DNA with oligonucleotides tlpBCompFBamHI and tlpBCompREcoRI using Roche High Fidelity Polymerase. The resulting 2.1 kb band was gel purified and digested with EcoRI and BamHI and cloned into a similarly digested pUC19 such that *tlpB* would be under the P_{lac} promoter of the plasmid. This yielded pUC*tlpB*. The ligation was transformed into $CaCl_2$ competent *E. coli* JM109 and selected on LB supplemented with ampicillin, X-gal and IPTG. The resulting white colonies were picked, grown overnight and the proper insertion of *tlpB* was confirmed by restriction digestion and DNA sequencing.

P. mirabilis was grown overnight in nutrient broth supplemented with 10 mM nicotinic acid. Electrocompetent cells were made by diluting an overnight culture 1:100 in 50 ml fresh nutrient broth supplemented with nicotinic acid and allowed to grow until mid-log phase (OD_{600} ~0.5-0.6). The cells were harvested by centrifugation at 5,000 x *g*

for 4 minutes and washed 2x with cold, sterile 10% glycerol solution supplemented with 10 mM $MgCl_2$. After the washes, the final pellet was resuspended in 5 ml of 10% glycerol with $MgCl_2$ and 500 µl aliquots were made and frozen at -70 °C. Two hundred microlitres of the electroccompetent cells was mixed with 1 µl of plasmid DNA (pUC19 or pUC*tlpB*) and subjected to a 1.0 kV pulse (4-5 ms) in a Bio-Rad MicroPulser™ in a 0.2 ml electroporation cuvette (MolBio Products). After the addition of 800 µl of fresh nutrient broth and a 2 hour recovery at 37 °C, the cells were plated on LSW^- plates supplemented with ampicillin. Colonies were picked the next day and plasmids were isolated from *P. mirabilis* using the QIAprep Mini Prep Kit and checked by restriction digestion.

3.23 Cloning of Antisense-*ahpC*

The full length *ahpC* gene (*hp1563*) was PCR amplified using primers ahpCA2F and ahpCA2R from *H. pylori* KE26695 chromosomal DNA with Roche High Fidelity Polymerase. Similarly, a 300 bp upstream region containing the native promoter of *ahpC* (P_{ahpC}) was PCR amplified with primers ahpCAF and ahpCAR. After EcoRI digestion, the two fragments were ligated together with T4 ligase and 1 µl of the ligation mix was subjected to PCR to amplify the *ahpC* promoter driving the *ahpC* gene in the reverse orientation using primers ahpCAF and ahpCA2F. After gel purification, the resulting amplicon was digested with SmaI and blunt-end ligated into similarly cut pBlueScript SK+ (pBSK) yielding pASAHPC and transformed into $RbCl_2$ competent *E. coli* DH5α and selected on LB ampicillin plates. After plasmid isolation, the construct was confirmed by restriction digest and DNA sequencing.

3.24 Construction of Varying Length Antisense-*ahpC*

pASAHPC was used as a template, with primer aahpCFBHI to PCR amplify a 100 and 250 bp antisense-*ahpC* with primers aahpC100BPE and aahpC250BPE respectively with Roche High Fidelity Polymerase. The resulting amplicon was gel purified and partially digested with EcoRI. After a full BamHI digest, the DNAs were cloned into an EcoRI and BamHI digested pBSK yielding pASAHPC100 and pASAHPC250 and transformed into $RbCl_2$ competent *E. coli* DH5α cells and selected on LB ampicillin plates. All constructs were confirmed with restriction digest and DNA sequencing.

3.25 Testing Various Length Antisense-*ahpC* in *E. coli*

The *ahpC* gene from *H. pylori* KE26695 was PCR amplified with Roche High Fidelity Polymerase using primers ahpCFNdeI and ahpCRXhoI. The resulting amplicon was gel purified, digested with BamHI and XhoI for in-frame cloning with the 6x C-terminal His-tag into the similarly cut pET-29b (Novagen) expression vector yielding pET-29b::*ahpC*. After confirmation with restriction digestion and DNA sequencing, pET-29b::*ahpC* was transformed into $CaCl_2$ competent *E. coli* BL21 CodonPlus®(DE3)-RIL (Stratagene) and plated on LB containing chloramphenicol and kanamycin. The resulting strain was made $CaCl_2$ competent and transformed with either pBlueScript SK+, pASAHPC100, pASAHPC250 or pASAHPC and selected on ampicillin (150 μg/ml), chloramphenicol and kanamycin (50 μg/ml). The resulting colonies were grown overnight in 5 ml of LB broth containing the same antibiotic selection. The next day, a 1:100 dilution of the overnight growth was started in fresh 5 ml of LB containing the antibiotic mix and shook at 150 rpm at 37°C. After the cultures reached an OD_{600} of ~0.6,

1 mM IPTG was added and the cultures were put back in the shaker for an additional hour. Protein extracts were prepared and run on a SDS-PAGE as described above.

3.26 Introduction of Antisense-*ahpC* Constructs in *H. pylori*

The full length antisense-*ahpC* construct was digested from pASAHPC with SmaI. The fragment was cloned into an NruI digested pDH37, an *E. coli* / *H. pylori* shuttle vector a gift from Dr. Rainer Haas (Heuermann and Haas, 1998), yielding pHASAHPC. The 100 and 250 bp antisense-*ahpC* constructs were cut from pASAHPC100 and pASAHPC250 with a BamHI and a partial EcoRI digest and had the ends filled in with T4 Polymerase. After gel purification, the fragments were cloned into a SacI digested/T4 Polymerase treated pDH37 yielding pHASAHPC100 and pHASAHPC250. All three plasmids were transformed into $RbCl_2$ competent *E. coli* DH5α cells and selected on LB kanamycin plates. After plasmid isolation and restriction digestion, each plasmid was naturally transformed into *H. pylori* 1061 and selected on brucella agar supplemented with kanamycin. After confirming the resulting colonies, protein extracts were made and protein profiles run on an SDS-PAGE.

3.27 Construction of pRDX-K+ Vector

Approximately 580 bp flanking regions upstream and downstream of *rdxA* (*hp0956*) was amplified with PCR using primers RDXAI+SacI / RDXAI+XbaI and RDXAII+XhoI / RDXAII+KpnI respectively. The SacI and XbaI digest of the upstream amplicon was cloned into a similarly cut pBC KS+ (Stratagene) yielding pBC::RDXAI. After transformation into $RbCl_2$ competent *E. coli* DH5α and selection on LB chloramphenicol, plasmid was isolated and the insert was confirmed with restriction

digestion. The XhoI and KpnI digested downstream amplicon was then cloned into an XhoI/KpnI digested pBC::RDXAI yielding pRDX+, transformed into $RbCl_2$ competent *E. coli* DH5α and selected on LB chloramphenicol. The isolated plasmid was confirmed by restriction digestion and DNA sequencing. A non-polar kanamycin resistance cassette from *C. coli* (*aphA3*) was excised from the *E. coli* / *H. pylori* shuttle plasmid pHP1 (Kleanthous *et al.*, 1991) with EcoRI. Following T4 Polymerase treatment of the *aphA-3* cassette, the fragment was blunt-end cloned into the EcoRV site of pRDX+ yielding pRDX-K+ and transformed into $RbCl_2$ competent *E. coli* JM109 on LB supplemented with chloramphenicol and kanamycin. After plasmid isolation, the forward orientation (same direction as the *rdxA* flanking regions) was confirmed by restriction analysis and DNA sequencing.

3.28 Construction of a Promoterless *gfp* Chromosomal Insertion Vector

The green fluorescent protein gene *gfpmut3* without promoter was digested with BamHI and PstI from pGFPmut3 (gift from Dr. Valdivia; Cormack *et al.*, 1996) and cloned into a similarly cut pRDX-K+ yielding pGFP3K. After transformation into $RbCl_2$ competent JM109, selection on LB chloramphenicol and kanamycin and plasmid isolation, the construct was confirmed with restriction digest and DNA sequencing. Promoters can be cloned upstream of the *gfpmut3* gene and a KpnI / SacI fragment including the promoter-*gfpmut3*, *aphA3* (kanamycin cassette) and upstream and downstream flanking region of *rdxA* can be naturally transformed into *H. pylori* G27. Transformants were selected on kanamycin and proper insertion of the promoter-*gfpmut3* construct into the *rdxA* gene was confirmed with PCR using rdxAISacI/RDXAII+KpnI and rdxASacI/pKMR.

Cells were washed 2 x in PBS to remove any secreted or excess GFP in the supernatant and diluted to an OD_{600} of 0.2 in 2 ml. Flourometry was performed on a Bio-Rad VersaFluor Fluorometer excited at 488 nm on the medium sensitivity level after being blanked with PBS.

3.29 Insertion of Antisense-*ahpC* (AS1563) into the Chromosome of *H. pylori*

An 81 bp PstI/EcoRI fragment of pHel2 (Heuermann and Haas, 1998) containing the *fd*-bacteriophage transcriptional terminator was cloned into a PstI/EcoRI digested pBC KS+ yielding pFD. Two antisense-*ahpC* constructs were designed. First, pBKS::antisense-*ahpC* was digested with BamHI and PstI to drop out the full length antisense-*ahpC* and the native promoter and cloned into a similarly cut pFD yielding pBC::AS1563. The P_{ahpC}, full length antisense and *fd*-terminator was excised from pBC::AS1563 with a partial EcoRI digest and complete BamHI digest into an EcoRI/BamHI digested pRDX-K+ yielding pAS1563K and transformed into $RbCl_2$ competent *E. coli* JM109 with selection on LB supplemented with chloramphenicol and kanamycin.

The second antisense-*ahpC* was constructed using the strong *ureA* promoter to drive expression. The P_{ureA} was PCR amplified with primers PureAFSpeI / PureARBamHI, digested with SpeI and BamHI and cloned into a similarly cut pFD yielding pFD::P_{ureA}. Secondly, a full length *ahpC* was PCR amplified to include the all of the 5' untranslated region with primers aahpCBFPstI / aahpCB670R. This full length *ahpC* amplicon was treated with T4 Polymerase and digested with PstI. Subsequently, pFD::P_{ureA} was digested with BamHI, treated with T4 Polymerase and then digested with PstI. The digested *ahpC* amplicon was cloned into pFD::P_{ureA} yielding pBC::AS1563-2.

The P_{ureA}-antisense *ahpC fd*-terminator was digested from pBC::AS1563-2 with SpeI and EcoRI and cloned into pRDX-K+ yielding pAS1563-2K. All constructs were confirmed by restriction digestion and DNA sequencing.

A SacI/KpnI digestion of pAS1563K or pAS1563-2K excised an ~3.5 kb fragment containing the flanking regions to *rdxA*, the respective antisense-*ahpC* construct and *aphA-3* cassette was naturally transformed into *H. pylori* X47. Colonies were selected on brucella agar supplemented with kanamycin and were designated *H. pylori* X47 *rdxA*::AS1563K or *H. pylori* X47 *rdxA*::AS1563-2K based on the respective antisense construct. Proper chromosomal replacement of *rdxA* with the antisense construct was verified with PCR using primer sets rdxAISacI/RDXAII+KpnI, rdxAISacI/aahpCB670R and rdxAISacI/pKMR. As a control, a 2.6 kb SacI/KpnI fragment of pRDX-K+ (*rdxA* flanking regions and the *aphA3* cassette) was naturally transformed into *H. pylori* X47 and designated *H. pylori* X47 *rdxA*::*aphA3*. Protein profiles were viewed with SDS-PAGE.

3.30 Densitometry

Densitometry was performed using Gel Pro v3.1.00.00 (Media Cybernetics Inc.) or TotalLab™ v2005 1D Gel Analysis (Nonlinear Dynamics Ltd.). Black and white scans were taken at 1200 dpi using an Epson Expression 1680 scanner and imported into the densitometry software. Normalization was made by referencing 3-4 bands of the lanes of interest to the wild type or control lanes (considered 100%). Bands of interest were selected and the densities were compared and adjusted according to the normalization.

3.31 In Vitro Growth Curves

H. pylori broth cultures were prepared as described above and grown to an OD_{600} of 0.6-0.7 (14-15 hours). The cultures were diluted to an OD_{600} of 0.1 in 10 ml brucella broth and OD_{600} were measured at 0, 4, 8, 12, 24 and 32 hours with a SpectraMax M2 spectrophotometer (Molecular Devices).

3.32 Peroxide Challenge

H. pylori strains were grown for 16 hours on brucella agar, normalized to an OD_{600} of 0.1 and plated for confluent growth on fresh brucella agar. After 2 hours recovery in the microaerophilic incubator, filter paper discs with a diameter of 7.5 mm saturated with 20 µl of various concentrations of hydrogen peroxide (H_2O_2), diluted in sterile ddH_2O, *tert*-butyl hydroperoxide, diluted in sterile ddH_2O, or cumene hydroperoxide, diluted in DMSO, were placed on the plates. After three days, zones of inhibition were measured around the disc. Challenges with peroxides were performed at least 3 times.

3.33 Determination of Minimal Bactericidal Concentration (MBC)

Fourteen hour broth cultures of *H. pylori* X47 WT, *H. pylori* X47 *rdxA*::*aphA3* and *H. pylori* X47 *rdxA*::AS1563K and *H. pylori* X47 *rdxA*::AS1563-2K (OD_{600} ~ 0.6) were normalized to an OD_{600} of 0.1 in 10 ml of brucella broth. Two hundred microlitres of each suspension was added to the first well of a 96 well microtitre dish. One hundred microlitres was added to each subsequent well. Two microlitres of the desired starting concentration of organic peroxides (either *tert*-butyl hydroperoxide or cumene hydroperoxide) was added to the first 200 µl suspension in the first well. Subsequently,

100 µl of the 200 µl suspension containing the highest concentration of the peroxides were 2-fold serially diluted through the remainder of the wells. After microaerophilic incubation at 37 °C for 1 hour and 24 hours, a small aliquot of the suspensions were spotted on brucella agar and allowed to incubate in the microaerophilic incubator for 4 days. The lowest concentration of tBOOH that prevented bacterial growth (small spot on the plate) was considered to be the minimal bactericidal concentration (MBC). Each challenge was performed 3 times.

3.34 Kill Curve

Fourteen hour broth cultures of *H. pylori* X47 WT, *H. pylori* X47 *rdxA*::*aphA3*, *H. pylori* X47 *rdxA*::AS1563K and *H. pylori* X47 *rdxA*::AS1563-2K (OD_{600} ~0.6) were normalized to an OD_{600} of 0.1 in 4 ml of brucella broth containing 10% NCS. Each sample was subjected to 0.5 mM *tert*-butyl hydroperoxide and 100 µl aliquots were removed at 0, 5, 10, 15 and 25 minutes, washed in PBS and plated on brucella agar after 10-fold serial dilutions. Colonies were scored after 4 days of microaerophilic incubation. Challenge to *tert*-butyl hydroperoxide was repeated 3 times.

3.35 Cloning, Over Expression and Purification of *hp0166* and *hp1043*

H. pylori regulators *hp0166* (recently termed; *arsR*) and *hp1043* were PCR amplified from *H. pylori* SS1 genomic DNA with primers HP0166FNdeI / HP0166RBamHI and HP1043FNdeI/HP1043RBamHI respectively using Roche Expand High Fidelity Polymerase. The resulting amplicons were digested with NdeI and BamHI and cloned, in-frame with the 10x N-terminal His-tag of the similarly digested pET-16b (Novagen) yielding pET16::*hp0166* and pET16::*hp1043*. The ligations were transformed

into $RbCl_2$ competent *E. coli* DH5α and selected on LB-ampicillin plates. Transformants were confirmed by restriction digestion and sequencing. The confirmed constructs were then transformed into $CaCl_2$ competent *E. coli* BL21 CodonPlus®(DE3)-RIL (Stratagene) and selected on LB containing both ampicillin and chloramphenicol.

Overnight cultures of *E. coli* BL21 CodonPlus®(DE3)-RIL containing either pET16::*hp0166* or pET16::*hp1043* was grown overnight in 5 ml of LB broth containing carbenicillin and chloramphenicol. The following morning, the overnight culture was diluted 1:1,000 in 500 ml of fresh LB containing carbenicillin and chloramphenicol and allowed to grow to an OD_{600} of ~0.5 (approximately 2 ½ hours) before the addition of IPTG to a final concentration of 1 mM. After 2 hours of induction, the cells were harvested by centrifugation at 5,000 x *g* for 6 minutes. Over expression of *hp0166* and *hp1043* were visualized on an SDS-PAGE comparing a small sample of induced versus non-induced cells.

Purification of the 10x N-terminal His-tagged was performed on 2 ml of Ni^{2+}-NTA resin (Qiagen) following the manufacturer's instructions. Imidazole was removed using Spectra/Por® 1 Molecularporous Dialysis Membrane (6-8,000 molecular weight cutoff; Spectrum Laboratories Inc.) in 1L of PBS for 2 hours, followed by an overnight dialysis in 1L PBS + 10% glycerol. The eluted protein was subjected to a second pass through 1 ml of Ni^{2+}-NTA resin to remove contaminating proteins as described in the manufacturer's manual except three washes of 20, 40 and 60 mM imidazole were used before the final elution. After dialysis, a small sample was run on SDS-PAGE to determine purity and protein concentrations were calculated with the BCA assay (Pierce).

When preparing the samples to be injected into mice for antibody production, approximately 150 μg/ml of protein was loaded and run on an SDS-PAGE. The first two lanes of the gel (containing the molecular weight marker and a small amount of purified protein) were separated from the rest of the gel with a sterile scalpel. The two lanes were briefly stained with Coomassie stain and destained to determine the distance of migration of the purified band. The two lanes were rehydrated in ddH_2O as the destain shrinks the gel. The stained lanes were then aligned with the unstained gel containing the rest of the protein samples. Using a sterile scalpel, the SDS-PAGE was cut above and below the distanced in which the purified protein ran and the strip was placed in a sterile 2 ml Eppendorff containing 200 μl elution buffer (50 mM Tris, 150 mM NaCl, 0.1 mM EDTA, pH 7.5). After crushing the strip into small pieces, the elution was allowed to occur overnight, rocking at room temperature. The following day, the small pieces of gel were centrifuged for 5 minutes at 16,000 x *g* and the supernatant was saved. Protein concentrations were determined with the BCA assay (Pierce) to ensure sufficient protein concentration for injection into mice. A 65-75% recovery of protein was typical.

3.36 Production of Polyclonal Antisera

Polyclonal antisera were made in BALB/c mice with the help of Dr. Mohammad Samiul Alam (University of Virginia). Briefly, after taking a small sample of blood (pre-serum), 200 μl of ~0.1 mg/ml of purified HP0166 or HP1043 was mixed with 200 μl of Incomplete Freud's Adjuvant (Sigma) and injected intraperitoneally into two mice (per protein sample) to prime the mice. After 14 days, a small sample of blood was taken from the tail with a 75 mM special heparinized micropipette (Drummond Scientific), centrifuged at 1,500 x *g* for 5 minutes and the supernatant (serum) was tested with

immunoblotting to check against the respective purified protein. Immunization boosts were given after 6 weeks of the initial injection with the same concentration of protein. Following this booster, two subsequent boosts were given at three week intervals. After the final boost, a small sample of serum was isolated and checked with immunoblotting against the respective purified protein. The mice were sacrificed and serum collected. Antiserum samples were stored at -70 °C in 10 µl aliquots.

3.37 Cloning of Antisense-*hp0166* (AS0166) and Introduction in *H. pylori*

A 341 bp fragment of *hp0166* containing the 5' untranslated region and Shine-Dalgarno was PCR amplified with primers anti0166FPstI/anti0166FBamHI using Roche High Fidelity Polymerase. After digestion with PstI and BamHI, the fragment was cloned in the antisense direction under the P_{ureA} promoter in the similarly digested pBC::P_{ureA} yielding pBC::P_{ureA}-*hp0166*. After transformation into $RbCl_2$ competent *E. coli* DH5α, transformants were selected on LB chloramphenicol. After plasmid isolation and confirmation by restriction digest, both the P_{ureA} promoter and antisense-*hp0166* were digested from pBC::P_{ureA}-*hp0166* with SpeI and PstI and cloned in the similarly digested pFD yielding pBC::AS0166, transformed into $RbCl_2$ competent *E. coli* DH5α and selected on LB chloramphenicol. After isolation of the plasmid from a transformant, the construct was confirmed by restriction digestion and DNA sequencing.

The antisense construct containing the P_{ureA} promoter, antisense *hp0166* and *fd*-bacteriophage terminator was digested from pBC::AS0166 with SpeI and EcoRI and cloned into the similarly digested pRDX-K+ yielding pAS0166K. After transformation into $RbCl_2$ competent *E.* coli DH5α and selection on LB chloramphenicol and

kanamycin, the proper insertion of the antisense was confirmed by restriction digest following plasmid isolation.

H. pylori SS1 was naturally transformed with an approximate 3.0 kb KpnI / SacI fragment of pAS0166K which contains the antisense-*hp0166* construct, *aphA-3* (kanamycin cassette) flanked by upstream and downstream regions to *rdxA*. Transformants were scored on brucella agar supplemented with kanamycin. Proper insertion of the antisense-*hp0166* construct into the *rdxA* gene was confirmed with PCR using primer sets rdxAISacI / RDXAII+KpnI, rdxAISacI / anti0166FPstI and rdxAISacI / pKMR.

3.38 Acid Survival

Overnight *H. pylori* SS1, *H. pylori* SS1 *rdxA*::*aphA-3* and *H. pylori* SS1 *rdxA*::AS0166K broth cultures (OD_{600} ~0.6-0.7) were normalized to an OD_{600} of 0.1 and resuspended in 1 ml of sterile PBS pH 7.0 or PBS pH 3.0, with or without 5 mM urea for 1 hour in a 37 °C microaerophilic incubator. Following incubation, the cells were harvested by centrifugation at 3,300 x *g* for 5 minutes and washed 2 x with sterile PBS pH 7.4 before being 10-fold serially diluted and plated on brucella agar. Colonies were scored 2 days later. Challenges were repeated an additional two times.

3.39 Cloning of Antisense-*hp1043* (AS1043) and Introduction in *H. pylori*

Four different antisense constructs for *hp1043* were cloned. First, the *hp1043* promoter (P_{hp1043}) was PCR amplified with Roche High Fidelity Polymerase using primers Php1043FSpeI and Php1043RBamHI. The amplicon was digested with SpeI and BamHI, cloned into a similarly cut pBC yielding pBC::P_{hp1043}. The resulting plasmid was

transformed into $RbCl_2$ competent *E. coli* JM109 and selected on LB chloramphenicol plates. Secondly, a 341 bp and a 701 bp (full length) fragment of *hp1043* was PCR amplified with Roche High Fidelity Polymerase using primer sets anti1043FPstI / anti1043RBamHI and anti1043FPstI / HP1043RBamHI respectively. The cloning of the resulting amplicons was similar to the cloning of the antisense-*hp0166* except both the 341 bp and 701 bp fragment were also cloned under the P_{hp1043} promoter in pBC::P_{hp1043}. After cloning the promoter-antisense constructs into pFD to add the *fd*-bacteriophage promoter, the constructs were all confirmed with restriction digestion and DNA sequencing. The constructs were then cloned into pRDX-K+ yielding the following plasmids: pAS1043K (P_{ureA} driving expression of 341 bp antisense-*hp1043*); pAS1043-2K (P_{hp1043} driving expression of 341 bp antisense-*hp1043*); pFAS1043K (P_{ureA} driving the 701 bp antisense-*hp1043*) and pFAS1043-2K (P_{hp1043} driving the 701 bp antisense-*hp1043*).

H. pylori SS1 was electroporated with a 3.0 kb (341 bp antisense-*hp1043* construct) or 3.4 kb (701 bp antisense-*hp1043* construct) KpnI / SacI fragments digested from pAS1043K, pAS1043-2K, pFAS1043K or pFAS1043-2K. After transformant selection on kanamycin, proper insertion of the antisense-*hp1043* construct into the *rdxA* gene was confirmed with PCR using primer sets rdxAISacI / RDXAII+KpnI, rdxAISacI / anti1043FPstI and rdxAISacI/pKMR.

3.40 Cloning of Antisense-*hp1043* (AS1043) into pHel3

The full length antisense-*hp1043* cloned under either the P_{ureA} or P_{hp1043} promoter (FAS1043 and FAS1043-2 respectively) were subcloned from pBC::FAS1043 and pBC::FAS1043-2 as a SacI/XhoI fragment into the SacI/XhoI sites of pHel3 (gift from

Dr. Rainer Haas; Heuermann and Haas, 1998) yielding pHel3::FAS1043 and pHel3::FAS1043-2. The resulting plasmids were transformed into $RbCl_2$ competent *E. coli* DH5α and selected on LB supplemented with kanamycin. After plasmid isolation and confirmation with restriction digestion, the plasmids were transformed into *H. pylori* 1061 via natural transformation and selected on brucella agar supplemented with kanamycin.

Chapter 4: Results

Microorganisms with small genomes that encode most of the metabolic pathways of bacteria with much larger genomes do so at the expense of redundancy in pathways, regulatory mechanisms and nutritional diversity. Therefore, much of the gene content of these organisms becomes essential for viability, which often restricts them to a particular niche. In the case of *H. pylori,* which is limited to the gastric mucosal niche, progress in elucidating molecular determinants of virulence has been hindered by both the high percentage of essential genes and by the lack of molecular tools required to study these genes. In particular, the function of genes associated with acid survival, mucosal colonization, oxidative defense and persistence have not been rigorously investigated.

This dissertation is divided into two areas where novel molecular tools have been applied to elucidate fundamental mechanisms associated with: (i) colonization through sensing of pH and; (ii) the importance of the oxidative response (AhpC) in colonization. As part of these studies, I developed and validated an antisense RNA interference strategy that can be broadly applied to the study of gene function in *H. pylori.*

4.1 Part I: Is *H. pylori* pH Tactic?

Based on the work of Suerbaum's group (Schreiber *et al.*, 2004), it was hypothesized that *H. pylori* senses pH and uses gradients of acid to direct both initial colonization and life long persistence in the gastric mucosa. In this section, we sought to examine the behavior of *H. pylori* when exposed to different pH and determine what factors, if any, are involved in these observations. The following work has been published in the Journal of Bacteriology (Croxen *et al.*, 2006) and included herein with permission (see Appendix).

4.1.2 *H. pylori* Displays pH Taxis But Not Aerotaxis

To test the hypothesis that *H. pylori* senses and responds to pH, we set up a slide-based assay, optimized by Gary Sisson, to monitor a small suspension of broth grown *H. pylori* SS1 (wild type). Recorded videos and tracking were done with the assistance of Gary Sisson. When motile *H. pylori* were viewed microscopically and their motility recorded random motility was observed. Figure 4A shows a linear motility pattern tracked with ImagePro software of one bacteria (also see video S1; http://intmed.med.virginia.edu/hoffmanlab/). Several bacteria were monitored and demonstrated similar results. After exposure to acid, an increase in bacterial motility with clockwise arcing was observed and tracked with software (Figure 4B). The overall result of this acid exposure is shown in Figure 5. Following acid exposure and increased rate of clockwise motility (Figure 5B), a collective movement of the bacterial population away from the acid can be seen (see video S2; http://intmed.med.virginia.edu/hoffmanlab/). Within a few minutes, the bacterial population moved away from the acid (and out of the field of view) to form a dense band (wall) where motility seemed slow to non-existent (Figure 5C) (see video S3; http://intmed.med.virginia.edu/hoffmanlab/). Upon addition of more acid, the wall became highly motile again with frenetic arcing and movement away from the acid suggesting that the organisms that made up the wall were still viable. When tested with 0.1 M HCl + 5 mM urea, no changes in the tactic response were observed, other than a larger population of cells survived the initial exposure to the acid (closer to the injection point). Tactic behavior was not observed in pH > 3.0 or to strong bases. As a control, *P. mirabilis*, which does not display pH or negative taxis was used (Williams *et al.*, 1976). Upon exposure to acid, *P. mirabilis* quickly became non-motile. It is also

noteworthy that neither *C. jejuni* or *H. hepaticus* displayed tactic behavior to acids or bases.

To determine if *H. pylori* displays aerotaxis, we examined *H. pylori* SS1 in semi-solid media in a test tube for the presence of an aerotactic band, a dense population of bacteria that migrates to an optimal oxygen tesnsion with the help of Gary Sisson. *C. jejuni*, previously shown to be aerotactic (Hazeleger *et al.*, 1998) was used as a positive control. Although a band was formed by *C. jejuni* H840, no such band was observed with *H. pylori* and therefore judged to be non-aerotactic and therefore, further studies were not pursued (data not shown).

4.1.3 Creation of pH Taxis Mutants

In other bacteria, MCPs are involved in the sensing of a repellant or an attractant. The annotated genomes of *H. pylori* reveal four orthologs of MCPs called TlpA, TlpB, TlpC and TlpD (Alm *et al.*, 1999; Oh *et al.*, 2006; Tomb *et al.*, 1997). To examine the role of Tlps in pH taxis, isogenic mutants were made in *H. pylori* SS1 using a PCR-based method to create the knockout constructs as depicted in Figure 6. Mutants of *tlpB* and *tlpC* were made by Gary Sisson and Dr. Roberto Melano, respectively. Following natural transformation of the PCR amplicons and antibiotic selection, proper replacement of the target gene with the antibiotic cassette (*aphA3* or *cat*) was confirmed by comparing PCR reactions from wild type and mutant chromosomal DNA (data not shown). As none of the PCR reactions suggested a doublet (wild type amplicon and the mutant amplicon), along with other PCR reactions, all four *tlp* mutants were judged to be authentic.

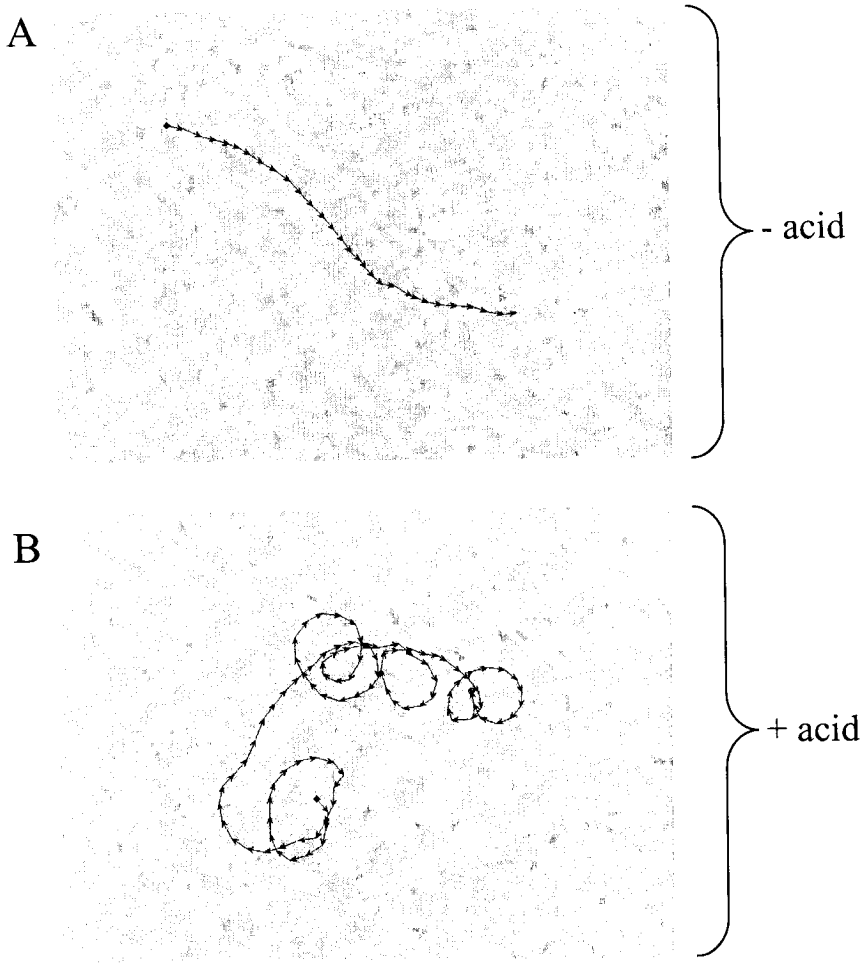
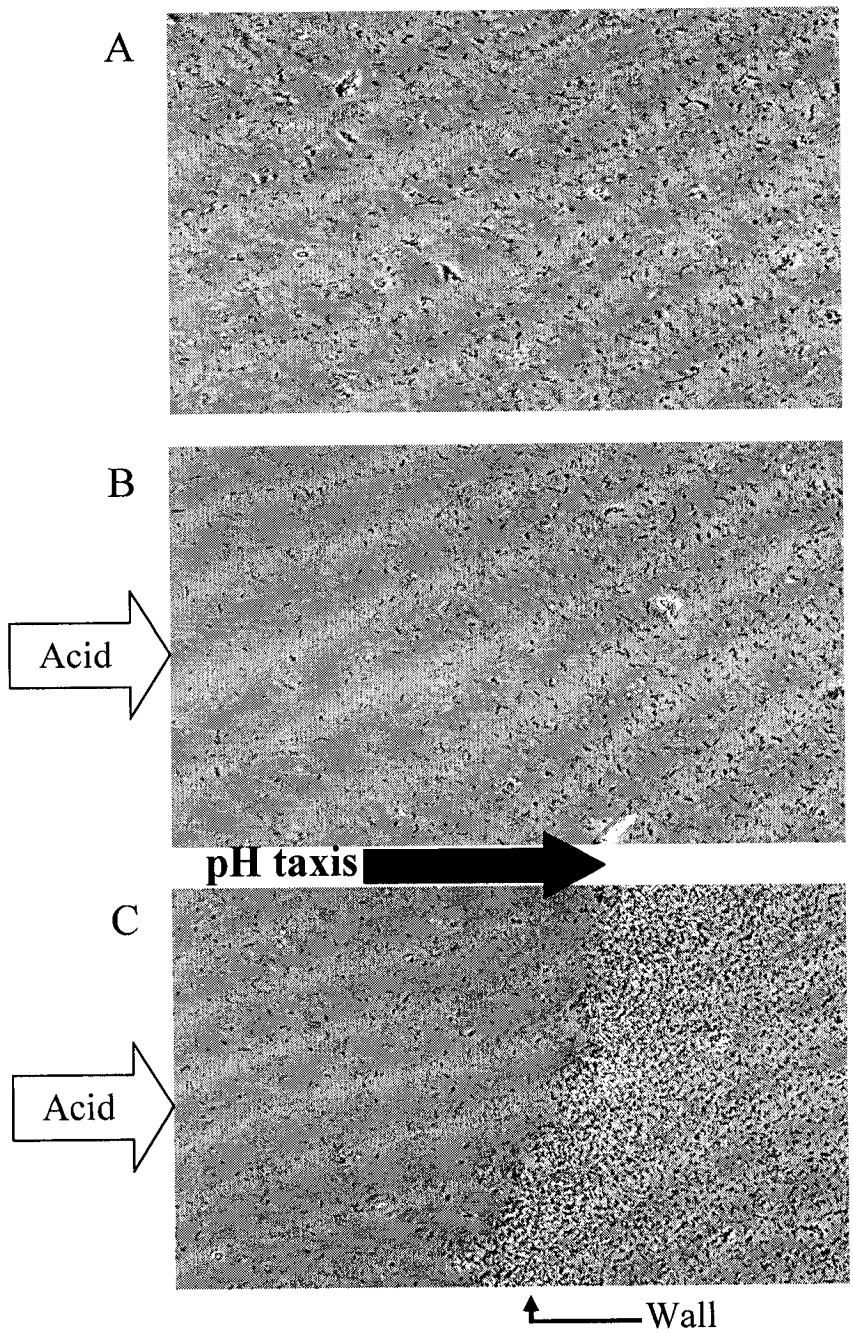


Figure 4. *H. pylori* SS1 Motility Patterns. Five individual bacteria were tracked for motility patterns using the tracking function of ImagePro software. Only one bacterium is represented in the above figure. (A) Under normal conditions *H. pylori* swam in a linear fashion and did not arc. (B) Under acidic conditions (0.1 M HCl), *H. pylori* was motile and swam in a series of arcs that resulted in the collective movement of the bacterium away from the influx of acid (from left to right). Multiple trackings under the same conditions yielded similar results summarized in Table 3.

Figure 5. pH Tactic Behavior of *H. pylori* SS1. Bacteria were placed on a wet-mount and observed by phase constrast microscopy. (A) Normal conditions. Random distribution of wild type *H. pylori* SS1 display random motility. (B) Acidic conditions (0.1 M HCl). Collective movement of bacteria from left (more acidic) to right (less acidic) by a clockwise arcing in response to the acid after 2 minutes. Movement of bacteria is indicated by the thick black arrow. (C) Acidic conditions after approximately 5 minutes. A highly concentrated density of bacteria forms (wall; indicated by the arrow). Bacteria are not as motile in the wall. The bacteria in the wall become motile again after the addition of more acid and the the bacteria became motile again and formed a wall away from the acid. It should be noted that *H. pylori* did not display chemotactic behavior to base (0.1 M NaOH or KOH).


A
B
Acid
pH taxis
C
Acid
Wall

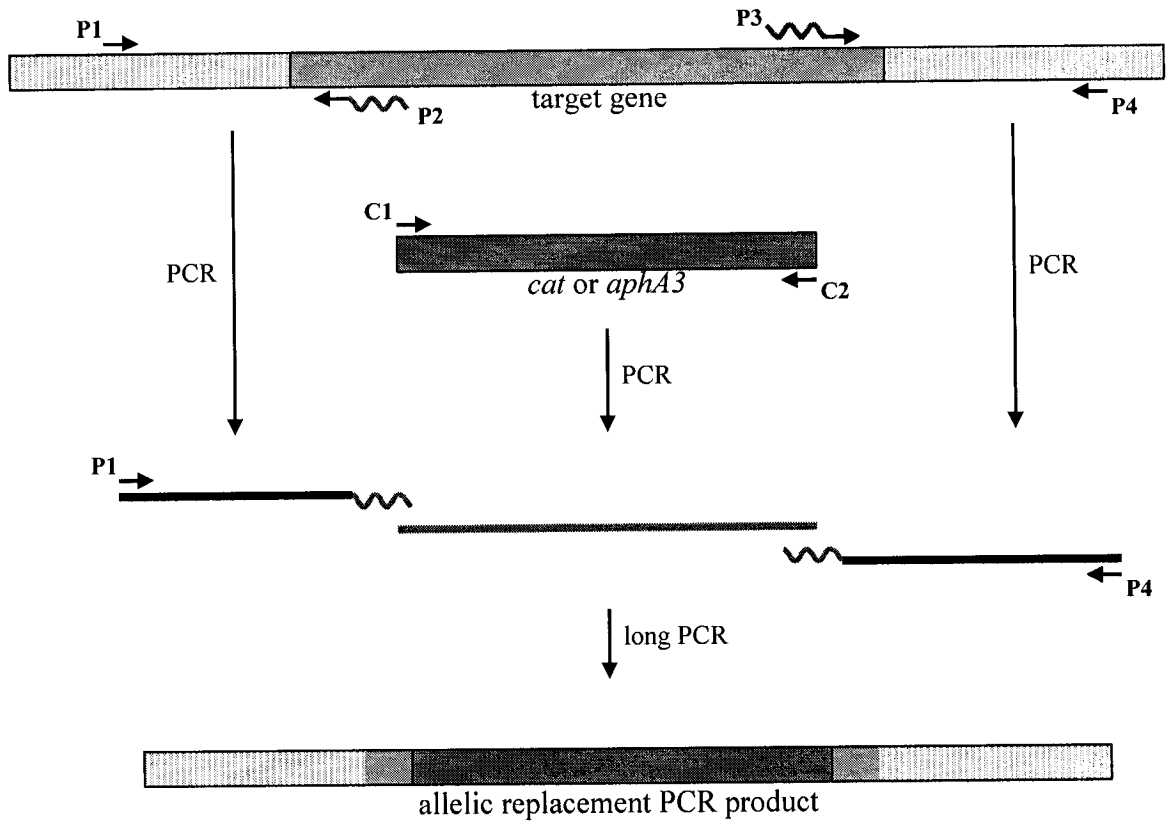


Figure 6. Schematic of PCR Based Allelic Replacement Fragment. Approximately 500 bp of DNA flanking the target genes is amplified with primer sets P1/P2 (upstream to target gene) and P3/P4 (downstream to target gene). Additionally, primers P2 and P3 contain a 5' leader sequence (represented by squiggly lines) that is complementary to primers C1 or C2, respectively of the chloramphenicol (*cat*) or kanamycin (*aphA3*) cassettes. The 5' upstream flanking sequence encompasses any intergenic space (e.g. promoters) and ends just inside of the coding region of the target gene. The 3' downstream flanking sequence contains a part of the coding sequence of the target gene, transcriptional terminators and any intergenic spacing between the target and downstream gene. The three generated PCR amplicons were mixed and subjected to a long-PCR with primers P1 and P4. The resulting amplicon was used for transformation into *H. pylori.*

4.1.4 Motility and Chemotaxis of *tlp* Mutants

Since motility is required for tactic behavior, we wanted to ensure that all of the *tlp* mutants were motile. Motility of each *tlp* mutant was assessed by microscopy and all were judged to be motile. Additionally, we checked the chemotaxis of each mutant in a brucella-based soft agar assay. As seen in Figure 7, all *tlp* mutants (Δ*tlpA* is not depicted) displayed chemotactic behavior as shown by an outward migration from the central inoculum. Measurements of diameters and rates of migration can be seen in Table 3. No significant differences in diameter sizes were observed between wild type, Δ*tlpA* or Δ*tlpC* after 5 days. However, both Δ*tlpB* and Δ*tlpD* demonstrated large differences in diameter sizes as they were approximately 70% of wild type, Δ*tlpA* and Δ*tlpC*. Rates of migration were also measured daily over a five day period. Consistent with the visual diameter size differences in Figure 7, Δ*tlpB* and Δ*tlpD* had a slower rate of migration per day (1.4 mm ± 0.3 and 1.5 mm ± 0.3 respectively) than wild type, Δ*tlpA* or Δ*tlpC* mutants. Therefore, both the Δ*tlpB* and Δ*tlpD* mutants seem to be less chemotactic than wild type, Δ*tlpA* and Δ*tlpC* when measured in the soft agar assay.

4.1.5 Defects in pH Taxis

Since chemotactic behavior of some *tlp* mutants differed from wild type in the soft-agar assay, we sought to test the role of each Tlp in the pH tactic response. Each *tlp* mutant was observed in our pH taxis assay while swimming velocities were measured with software by Gary Sisson. Although there were some noted differences in swimming behavior, Δ*tlpA*, Δ*tlpC* and Δ*tlpD* all displayed a similar pH tactic response to acid as wild type *H. pylori* SS1 and formed the wall (Table 4). As observed previously, the

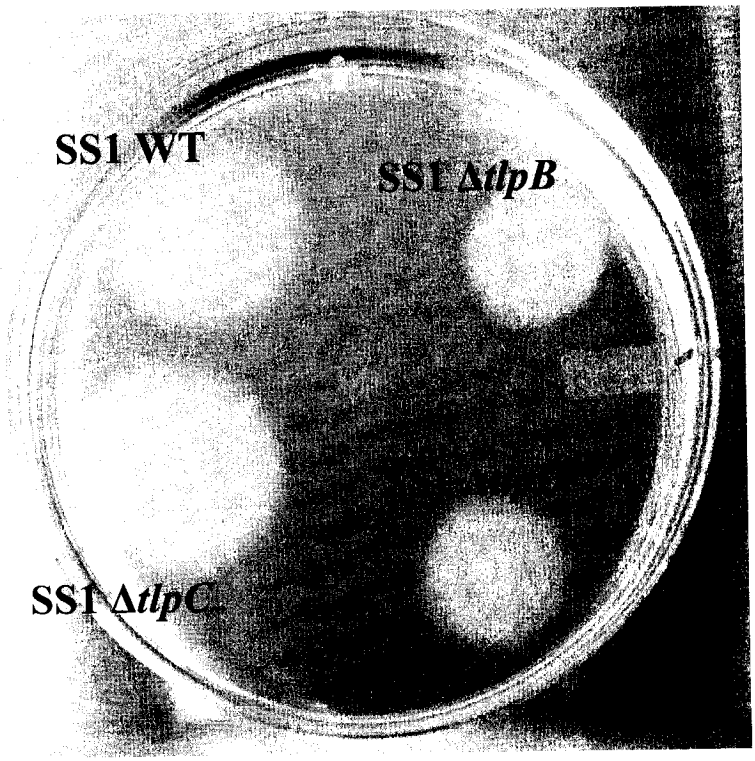


Figure 7. Qualitative Motility/Chemotaxis Assay. Small inoculums of *H. pylori* SS1 wild type, *H. pylori* SS1 Δ*tlpB*, *H. pylori* SS1 Δ*tlpC* and *H. pylori* SS1 Δ*tlpD* were spotted in 0.35% soft brucella agar. The plates were put in the micoraerophilic incubator and the ability to swarm was assessed after 5 days. Diameter sizes of Δ*tlpA* (not depicted) and Δ*tlpC* was similar to wild type while the Δ*tlpB* and Δ*tlpD* mutants which displayed a small diameter. Non-motile bacteria do not swarm and develop a concentrated spot at the place of inoculation. See Table 3 for the daily rates of swarming and diameter sizes.

Table 3. Swarming Rates of Motility/Chemotaxis Assay

Strain	Diameter (mm) ± SD	mm / Day ± SD
SS1 WT	18.7 ± 1.26	1.9 ± 0.1
SS1 Δ*tlpA*	17.6 ± 0.6	1.88 ± 0.1
SS1 Δ*tlpB*	13.5 ± 1.0	1.4 ± 0.3
SS1 Δ*tlpC*	19.0 ± 0.82	2.5 ± 0.2
SS1 Δ*tlpD*	13.5 ± 1.0	1.5 ± 0.3

Small inocula of *H. pylori* SS1 wild type, *H. pylori* SS1 Δ*tlpA*, *H. pylori* SS1 Δ*tlpB*, *H. pylori* SS1 Δ*tlpC* and *H. pylori* SS1 Δ*tlpD* were spotted in 0.35% soft brucella agar. The diameter of the swarming zone was measured every 24 hours for 5 days and the change in diameter size per day was recorded as the daily swarming rate (mm / Day). The final diameter size was measured after 5 days and reported. Results are an average of of 5 independent experiments and are reported as average ± standard deviation (SD).

Table 4. Measurements of *H. pylori* Wild Type and *tlp* Mutant pH Tactic Behaviors

Strain	Acid (+/-)	Swimming Observation	Linear Velocity (μm/s)	Mean Arc Radius (μm)	Wall
SS1 WT	-	Random	11.4 ± 1.7	None	
	+	Arc	14.1 ± 1.4	3.35 ± 0.49	Yes
SS1 Δ*tlpA*	-	Random	ND	ND	
	+	Arc	ND	ND	Yes
SS1 Δ*tlpB*	-	Random	13.6 ± 1.8	None	
	+	Random	16.3 ± 2.9	None	No
SS1 Δ*tlpC*	-	Random	57.4 ± 4.5	Linear	
	+	Linear/Arc	No Change	ND	Yes
SS1 Δ*tlpD*	-	Arc/Tumble	17.8 ± 1.6	None	
	+	Arc/Tumble	9.3 ± 2.6	2.67 ± 0.71	Yes

H. pylori SS1 wild type, *H. pylori* SS1 Δ*tlpA*, *H. pylori* SS1 Δ*tlpB*, *H. pylori* SS1 Δ*tlpC* and *H. pylori* SS1 Δ*tlpD* were observed with microscopy in the pH taxis assay with and without 0.1 M HCl by Gary Sisson. Software was used to determine swimming patterns (random or arc), linear velocities and mean arc radiuses. Wall formation was also noted. ND = not determined.

velocity of swimming of *H. pylori* SS1 wild type increased in the presence of acid. Similar results were seen with Δ*tlpA* mutants. Mutants of Δ*tlpC* showed a linear velocity nearly 5x that of wild type (57.4 μm/s ± 4.5 vs 11.4 μm/s ± 1.7) which was the same in the presence or absence of acid. In terms of swimming behavior, Δ*tlpD* mutants consistently arced or tumbled in the presence or absence of acid. When Δ*tlpD* mutants were exposed to acid, the motility decreased and thus was less robust in wall formation.

In contrast to all of the other *tlp* mutants, Δ*tlpB* mutants, although displaying increased motility in response to acid (Table 4), did not display any pH tactic responses or wall formation (Figure 8) (see video S4; http://intmed.med.virginia.edu/hoffmanlab/). As time progressed, the *H. pylori* SS1 Δ*tlpB* mutants became non-motile and eventually formed coccoid bodies. There was no evidence of the bacterial population moving away from the acid or wall formation. Since Δ*tlpB* was unable to respond to acid, TlpB may play a role in sensing pH and may be important in the pH tactic response.

4.1.6 *tlpB* Mutants are Unable to Colonize Mice

Since mutants in chemotaxis proteins of *H. pylori* were unable to colonize mice (Foynes *et al.*, 2000), we wanted to determine if mutants in *tlp* displayed any deficiencies in colonization of the highly permissive IL-12-deficient mouse model (Hoffman *et al.*, 2003). *H. pylori* SS1 Δ*tlpA* was not tested as it was reported to fully colonize mice (Andermann *et al.*, 2002). Results of the mouse colonization can be seen in Table 5. Bacterial densities from Δ*tlpC* and Δ*tlpD* were comparable to that of wild type *H. pylori* SS1 as all of the test mice were colonized with over 10^7 CFU/(g stomach). In contrast, only 3 of the 5 mice were colonized with the Δ*tlpB* mutant with a >5 fold lower recovery than the other *tlp* mutants or wild type. As a control, no *H. pylori* was recovered from the

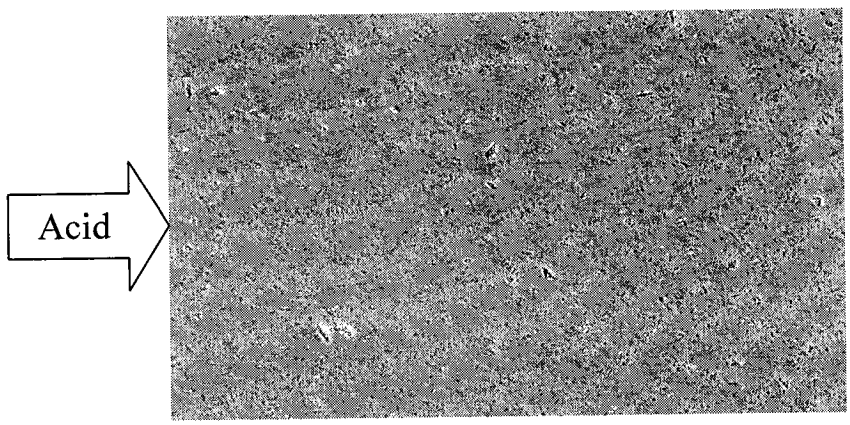


Figure 8. pH Tactic Behavior of *H. pylori* SS1 Δ*tlpB*. Bacteria were spotted on a wet-mount and observed by phase contrast microscopy. After the addition of acid (0.1 M HCl) the *H. pylori* SS1 Δ*tlpB* continued to swim in a random pattern of linear motility pulses, seemingly unable to respond to the acid. After approximately 5-10, the cells became non-motile and formed coccoid bodies. Tactic behavior of all *tlp* mutants are reported in Table 4.

Table 5. Gastric Colonization of Mice by *H. pylori* SS1 and *tlp* Mutants

Strain	**No. Infected / No. Mice**	**C.F.U / g stomach ± SD**
Mock (sterile PBS)	0 / 5	0
SS1 WT	5 / 5	$3.87 \times 10^7 \pm 1.6 \times 10^7$
SS1 Δ*tlpB*	3 / 5	81 ± 61
SS1 Δ*tlpC*	5 / 5	$2.59 \times 10^7 \pm 1.1 \times 10^7$
SS1 Δ*tlpD*	5 / 5	$3.15 \times 10^7 \pm 1.4 \times 10^7$

Five IL-12-deficient mice were infected with *H. pylori* SS1 WT, *H. pylori* SS1 Δ*tlpB*, *H. pylori* SS1 Δ*tlpC* and *H. pylori* SS1 Δ*tlpD*. Mouse infections were done with the help of Gary Sisson and Donna Hutchison. Three weeks post infection the stomachs were isolated, weighed and homogenized, and the homogenate was plated for bacterial loads (CFU/g stomach). SD, standard deviation.

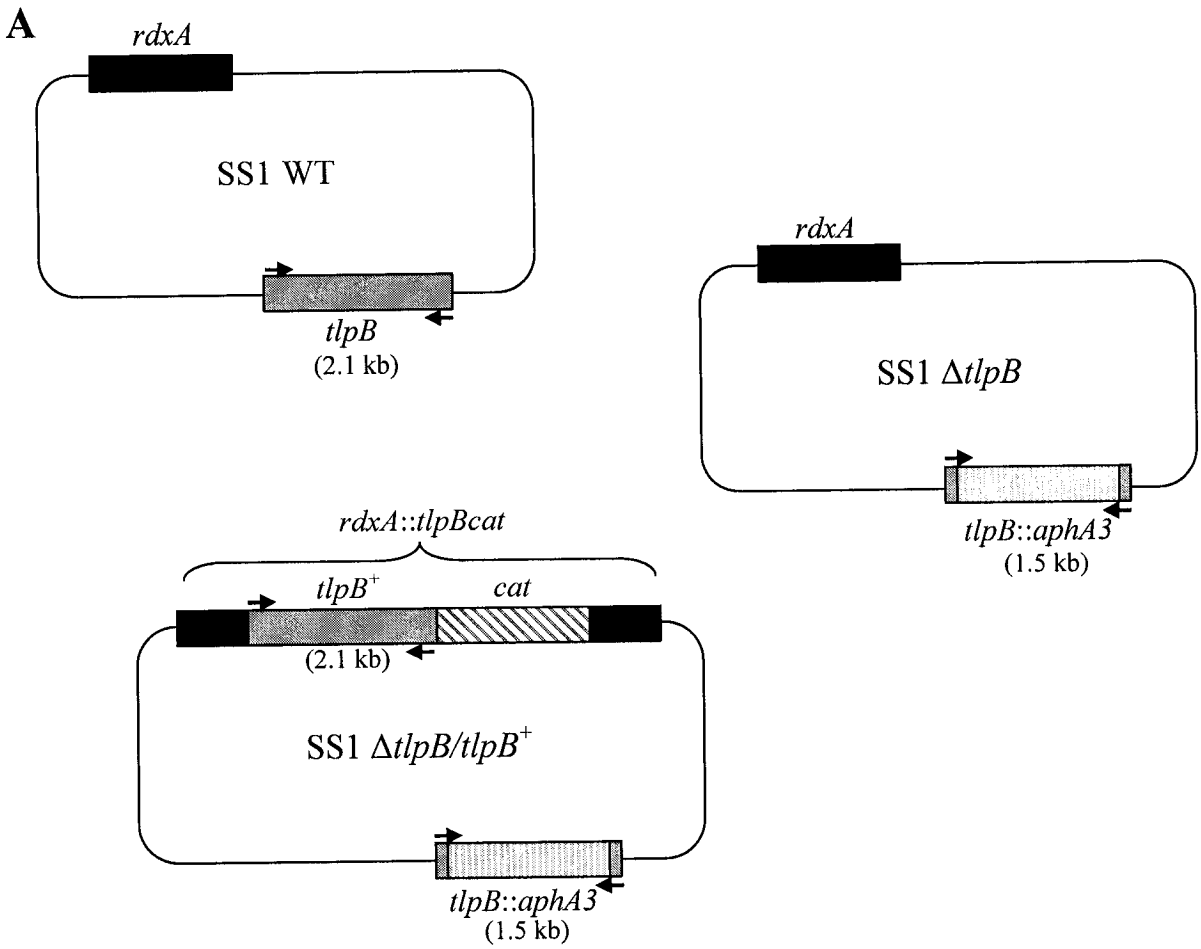
mock-treated (sterile PBS) mice. Therefore, TlpB, and the ability to sense/respond to pH, seems to be an important factor for *H. pylori* in colonization of the gastric mucosa.

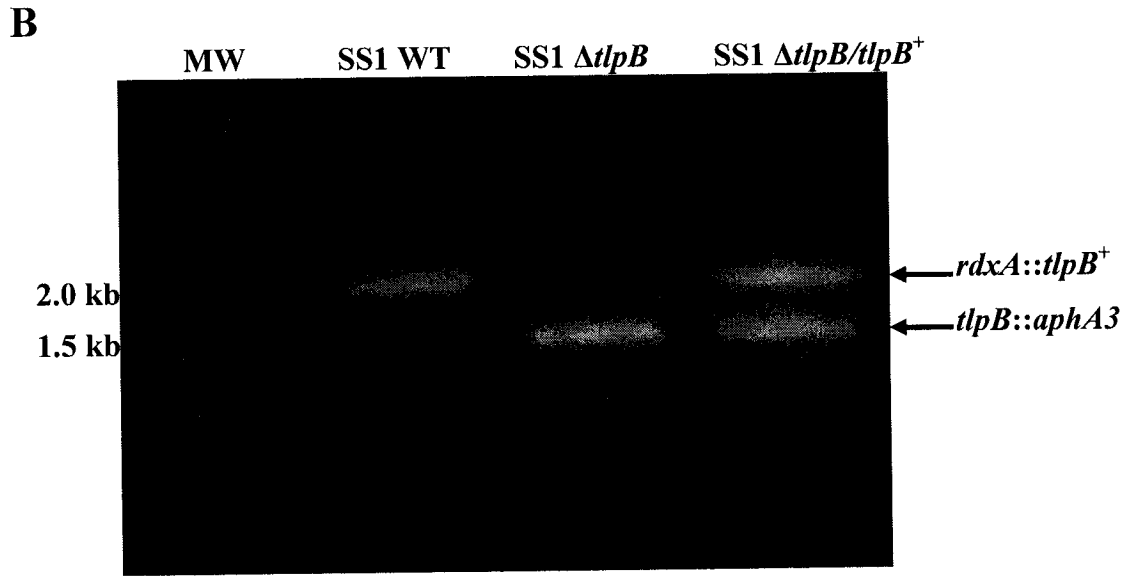
4.1.7 Allelic Complementation of *tlpB*

To ensure that the function of TlpB was involved in the pH tactic behavior, genetic complementation into the chromosome of *H. pylori* was performed to determine if the pH tactic phenotype and mouse colonization could be restored. Wild type *tlpB* was cloned into pRDX-C, a variant of pRDX originally designed by Dr. Johannes Kusters' group (Smeets *et al.*, 2000), and used to deliver wild type *tlpB* into the *rdxA* gene. Figure 9A depicts the *rdxA* locus and *tlpB* genes of *H. pylori* SS1 wild type, *H. pylori* SS1 Δ*tlpB* and *H. pylori* SS1 Δ*tlpB/tlpB*$^+$ (complementing strain) and arrows depict primer binding sites for the *tlpB* gene. Figure 9B shows the results of one of the analytical PCR reactions. The single, 2.1 kb band in the second lane (*H. pylori* SS1) is the wild type size of *tlpB*. The third lane shows the smaller, 1.5 kb *tlpB* mutant amplicon in *H. pylori* SS1 Δ*tlpB* (*tlpB*::*aphA3*) while the last lane shows both the *tlpB* mutant amplicon (1.5 kb; *tlpB*::*aphA3*) and the wild type *tlpB* (2.1 kb) found in the *H. pylori* SS1 Δ*tlpB/tlpB*$^+$. Further PCR reactions were performed to demonstrate that the wild type *tlpB* was properly integrated into the *rdxA* gene (data not shown)

In the pH taxis assay, *H. pylori* SS1 Δ*tlpB/tlpB*$^+$ regained the ability to sense pH and form a wall, though not as robustly as the wild type. Additionally, in the brucella-based soft agar chemotaxis assay, *H. pylori* Δ*tlpB/tlpB*$^+$ was just as chemotacitc as the wild type strain (Figure 10). Finally, C57BL/6 mice were infected with *H. pylori* SS1 Δ*tlpB/tlpB*$^+$ to determine if it could regain the ability to colonize the stomach of mice. As

Figure 9. Schematic Representation of the PCR Procedure to Confirm the Δ*tlpB/tlpB*$^+$ Construct. (A) Schematic of *tlpB* and *rdxA* genes and sizes in *H. pylori* SS1 wild type (WT), *H. pylori* SS1 Δ*tlpB* and *H. pylori* SS1 Δ*tlpB/tlpB*$^+$ (complementing strain). *H. pylori* SS1 contains a wild type copy of *rdxA* and *tlpB*. *H. pylori* SS1 Δ*tlpB* contains a wild type *rdxA* gene and *tlpB* replaced with a kanamycin (*aphA3*) cassette. *H. pylori* SS1 Δ*tlpB/tlpB*$^+$ contains the *tlpB* replaced with *aphA3* and *rdxA* replaced with a wild type *tlpB-cat* cassette. A primer set that flanked the *tlpB* gene is marked by arrows and annealing spot depicted in all genotypes. Predicted amplicon sizes are shown in parenthesis. (B) PCR using primer set (shown in A) that anneal outside of *tlpB*. MW, DNA marker. *H. pylori* SS1 WT amplifies the wild type *tlpB* gene (2.1 kb) only. *H. pylori* SS1 Δ*tlpB* amplifies the *tlpB*::*aphA3* (1.5 kb) only. *H. pylori* SS1 Δ*tlpB/tlpB*$^+$ amplifies both the *tlpB*::*ahpA3* (1.5 kb) and the wild type *tlpB* in the *rdxA* locus (*rdxA*::*tlpB*$^+$; 2.1 kb).


A
rdxA
SS1 WT
tlpB
(2.1 kb)
rdxA
SS1 ΔtlpB
tlpB::aphA3
(1.5 kb)
rdxA::tlpBcat
tlpB+
cat
(2.1 kb)
SS1 ΔtlpB/tlpB+
tlpB::aphA3
(1.5 kb)


B
MW
SS1 WT
SS1 ΔtlpB
SS1 ΔtlpB/tlpB+
2.0 kb
1.5 kb
rdxA::tlpB+
tlpB::aphA3

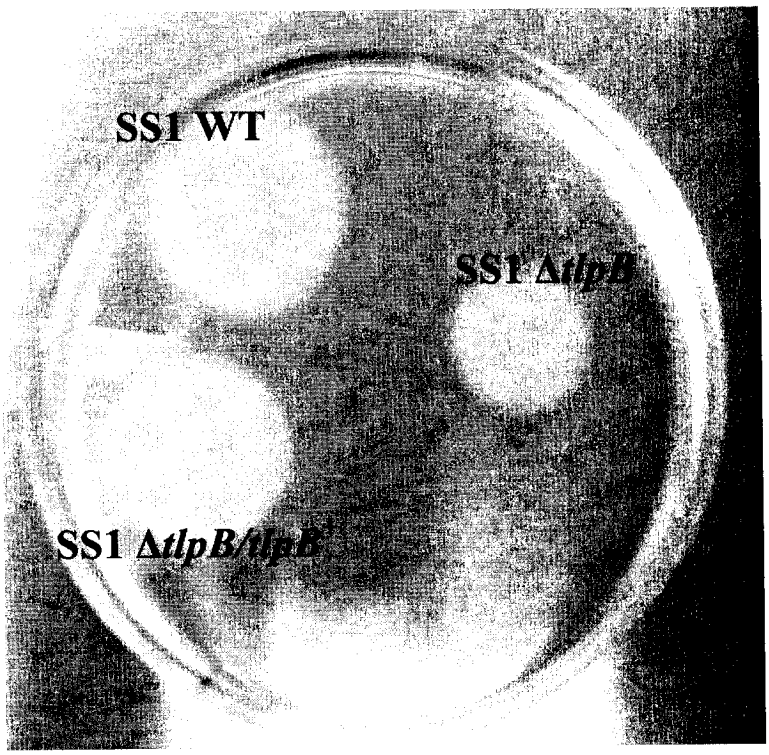


Figure 10. Qualitative Motility/Chemotaxis Assay of Δ*tlpB/tlpB*$^+$. Small inoculums of *H. pylori* SS1 wild type, *H. pylori* SS1 Δ*tlpB* and *H. pylori* SS1 Δ*tlpB/tlpB*$^+$ were spotted in 0.35% soft brucella agar. The plates were incubated under microaerobic conditions and the diameter of the swarm zone was measured at 5 days.

shown in Table 6, *H. pylori* SS1 Δ*tlpB*/*tlpB*$^{+}$ regained the ability to colonize the stomachs of mice as bacterial densities were within one log of the wild type strain (4.6 x 10^4 CFU/g vs 3.3 x 10^5 CFU/g). These studies demonstrate the importance of TlpB in pH taxis and mouse colonization.

4.1.8 Expression of *H. pylori tlpB* in *P. mirabilis*

Previous work in our lab used *P. mirabilis* as a host for urea transport into the cell via *H. pylori* UreI (unpublished data). Additionally, *P. mirabilis* does not display pH or negative taxis and therefore is unable to respond to repellants (Williams *et al.*, 1976). We wondered if the introduction of *H. pylori tlpB* into *P. mirabilis* would result in the gain of the ability to become pH tactic and therefore, could be used to study *tlpB* function.

H. pylori tlpB was cloned under the P_{lac} promoter of pUC19 and introduced into *P. mirabilis* PMS2. Although slightly more enfeebled for growth, the pUC*tlpB*-containing *P. mirabilis* was motile and exhibited some tumbling behavior; however, it quickly became non-motile as with the wild type and plasmid-control (pUC19) controls upon exposure to acid. Therefore, *P. mirabilis* did not gain pH tactic sensing ability from *H. pylori tlpB* and it may not a suitable host for the study of *tlpB* function. The expression level of TlpB in *P. mirabilis* was not determined and may contribute to the results.

Table 6. Gastric Colonization of Mice by *H. pylori* SS1 and Δ*tlpB/tlpB*$^{+}$

Strain	No. Infected / No. Mice	C.F.U / g stomach ± SD
Mock (1x sterile PBS)	0 / 4	0
SS1 WT	4 / 4	$3.3 \times 10^5 \pm 2.1 \times 10^5$
SS1 Δ*tlpB/tlpB*$^{+}$	4 / 4	$4.6 \times 10^4 \pm 2.0 \times 10^4$

Four C57BL/6J mice were infected with *H. pylori* SS1 Δ*tlpA* and *H. pylori* SS1 Δ*tlpB/tlpB*$^{+}$. Three weeks post infection the stomachs were isolated, weighed and homogenized and the homogenate was plated for bacterial loads (CFU/g stomach). SD, standard deviation.

4.2 Part II: Oxidative Stress in *H. pylori*

AhpC provides an essential function in protecting bacteria from oxidative stress under normal growth conditions (Baker *et al.*, 2001; Lundstrom and Bolin, 2000). Mutants of *ahpC* are enfeebled for in vitro growth and for mouse infection (Olczak *et al.*, 2002; Olczak *et al.*, 2003). Since AhpC is one of the most abundant proteins expressed by *H. pylori,* we tested the feasibility of an antisense technology to knockdown levels of AhpC and to assess its role in protection from oxidative stress and in mouse colonization. Some of the following work has been accepted for publication in the Journal of Bacteriology (Croxen *et al.*, 2007) and included herein with permission (see Appendix).

4.2.1 Antisense-*ahpC* Knocks Down *H. pylori* AhpC in *E. coli*

To demonstrate that antisense RNA can knockdown protein levels, we set up a system in *E. coli* that harbored the pET-29b::*ahpC* over expression plasmid and pBSK, pASAHPC100, pASAHPC250 or pASAHPC that contains the 100, 250 bp and full length antisense-*ahpC* constructs, respectively (Figure 11). Cultures were set up for IPTG inductions and crude lysates were subjected to SDS-PAGE as shown in Figure 12. In the absence of IPTG, none of the cultures show an over expression of AhpC. However, a strong expression of AhpC can be seen in the induced cultures of *E. coli* harboring pET-29b::*ahpC* alone (lane A+) and *E. coli* harboring both pET-29b::*ahpC* and pBSK (lane B+). Except for the 250 bp antisense-*ahpC* construct (lane D+), both the 100 bp antisense-*ahpC* (lane C+) and full length antisense-*ahpC* (lane E+) shows nearly a 100% knockdown of AhpC from the pET-29b plasmid. These results suggest that antisense RNA interference targeted to *H. pylori ahpC* is capable of knocking down *H. pylori* AhpC in *E. coli.*

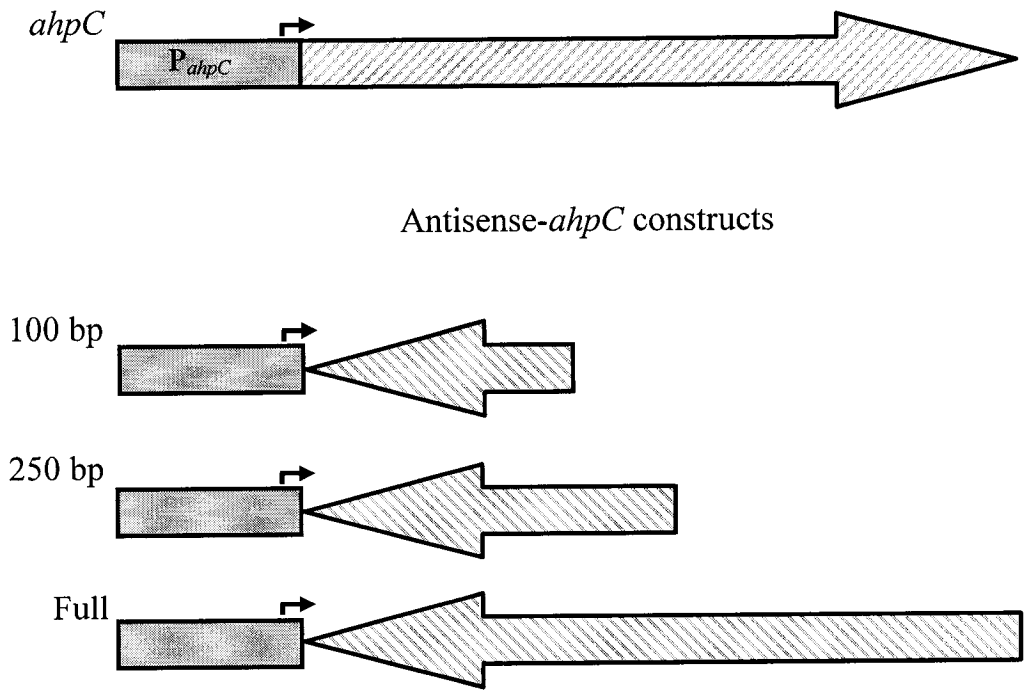


Figure 11. Schematic Representation of the Sense and Antisense-*ahpC* Constructs Used in Part II of this Work. The sense gene is depicted as found within the chromosome of *H. pylori,* under the control of the native *ahpC* promoter (P_{ahpC}). The three varying sized antisenses are presented in the 100, 250 bp and full length constructs, also driven by P_{ahpC}. These constructs were used in testing of antisense in *E. coli* and were also subcloned into the pDH37 *E. coli* / *H. pylori* shuttle vector for transformation into *H. pylori* 1061.

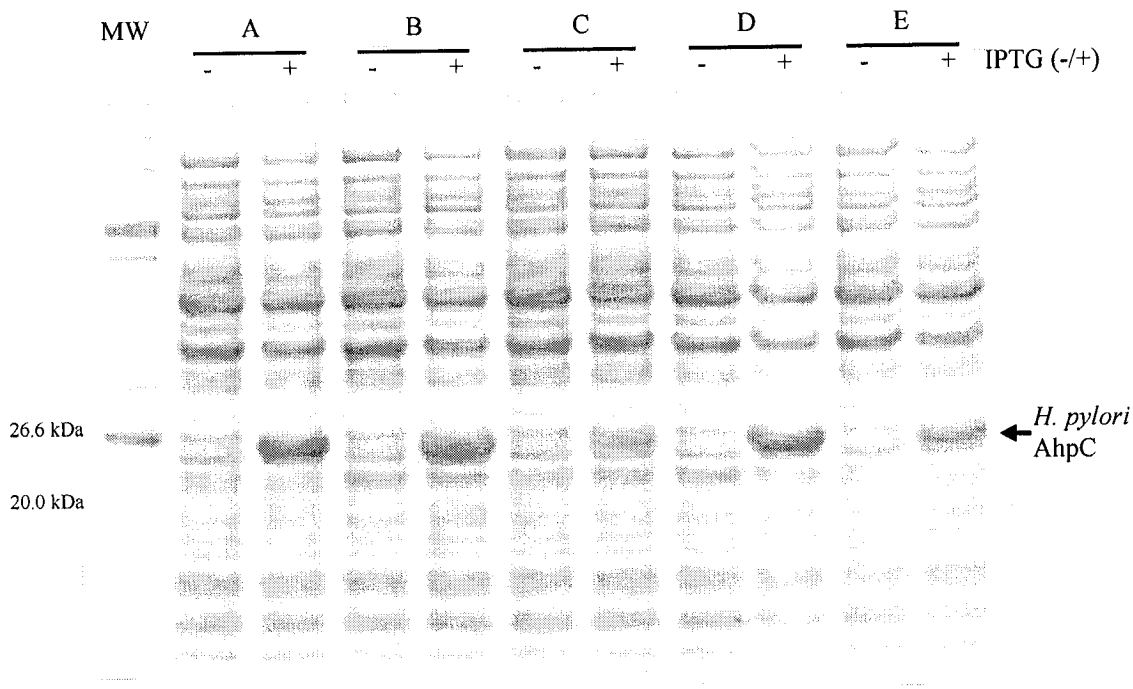


Figure 12. Antisense-*ahpC* in *E. coli*. *E. coli* BL21 CodonPlus®(DE3)-RIL cells harboring pET-29b::*ahpC* and the 100, 250 bp and full length antisense-*ahpC* constructs cloned into pBSK were grown to mid-log phase and induced for 1 hour with 1 mM IPTG. Cellular extracts were prepared by lysing the cells with LDS and boiling. Each extract was run on an SDS-PAGE. MW, molecular mass standard; column A (lanes 2 and 3), pET-29b::*ahpC* over expression control; column B (lanes 4 and 5), pET-29b::*ahpC* + pBSK vector control; column C (lanes 6 and 7), pET-29b::*ahpC* + pASAHPC100; column D (lanes 8 and 9), pET-29b::*ahpC* + pASAHPC250; column E (lanes 10 and 11), pET-29b::*ahpC* + pASAHPC. *H. pylori* AhpC is represented by the arrow, the absence or presence of IPTG is designated by – or +, respectively.

4.2.2 Antisense-*ahpC* Knocks Down AhpC in *H. pylori*

Since antisense-*ahpC* was able to significantly knockdown levels of *H. pylori* AhpC in the *E. coli* test system, we sought to confirm that antisense could similarly function in *H. pylori.* After natural transformation of pDH37, pHASAHPC100, pHASAHPC2500 or pHASAHPC into *H. pylori* 1061 and selection on antibiotics under normal culture conditions (7% O_2), crude lysates were prepared and run on an SDS-PAGE (Figure 13). No change in AhpC levels were seen in *H. pylori* 1061 wild type or *H. pylori* 1061 harboring the empty pDH37 plasmid (lanes A and B, respectively). However, all of the antisense-*ahpC* constructs (lanes C-E) show a knockdown level of AhpC. The full length antisense-*ahpC* (lane E) and 100 bp antisense-*ahpC* (lane C) were the best at knocking down AhpC levels by 72% and 42%, respectively, as calculated by densitometry. Unlike the results found in *E. coli*, the 250 bp antisense-*ahpC* (lane D) was able to knockdown AhpC levels by 34%. This data shows that antisense-*ahpC* is able to efficiently knockdown AhpC in *H. pylori* which can vary depending on the length of the construct. The increase in catalase (KatA) in lane B was noted and will be addressed in section 4.2.4.

4.2.3 Knockdown of AhpC Does Not Enfeeble Growth

Reports showed that isogenic mutants in *ahpC* were enfeebled for growth at oxygen tensions of 2% (Olczak *et al.*, 2002) while we showed that a 72% knockdown of AhpC was able to grow under normal culture conditions (7% O_2). Therefore, in vitro growth was measured spectrophotometrically and the optical densities were plotted over time. As seen in Figure 14, there is no difference in growth rates between wild type and control *H. pylori* 1061 strains when compared to the three antisense-*ahpC* harboring

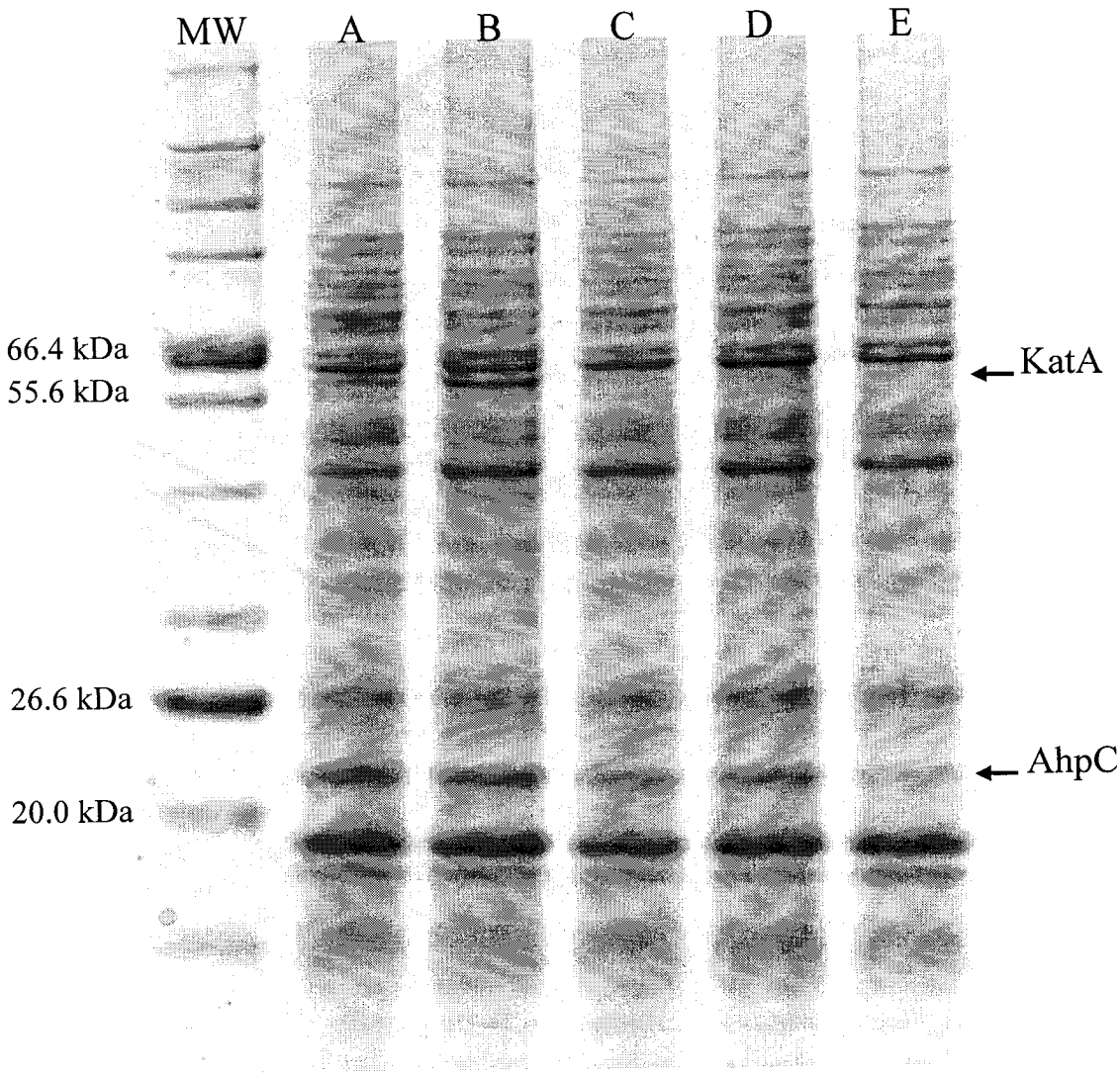


Figure 13. Antisense-*ahpC* in *H. pylori* 1061. *H. pylori* 1061 was transformed with 100, 250 bp or full length antisense-*ahpC* cloned into pDH37 were grown overnight (mid-log phase) and lysed with LDS and boiling. Cellular extracts were loaded and run on an SDS-PAGE. MW, molecular mass standard; lane A, wild type *H. pylori* 1061; lane B, *H. pylori* 1061 pDH37 vector control; lane C, *H. pylori* 1061 pHASAHPC100; lane D, *H. pylori* 1061 pHASAHPC250; lane E, *H. pylori* 1061 pHASAHPC. AhpC and KatA are represented by arrows. Note a strong over expression of KatA in the pDH37 vector control.

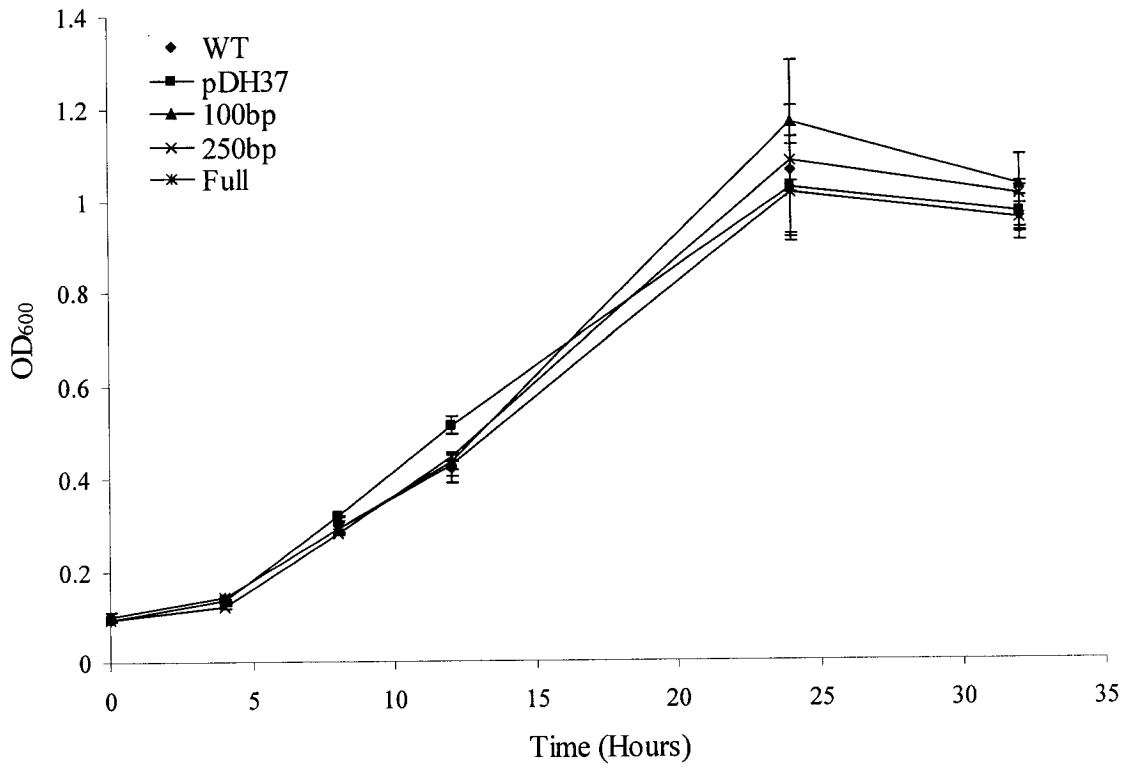


Figure 14. In Vitro Growth Curve of *H. pylori* 1061. *H. pylori* 1061 wild type (♦), *H. pylori* 1061 pDH37 (■), *H. pylori* 1061 pHASAHPC100 (▲), *H. pylori* 1061 pHASAHPC250 (x), *H. pylori* 1061 pHASAHPC (*) was seeded with an OD_{600} ~ 0.1 into fresh brucella broth and OD_{600} was measured at 4, 8, 12, 24 and 32 hours. The averages of three growth curves are shown with ± standard deviations.

strains. Moreover, colony forming units determined by serially diluting samples showed no difference in the number of colonies (data not shown). Therefore, it was determined that the viability and growth rate of *H. pylori* was not negatively affected by knockdown levels of AhpC. Higher oxygen tensions were not tested.

4.2.4 *H. pylori* Expressing Antisense-*ahpC* is More Sensitive to Peroxides

Although the antisense-*ahpC* constructs knocked down levels of AhpC as judged by SDS-PAGE and densitometry, we wished to determine if *H. pylori* harboring antisense-*ahpC* was more sensitive to peroxide stress. Zones of growth inhibition were measured around peroxide-saturated disks. Figure 15 shows that the antisense-*ahpC* harboring strains of *H. pylori* 1061 are more sensitive to H_2O_2, *tert*-butyl hydroperoxide (tBOOH) and cumene hydroperoxide than that of wild type strain or the pDH37 control. This data demonstrates that AhpC is important in protecting *H. pylori* from peroxide and organic hydroperoxide stress.

Although it appears that AhpC is important in protection of H_2O_2, this conclusion can not be drawn with confidence based on the results. The over expressed catalase (KatA) seen in lane B of Figure 13 (*H. pylori* 1061 pDH37) serves to distort the H_2O_2 results in Figure 14. Resistance to H_2O_2 at 100 mM was observed in *H. pylori* 1061 pDH37 only, as no zone of growth inhibition was observed. This observation was consistent at even higher concentrations of H_2O_2 (data not shown). Transformation of *H. pylori* 1061 with pDH37 results in this over expression of KatA which enhances the resistance of *H. pylori* to H_2O_2.

To further complicate matters, cloning into the multiple cloning site (MCS) of pDH37, prior to transformation into *H. pylori* 1061, nearly abolishes all KatA activity

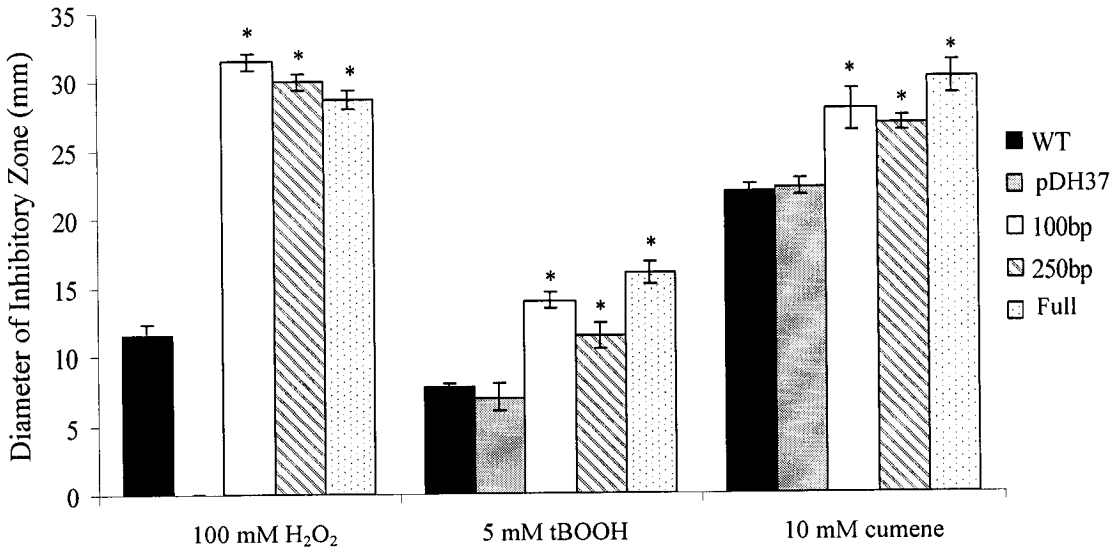


Figure 15. Zone of Growth Inhibition of *H. pylori* 1061 Challenged with Peroxides. *H. pylori* 1061 wild type (black), *H. pylori* 1061 pDH37 (grey), *H. pylori* 1061 pHASAHPC100 (white), *H. pylori* 1061 pHASAHPC250 (hash-mark) and *H. pylori* 1061 pHASAHPC (polka-dots) were plated for confluent growth on brucella agar. Sterile filter paper discs (7.5 mm) were saturated with either 100 mM H_2O_2, 5 mM *tert*-butyl hydroperoxide (tBOOH) or 10 mM cumene hydroperoxide and placed in the middle of the plated bacteria. Following 2 day incubation, the diameters of the inhibitory growth zone around the discs were measured. No zones of growth inhibition were observed with the diluents (ddH_2O for H_2O_2 and tBOOH or DMSO for cumene hydroperoxide). Results are the average of 4-5 separate experiments. Error bars represent standard deviations, asterisks represent statistical significance based on the Students' *t*-test (p-value < 0.001).

(data not shown). Therefore, the cloning of the three antisense-*ahpC* constructs into pDH37 resulted in this abrogation of activity and explains the hypersensitivity to 100 mM H_2O_2 (Figure 14). Moreover, cloning of other genes (e.g. chicken ovalbumin) or removal of the MCS from pDH37 also resulted in this abolishment of activity. Introduction of pDH37 into isogenic *katA* mutants of *H. pylori* 1061 did not exhibit any catalase activity (data not shown). Additionally, catalase activity of *H. pylori* 1061 pDH37 (over expressed *katA*) could be abolished by allelic replacement of *katA*. Therefore, some unknown aspect of pDH37 was able to over express *katA* or abolish activity with disruption of the MCS. Bioinformatic, and sequence analysis of pDH37 did not reveal any obvious clues for the perversity of this plasmid.

Despite the anomalous results dealing with pDH37 and H_2O_2, the antisense-*ahpC* harboring strains render *H. pylori* more sensitive to organic hydroperoxides, thus suggesting that antisense can function to knockdown protein levels of its target which can be correlated with a phenotype.

4.2.5 Initial Attempts at Chromosmal Integration of Antisense-*ahpC*

By showing that antisense can be used as a tool to specifically knockdown protein levels of target genes we wanted to extend the proof for a widespread application. Many strains of *H. pylori* are unable to stably maintain a plasmid (Aras *et al.*, 2002) and plasmids are cured during in vivo infections (personal communications with Dr. Rainer Haas). Therefore there was a need to stably integrate the antisense constructs into the chromosome of *H. pylori.*

A pilot *H. pylori* 1061 WT colonization of our permissive IL-12-defecient mouse model (Hoffman *et al.*, 2003) was unsuccessful (data not shown), so strains known to

colonize the stomachs of mice, *H. pylori* 88-3887 (motile variant of KE26695) and *H. pylori* SS1 were used for the integration of chromosomal antisense-*ahpC*. We chose to use the *rdxA* locus as the place of integration. RdxA encodes an oxygen-insensitive NADPH nitroreductase first discovered and characterized by our lab (Goodwin *et al.*, 1998). Disruption of *rdxA* renders *H. pylori* resistant to higher levels of the prodrug, metronidazole (Mtz). RdxA converts Mtz, through nitroreduction, to an active hydroxylamine metabolite that fragments DNA and leads to mutations (Sisson *et al.*, 2000; Sisson *et al.*, 2002). Additionally, Mtz resistant *H. pylori* can be isolated clinically, so there was little concern that RdxA was an important colonization or virulence factor. Therefore, by inserting our antisense constructs in the *rdxA* gene, we would be able to use Mtz resistance as the selectable marker.

A PCR-based method was used to insert the antisense constructs into *rdxA*. . Approximately 500 bp of the upstream and downstream regions were fused with the antisense-*ahpC* constructs as outlined earlier in the thesis except the antibiotic cassette was replaced with the antisense-*ahpC* constructs (see Figure 6). The resulting construct was introduced into *H. pylori* 88-3887 and *H. pylori* SS1 and originally selected on Mtz18 plates. Resulting colonies were few, and when screened for the insert with primers to amplify the *rdxA* gene, all were judged to have a wild type sized amplicon (i.e. no antisense-*ahpC* insertion). As a control, the same upstream and downstream fragments were also fused with the chloramphenicol (*cat*) cassette from *C. coli*. Selection on Cm or Cm + Mtz resulted in all clones, albeit few, with the *cat* cassette integrated into the *rdxA* gene. However, selection on Mtz only yielded results similar to those with the antisense-*ahpC* constructs. Additional attempts were made while lowering Mtz concentrations and

most were empty for an insert. Reasoning for these failed attempts may be explained by certain strains, designated as type II strains, being hypersensitive to Mtz (Jeong and Berg, 2000; Jeong *et al.*, 2001; Mukhopadhyay *et al.*, 2003).

Seven clones of both the 100 and 250 bp antisense-*ahpC* constructs and one for the full length antisense-*ahpC* were obtained in *H. pylori* SS1 and *H. pylori* 88-3887 but knockdown levels of AhpC could not be distinguished from wild type as judged by SDS-PAGE (data not shown).

Since chromosomal copies of the antisense constructs are in single copy versus 4-6 copies of the pDH37 vector (Heuermann and Haas, 1998), it was thought that the stability of the antisense transcript might be a factor in the poor knockdown of AhpC levels. One possibility we considered is that the antisense mRNA was a runoff message without termination which might have compromised function. To remedy this possibility, the T7-bacteriophage transcriptional terminator was cloned downstream of the 100 bp antisense-*ahpC*, as transcriptional terminators are believed to stabilize RNAs (Grunberg-Manago, 1999). The new construct, called P150T7, was introduced into the NifS-like gene (*hp0405*) of *H. pylori* G27. HP0405-null mutants were shown to have no obvious phenotypes in vitro and in vivo and the P150T7 antisense-*ahpC* was cloned into pEU39cm, a suicide vector that introduces DNA into *hp0405* with selection on chloramphenicol (gift from Dr. Robert Maier; Olson *et al.*, 2000; Olson *et al.*, 2001). Furthermore, *hp0405* (pEU39cm) was used to overcome the described issues of cloning into the *rdxA* locus. *H. pylori* G27 was used as transformation efficiencies are much higher than with *H. pylori* SS1 or *H. pylori* 88-3887. Resulting colonies showed a slight decrease in AhpC level on SDS-PAGE which correlated with higher sensitivity to organic

peroxides; however, this strain failed to colonize the highly permissive IL-12-deficient mouse, negating testing this strain for any differences in colonization efficiency.

4.2.6 Design of a New Chromsomal Insertion Suicide Vector

Since we had difficulty with chromosomal insertions into *rdxA* of *H. pylori,* we sought to improve the introduction, and selection into this gene. We also sought to identify mouse colonizing strains that might be more receptive to genetic manipulation. We obtained strain *H. pylori* X47 as a gift from Dr. Douglas Berg (Handt *et al.*, 1995) and determined that it was easier to manipulate genetically than SS1 and other mouse colonizing strains. The only downside of *H. pylori* X47 was the fact that it fell into the type II, Mtz-hypersusceptible class (Jeong *et al.*, 2001). In addition, the pEU39cm vector is limited to only an EcoRV cloning site, so to overcome these obstacles a new pRDX+ vector series was created. This vector differs from pRDX (described earlier) by having approximately 580 bp of additional upstream sequence from the previous construct and includes a small portion of the 5' and 3' end of the actual *rdxA* gene. These two regions were cloned such that most of the pBC cloning site remained intact and its introduction into the chromosome of *H. pylori* would fully replace the *rdxA* gene with whatever DNA was cloned between the two flanks. In addition, unique SacI and KpnI sites lie just outside of the 5' and 3' *rdxA* flanks so that a linear construct including the necessary DNA can be transformed into *H. pylori.* Finally, to facilitate easy selection into the *rdxA* locus the kanamycin cassette from *C. coli* (Trieu-Cuot *et al.*, 1985) was cloned in the forward orientation with respect to the *rdxA* flanks yielding pRDX-K+ (Figure 16). Transformation of the SacI/KpnI fragment into *H. pylori* X47 was efficient in yielding kanamycin resistant colonies.

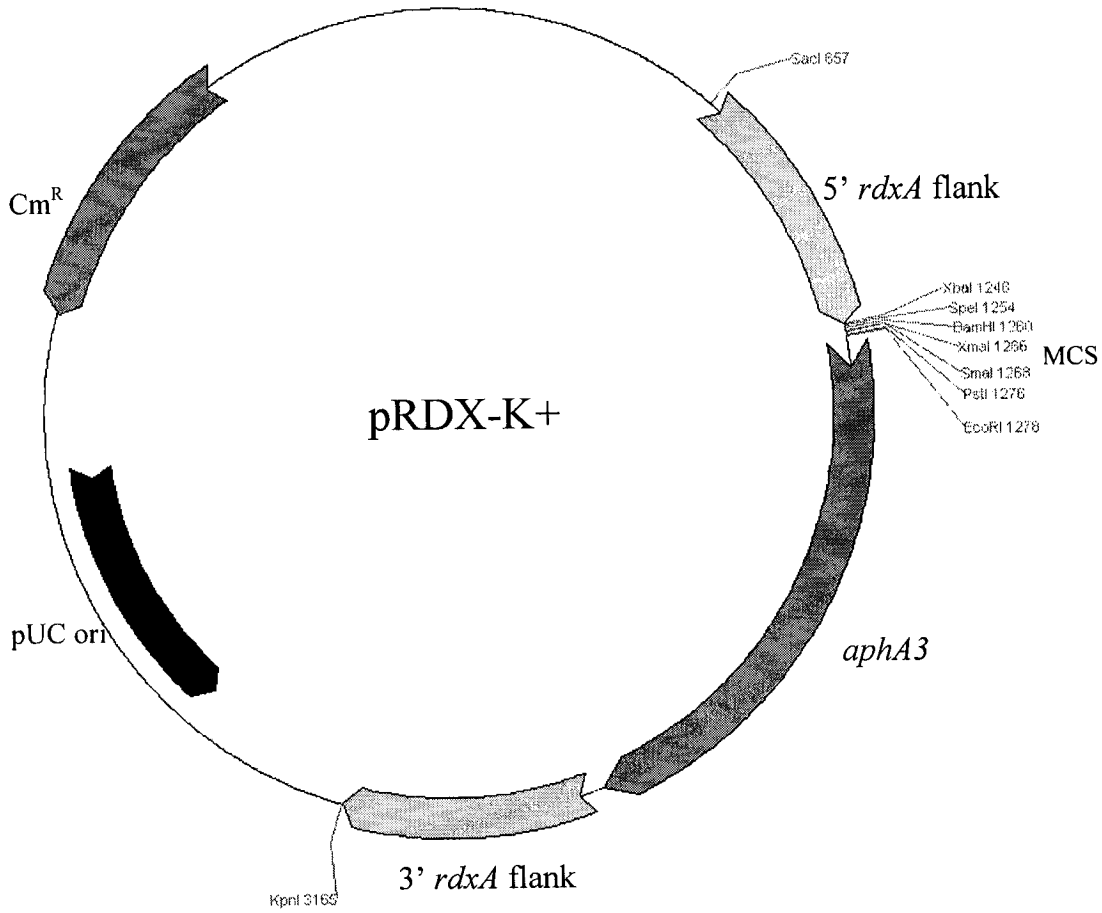


Figure 16. Schematic of pRDX-K+ Suicide Vector. Cm^R represents chloramphenicol resistance for *E. coli* but does not work in *H. pylori.* pUC ori is the origin of replication for the plasmid in *E. coli* and would not function in *H. pylori.* Approximately 580 bp of upstream and downstream sequences to *rdxA* was sequentially cloned into pBC KS+ thereby flanking the majority of the multiple cloning site (pRDX+). To facilitate easier selection of allelic replacement of *rdxA*, a kanamycin cassette (*aphA3*) from *C. coli* was cloned in the forward direction (same orientation as the *rdxA* flanks) between 3' and 5' *rdxA* flanks. Unique and relevant restriction sites are indicated in the MCS. SacI and KpnI site that lie outside of the two *rdxA* flanks were typically used to excise the complete construct for transformation or electroporation into *H. pylori.*

4.2.7 Design of a New Antisense-*ahpC* Constructs (AS1563)

Given that the full length antisense-*ahpC* was the most efficient in knocking down AhpC levels in *H. pylori* 1061, it was used as the base for the design of a new antisense construct. In addition to the full length, there is a 94 bp untranslated region upstream of the ATG start site of *ahpC* that contains the Shine-Dalgarno (Lundstrom and Bolin, 2000). Since a theme in the function of many small antisense and regulatory RNAs is to sequester the Shine-Dalgarno from translation initiation by ribosomes (Gollnick and Babitzke, 2002; Gottesman, 2004), the 94 bp untranslated region was also incorporated into the full length antisense design.

In order to find a strong promoter for driving expression of the antisense-*ahpC*, various promoters such as P_{rpoD}, P_{por}, P_{ureA}, P_{ahpC} from *H. pylori* were cloned upstream of the *gfpmut3* in pGFK3K and inserted into the *rdxA* gene of *H. pylori* G27. Fluorescence was measured and compared to the fluorescence of wild type *H. pylori* G27 and the promoterless *gfpmut3* cloned into the *rdxA* locus of *H. pylori* G27. The urease operon promoter (P_{ureA}) and P_{ahpC} gave the strongest fluorescence with the former being the better of the two (data not shown). Both were used in two separate constructs with the full length antisense-*ahpC* that contained the 94 bp upstream untranslated region.

The antisense-*ahpC* constructs were terminated with the *fd*-bacteriophage terminator that was shown to prevent read through transcription from the strong gonococcal *opa* promoter (P_{opa}) that drives expression of cat_{GC} from mini-TnMax, an *H. pylori* transposon (Kahrs *et al.*, 1995). Together with either the P_{ahpC} or P_{ureA} promoter, the new antisense-*ahpC* constructs were designated AS1563 and AS1563-2 respectively. Figure 17 depicts a schematic of the features of the improved antisense-*ahpC* constructs.

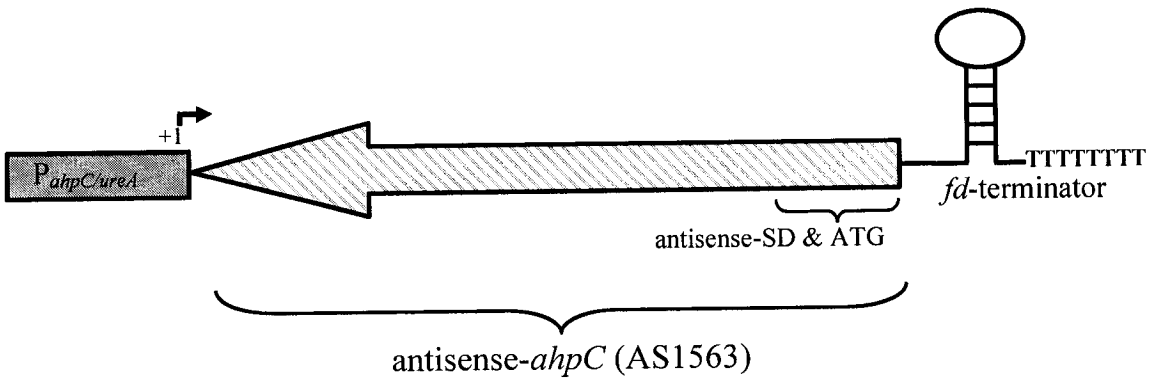


Figure 17. New Antisense-*ahpC* Design (AS1563). Redesigned antisense constructs were driven by either the *ahpC* (P_{ahpC}) or urease operon (P_{ureA}) promoters. The antisense *ahpC* was extended to encompass the 94 bp upstream untranslated region which contains complementary sequences to the Shine-Dalgarno and ATG start site. To prevent transcriptional read through and aid in potentially aid in stabilizing the antisense RNA, the *fd*-bacteriophage Rho-independent transcriptional terminator was included at the 3' end of the construct.

4.2.8 Introduction of AS1563 into Chromosome of *H. pylori*

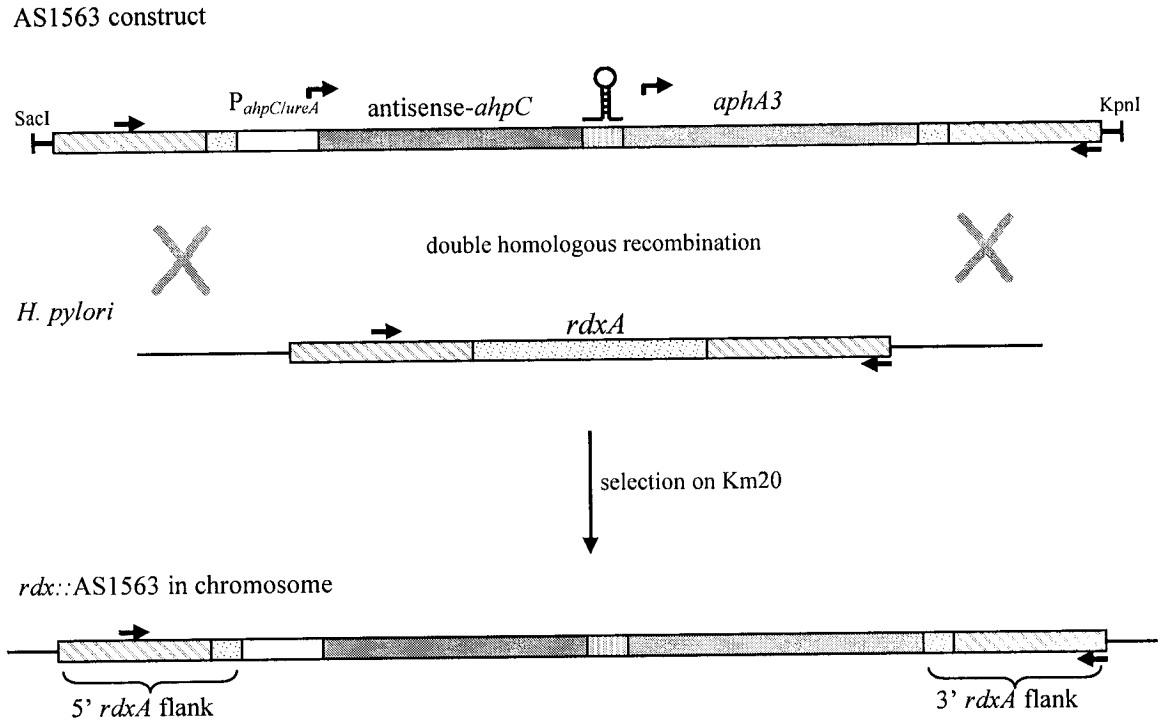
The newly designed antisense-*ahpC* constructs were introduced into the *rdxA* locus of *H. pylori* X47 via the pRDX-K+ vector as illustrated by Figure 18A. Following selection, proper insertion into the *rdxA* locus was confirmed with various PCR reactions. As an example, Figure 18B shows the results of one of these reactions. Primers designed to amplify the flanking regions of the *rdxA* gene were used and are depicted as small arrows in the various steps of Figure 18A. Lane A of Figure 18B shows the *H. pylori* X47 wild type *rdxA* gene (1.2 kb) and the *rdxA* replaced with the *aphA3* cassette (2.1 kb) is shown in lane B. Lanes C and D represent the *rdxA*::AS1563 (P_{ahpC}) and *rdxA*::AS1563-2 (P_{ureA}) antisense-*ahpC* constructs, respectively with respective sizes of 3.1 kb and 3.0 kb. Therefore, the proper insertions of the antisense-*ahpC* constructs into the *rdxA* locus were confirmed with PCR.

4.2.9 Single Copy AS1563 Knocks Down AhpC Levels

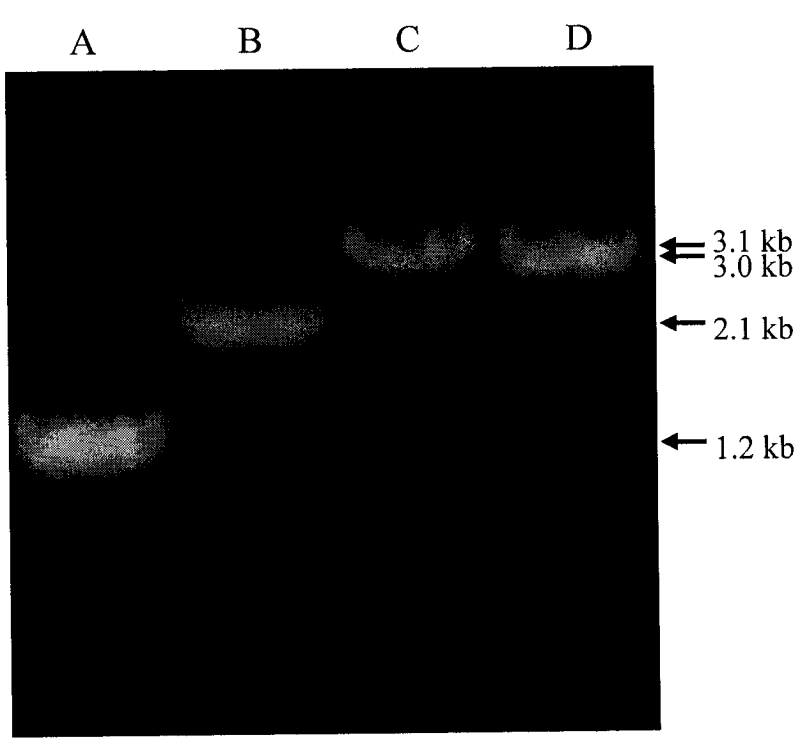
With the proper insertion of the two antisense-*ahpC* constructs into the *rdxA* locus of *H. pylori* X47, crude cell lysates were run on an SDS-PAGE to visualize the protein profiles (Figure 19). Lane A is the crude lysate of wild type *H. pylori* X47 and lane B is crude lysate of the control, *H. pylori* X47 *rdxA*::*aphA3* and it shows that there are high levels of AhpC expressed in both. Lane C is crude lysate from *H. pylori* X47 *rdxA*::AS1563 that shows a slight knockdown of AhpC (15% by densitometry) followed by lane D, lysate from *H. pylori* X47 *rdxA*::AS1563-2 that has approximately 32% knockdown of AhpC when compared to wild type and control (*rdxA*::*aphA3*) levels. This is consistent with the P_{ureA} displaying more fluorescence than P_{ahpC} in the fluorimetry measurements that demonstrated that P_{ureA} was a stronger promoter than P_{ahpC}.

Figure 18. Schematic Representaiton of the Chromosomal Insertion of AS156 and Screening by PCR. (A) After cloning AS1563 into pRDX-K+ the *rdxA* flanks, AS1563 construct and *aphA3* fragment was excised using the unique SacI and KpnI sites. Following transformation and double homologous recombination into the chromosome colonies were selected with kanamycin with *rdxA* potentially replaced with the AS1563 construct and *aphA3* (referred to as *rdxA*::AS1563). PCR primers that lie outside of the *rdxA* gene in the 3' and 5' flanks are represented as small arrows. (B) Sample PCR reaction for the confirmation of AS1563 replacement of *rdxA*. Lane A is wild type *H. pylori* X47 wild type amplifying a fragment of ~1.2 kb (see *H. pylori* chromosome in A); lane B represents X47 with *rdxA* replaced with *aphA3* cassette only (~2.1 kb; not depicted in 18A), lane C represents X47 with *rdxA* replaced with AS1563 (P_{ahpC} promoter) and *aphA3* amplifying a fragment ~3.1 kb; lane D represents X47 with *rdxA* replaced with AS1563-2 (P_{ureA} promoter) and *aphA3* amplifying a fragment of ~3.0 kb. Both AS1563 and AS1563-2 are depicted as AS1563 in A.

A


AS1563 construct
SacI
$P_{ahpC/ureA}$
antisense-*ahpC*
aphA3
KpnI
double homologous recombination
H. pylori
rdxA
selection on Km20
rdx::AS1563 in chromosome
5' *rdxA* flank
3' *rdxA* flank

B


A
B
C
D
3.1 kb
3.0 kb
2.1 kb
1.2 kb

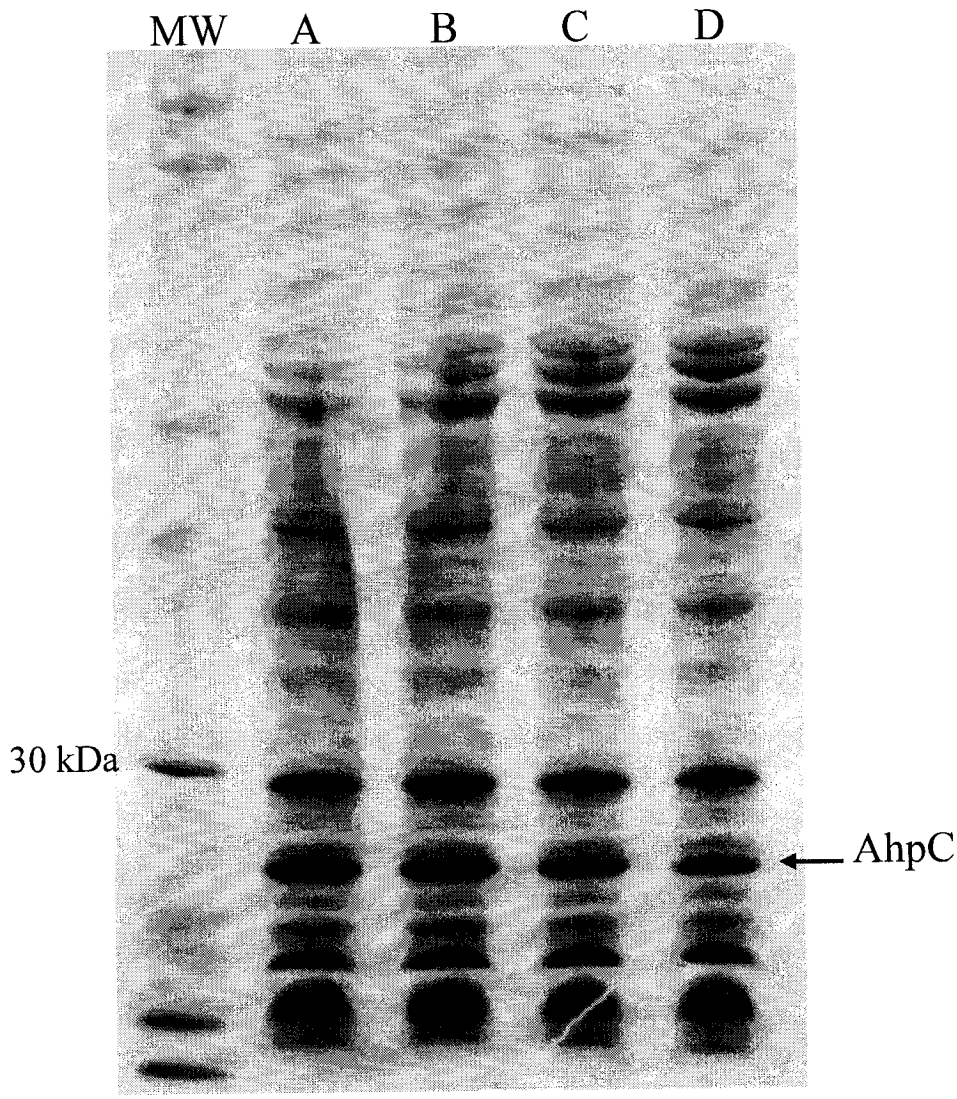


Figure 19. AS1563 in *H. pylori* X47. *H. pylori* X47 was transformed with AS1563 and AS1563-2 and integrated into the *rdxA* gene. Cellular extracts of overnight growth (mid-log phase) were prepared and run on an SDS-PAGE. MW, molecular mass standard; lane A; *H. pylori* X47 wild type; lane B, *H. pylori* X47 *rdxA*::*aphA3*; lane C, *H. pylori* X47 *rdxA*::AS1563 and; lane D, *H. pylori* X47 *rdxA*::AS1563-2. AhpC is represented by an arrow.

4.2.10 Knockdown of AhpC by AS1563 Does Not Enfeeble Growth

To ensure that the single copy of AS1563 and AS1563-2, in addition to the replacement of *rdxA* does not inhibit growth of *H. pylori,* in vitro growth was measured spectrophotometrically and plotted against time. As seen in Figure 20, *H. pylori* X47 wild type, *H. pylori* X47 *rdxA*::*aphA3*, *H. pylori* X47 *rdxA*::AS1563 and HpX47 *rdxA*::AS1563-2 did not display any difference in growth rates over a 32 hour time course. Colony forming units determined by serially diluting samples showed no difference in the number of colonies (data not shown). Neither allelic replacement of *rdxA* or introduction of the *aphA3* cassette affected the growth rate of *H. pylori.*

4.2.11 *H. pylori* Expressing AS1563 is More Sensitive to Peroxides

To verify that antisense-*ahpC* knockdown levels of AhpC rendered *H. pylori* more sensitive to hydroperoxide, the cells were challenged with H_2O_2, tBOOH and cumene peroxide in a disk diffusion assay. As an example of the assay, Figure 21A visually shows the zone of inhibition of *H. pylori* X47 wild type, *H. pylori* X47 *rdxA*::*aphA3*, *H. pylori* X47 *rdxA*::AS1563 and *H. pylori* X47 *rdxA*::AS1563-2 when challenged with 15 mM tBOOH. The zones of growth inhibition around the two antisense-*ahpC* containing strains are larger than that of *H. pylori* X47 wild type and *H. pylori* X47 *rdxA*::*aphA3* control. In correlation with the amount of knockdown levels between AS1563 and AS1563-2, *H. pylori* X47 *rdxA*::AS1563-2 displays a greater zone of growth inhibition than *H. pylori* X47 *rdxA*::AS1563.

Figure 21B shows the zones of growth inhibition of each strain challenged with H_2O_2, tBOOH and cumene hydroperoxides on a histogram. As shown visually in Figure 21A, the antisense-*ahpC* harboring strains are more sensitive to tBOOH but also to

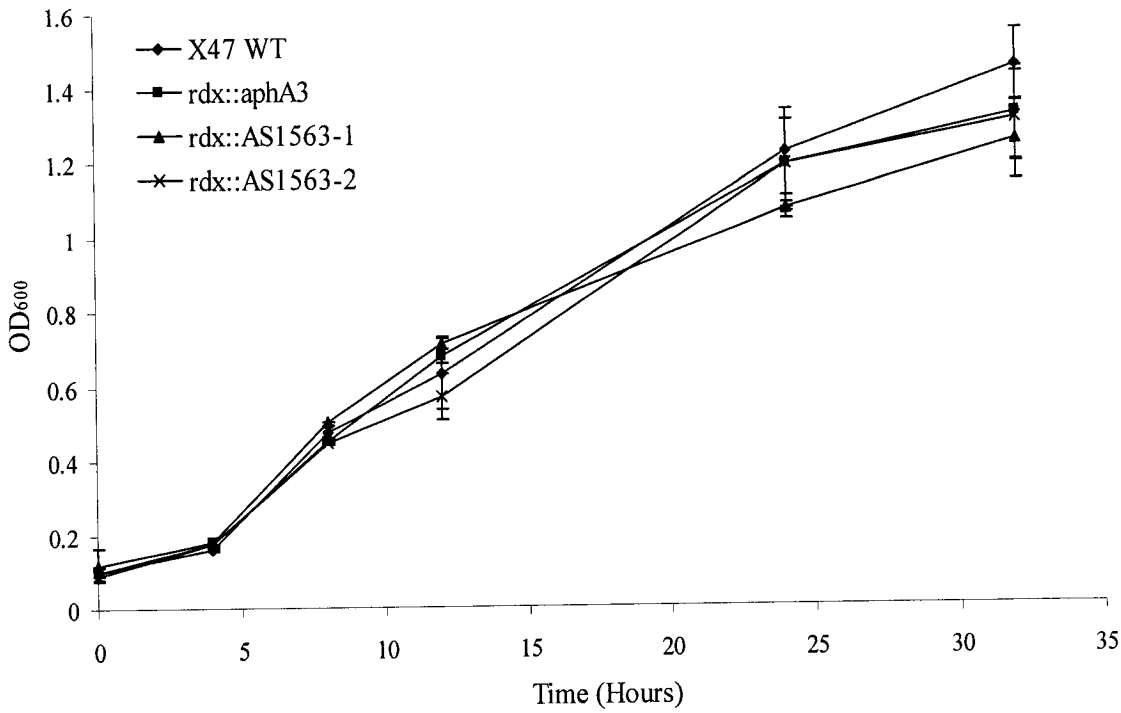
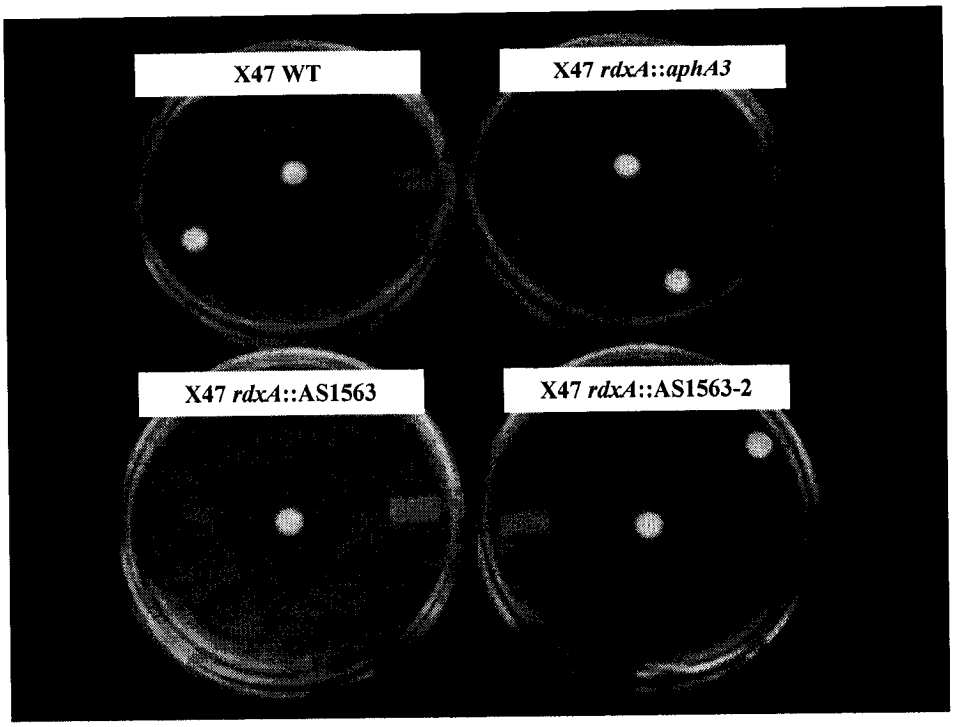


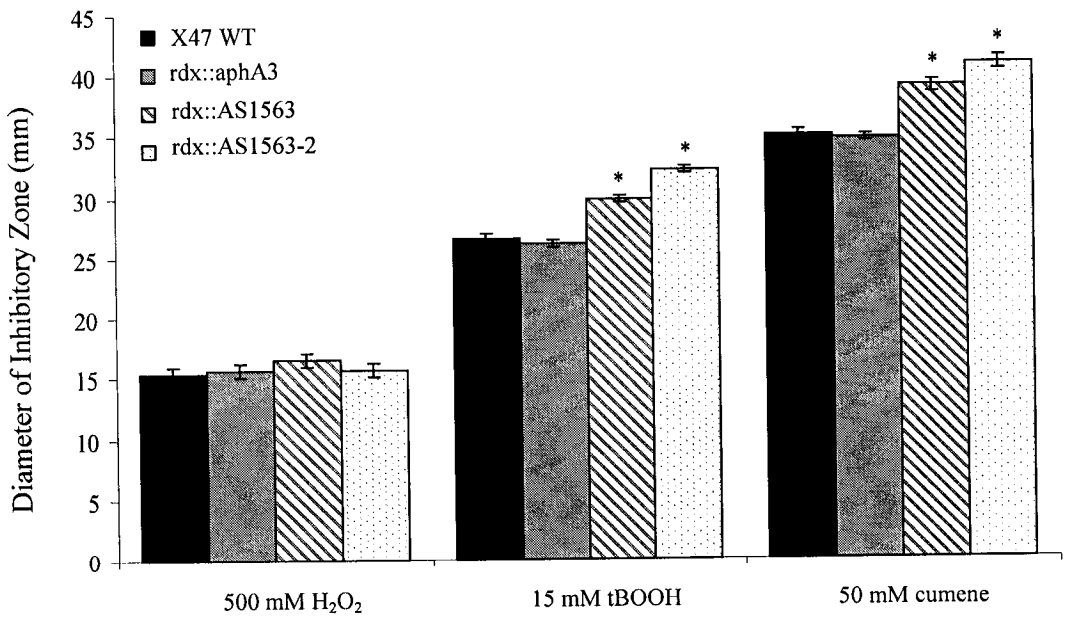
Figure 20. In Vitro Growth of *H. pylori* X47. *H. pylori* X47 wild type (♦), *H. pylori* X47 *rdxA*::*aphA3* (■), *H. pylori* X47 *rdxA*::AS1563 (▲) and *H. pylori* X47 *rdxA*::AS1563-2 (x) was seeded with an OD_{600} ~ 0.1 into fresh brucella broth and OD_{600} was measured at 4, 8, 12, 24 and 32 hours. The averages of three growth curves are shown with ± standard deviations.

Figure 21. Zone of Growth Inhibition of *H. pylori* X47 Challenged with Peroxides. (A) Representative plates of a zone of growth inhibition assay challenged with 15 mM *tert*-butyl hydroperoxide (tBOOH). (B) Zone of growth inhibition of *H. pylori* X47. *H. pylori* X47 wild type (black), *H. pylori* X47 *rdxA*::*aphA3* (grey), *H. pylori* X47 *rdxA*::AS1563 (hash-mark) and *H. pylori* X47 *rdxA*::AS1563-2 (polka-dots) were plated for confluent growth on brucella agar. Sterile filter paper discs (7.5 mm) were saturated with either 500 mM H_2O_2, 15 mM tBOOH or 50 mM cumene hydroperoxide and placed in the middle of the plated bacteria. After 3 days of incubation, the diameters of the inhibitory zone around the discs were measured. No zones of growth inhibition were observed with the diluents (ddH_2O for H_2O_2 and tBOOH or DMSO for cumene hydroperoxide). Results show the average of 3 independent challenges. Error bars represent standard deviations, asterisks represent statistical significance based on the Students' *t*-test (p-value < 0.001).

A


X47 WT
X47 rdxA::aphA3
X47 rdxA::AS1563
X47 rdxA::AS1563-2

B


X47 WT
rdx::aphA3
rdx::AS1563
rdx::AS1563-2
Diameter of Inhibitory Zone (mm)
0
5
10
15
20
25
30
35
40
45
*
500 mM H2O2
15 mM tBOOH
50 mM cumene

cumene hydroperoxide. In a similar manner to tBOOH, *H. pylori* X47 *rdxA*::AS1563-2 is more susceptible to cumene hydroperoxide than *H. pylori* X47 *rdxA*::AS1563 and both are more vulnerable than *H. pylori* X47 wild type and *H. pylori* X47 *rdxA*::*aphA3* control. Interestingly, no difference was observed when challenged with H_2O_2. The knockdown of AhpC by antisense-*ahpC* makes *H. pylori* more susceptible to organic hydroperoxides but not H_2O_2 and the sensitivity is correlated to the amount of knockdown.

4.2.12 *H. pylori* Expressing AS1563 is More Sensitive to Short Exposures of tBOOH

H. pylori with a decreased level in AhpC is more sensitive to organic hydroperoxides than wild type; however, we sought to determine if *H. pylori* was also more sensitive to short exposures of tBOOH. To find a suitable concentration of tBOOH, minimal bactericidal concentration (MBC) assays were performed with tBOOH serially diluted from 5 mM to ~4.88 μM in a culture of *H. pylori.* All *H. pylori* X47 strains (including those harboring the antisense-*ahpC*) were non-viable at 0.625 mM and viable at 0.3125 mM, therefore a concentration of 0.5 mM was chosen to be appropriate for the timed exposure.

Figure 22 shows the time dependent killing kinetics during exposure to 0.5 mM tBOOH. An accelerated rate of death was evident for *H. pylori* X47 *rdxA*::AS1563 and *H. pylori* X47 *rdxA*::AS1563-2 as there is a steeper slope of killing when compared to *H. pylori* X47 wild type and *H. pylori* X47 *rdxA*::*aphA3* control. In correlation with the above results that the greater the knockdown of AhpC, the more sensitive *H. pylori* is to organic hydroperoxides, *H. pylori* X47 *rdxA*::AS1563-2 exhibited a quicker rate of killing towards tBOOH than *H. pylori* X47 *rdxA*::AS1563. No differences in CFUs were

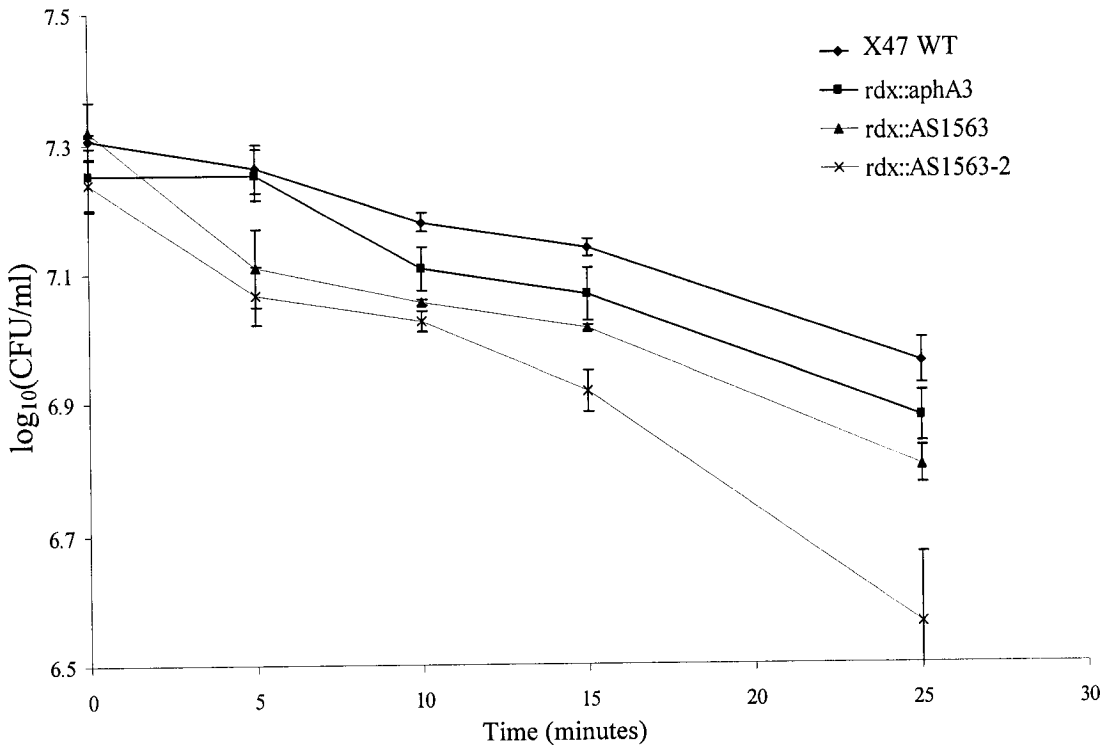


Figure 22. Killing of *H. pylori* X47 with tBOOH. *H. pylori* X47 wild type (♦), *H. pylori* X47 *rdxA::aphA3* (■), *H. pylori* X47 *rdxA*::AS1563 (▲), and *H. pylori* X47 *rdxA*::AS1563-2 (**x**) was subjected to 0.5 mM *tert*-butyl hydroperoxide (prepared in ddH_2O) and aliquots were taken at various time points, serially diluted 10-fold and plated on brucella agar. After four days of incubation, colonies were counted. No differences in CFUs were observed when challenged with ddH_2O. Results depict three separate experiments with ± standard deviations.

observed when challenged with ddH_2O. This data supports earlier findings that a decrease in AhpC renders *H. pylori* more susceptible to organic hydroperoxides and that the sensitivity is directly correlated to how much AhpC is available in the cell.

4.2.13 Full AhpC Function is Not Required for Colonization of Mice

Since *ahpC* mutants are defective for colonization of mice, AhpC was reported to be a colonization factor (Olczak *et al.*, 2003). However, the fact that the *ahpC* mutants are seriously enfeebled for in vitro growth, it is more likely that the reason for failed mouse colonization is simply impaired growth. If AhpC levels are critical for growth and colonization, then lower levels of expression should correlate with poor colonization efficiency in mice. To test this hypothesis, the X47 strain expressing antisense-*ahpC* was compared with controls for colonization efficiency in a mouse model. The result of the mouse colonization study is shown in Table 7. There was no difference in the ability of *H. pylori* X47 *rdxA*::AS1563 or *H. pylori* X47 *rdxA*::AS1563-2 to colonize the stomachs of mice when compared to *H. pylori* X47 wild type or *H. pylori* X47 *rdxA*::*aphA3* control. Additionally, the isolated colonies were passaged to confluence once and immediately tested for sensitivity to the organic hydroperoxide tBOOH. The results are depicted in Figure 23 and demonstrate that the sensitivity of the wild type, control and antisense-*ahpC* harboring *H. pylori* X47 strains were just as sensitive to tBOOH post-infection.

These results suggest that AhpC may not be a critical factor in the colonization of mouse stomachs and therefore may not be a virulence factor. Secondly, since the antisense-*ahpC* harboring strains exhibited the same tBOOH sensitivity pre- and post-infection suggests that the antisense is stabile in vivo.

Table 7. Gastric Colonization of Mouse by *H. pylori* X47 and AS1563 Harboring Strains

Strain	**No. Infected / No. Mice**	**C.F.U / g stomach ± SD**
Mock (sterile 1x PBS)	0 / 5	0
X47 WT	5 / 5	$1.015 \times 10^7 \pm 0.13 \times 10^7$
X47 *rdxA*::*aphA3*	5 / 5	$1.029 \times 10^7 \pm 0.18 \times 10^7$
X47 *rdxA*::AS1563-1	5 / 5	$0.979 \times 10^7 \pm 0.30 \times 10^7$
X47 *rdxA*::AS1563-2	5 / 5	$1.009 \times 10^7 \pm 0.05 \times 10^7$

CJB/6J mice were infected with *H. pylori* X47 wild type, *H. pylori* X47 *rdxA*::*aphA3*, *H. pylori* X47 *rdxA*::AS1563 and *H. pylori* X47 *rdxA*::AS1563-2. Stomachs were isolated 3 weeks later, weighed, and homogenized. The homogenate was plated for *H. pylori* by 10-fold serial dilutions on selective media. Colony counts were made three days later and presented as CFU/g stomach with standard deviations (SD).

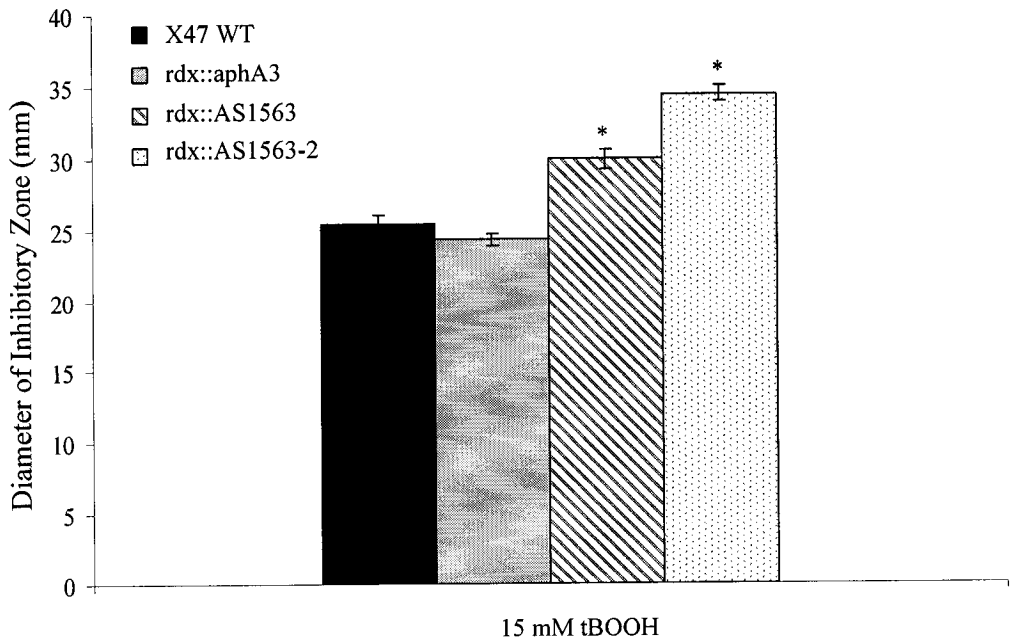


Figure 23. Sensitivity to tBOOH of *H. pylori* X47 and Derivatives After a 3 Week Mouse Infection. Three colonies, and a pool of the remaining colonies from the CJB/6J mouse infection were expanded with one pass on the culture dish and challenged with a sterile filter disc (7.5 mm) saturated with 15 mM *tert*-butyl hydroperoxide. *H. pylori* X47 wild type (black), *H. pylori* X47 *rdxA*::*aphA3* (grey) *H. pylori* X47 *rdxA*::AS1563 (hash-mark), and *H. pylori* X47 *rdxA*::AS1563-2 (polka-dots). No zones of growth inhibition were observed with the ddH_2O. Results show the average of three colonies and the remaining pooled colonies. Error bars represent the standard deviation, asterisks represent statistical significance based on the Students' *t*-test (p-value < 0.001).

4.3 Broadening of Antisense Application (HP0166 and HP1043)

Since knockdowns of AhpC were achieved with the antisense approach, we sought to knockdown two essential regulatory genes, *hp0166* (*arsR*) and HP1043. HP0166 has been studied in some detail and shown to be involved in acid resistance (Dietz *et al.*, 2002; Pflock *et al.*, 2006a; Wen *et al.*, 2003; Wen *et al.*, 2006) while HP1043 is an orphan regulator (lacks cognate histidine kinase) that has only been shown to bind upstream of the promoter of *tlpB*, but may be involved in its regulation (Delany *et al.*, 2002). Since global analysis of ArsR have only been looked at with ArsS mutants (cognate sensor of ArsR; not essential for viability) (Pflock *et al.*, 2006a), efforts were made to knock down protein levels of ArsR to see if we can identify new genes involved in both the acid-adaptive response and phosphorylation-independent viability. Additionally, no global studies on HP1043 regulons have been done so knockdown levels may also give a clue to its regulon and involvement, if any, in pH taxis.

4.3.1 Production of Antisera Against HP0166 (ArsR) and HP1043

Unlike AhpC, both HP0166 (ArsR) and HP1043 are not readily visible by SDS-PAGE. Since we also did not have a defined phenotype to look in knocked down levels of regulator, we produced antisera towards each regulator so that we can visualize knockdown levels of expression with immunoblots.

Each regulator was cloned in frame with the 10x His-tag of pET-16b because OmpR-like regulators have DNA binding domains at their C-terminus and in the event we would need purified regulator for electrophoretic mobility shift assays (EMSA) or DNA footprinting. Over expression of each regulator in *E. coli* BL21 CodonPlus®(DE3)-RIL can be visualized in Figure 24. Three out of three clones harboring the pET-

16b::*hp0166* plasmid strongly over expressed *hp0166* (ArsR) while only two of the three clones harboring pET-16b::*hp1043* strongly over expressed HP1043. One clone of each was selected for purification of the respective response regulator. Purification of HP0166 and HP1043 from Ni^{2+} NTA columns can be seen in Figures 25A and 25B.

After injection into mice, sera samples were taken periodically (every 2-3 weeks) until immunoblots displayed bands of proper sizes. Immunoblots with the final antisera isolation are shown in Figure 26. As seen with antisera to HP0166 (Figure 26A), it recognized purified 10x His-HP0166 (lane A) but not 10x His-HP1043 (lane B). Additionally, a single band was evident in crude lysates of *H. pylori* G27, *H. pylori* SS1 and *H. pylori* X47 (lanes C, D and E respectively). It should be noted that there appears to be a smaller product in lane A (purified 10x His-HP0166), perhaps a degradation product. Similarly with antisera towards HP1043 (Figure 26B), it did not recognized purified 10x His-HP0166 (lane A), but was bound to purified 10x His-HP1043 (lane B) as well as the crude lysates of *H. pylori* G27, *H. pylori* SS1 and *H. pylori* X47 (lanes C, D and E respectively). Lane B of Figure 26B also developed other bands that could be degradation products or protein aggregates.

4.3.1.1 Antisense-*hp0166* (AS0166) in *H. pylori*

Since we now had a way to determine protein levels of HP0166, an antisense-*hp0166* was designed to use the strong P_{ureA} promoter, an antisense to ~75% of the coding region of *hp0166* including the upstream untranslated region and the *fd*-terminator. The construct was called AS0166. The antisense construct was integrated into the *rdxA* locus of *H. pylori* SS1 using the pRDX-K+ vector. Resulting clones were grown up and

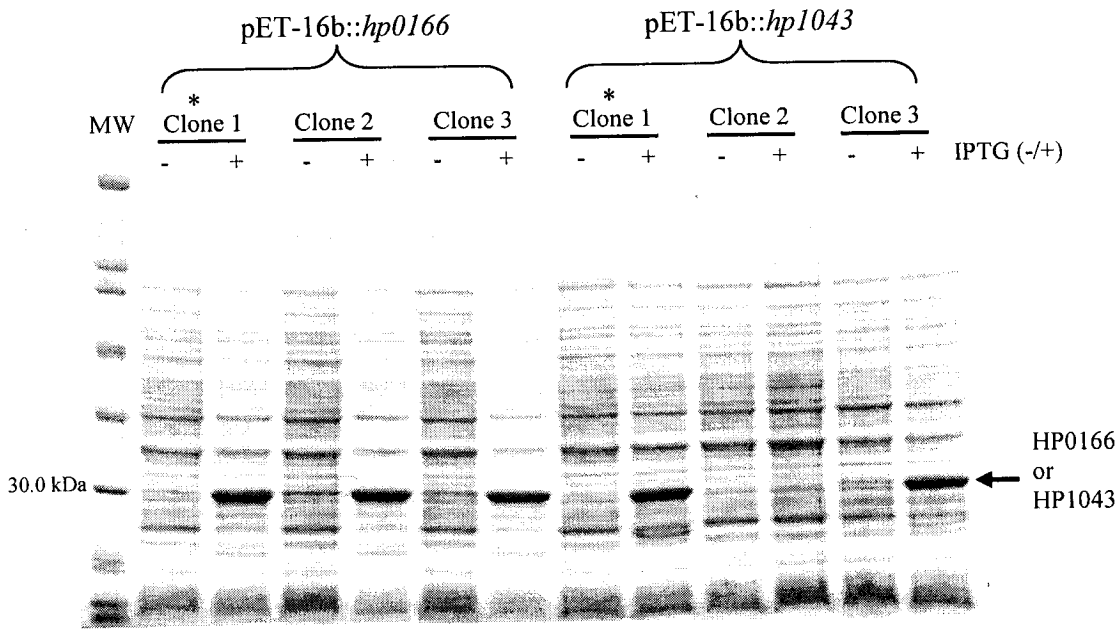
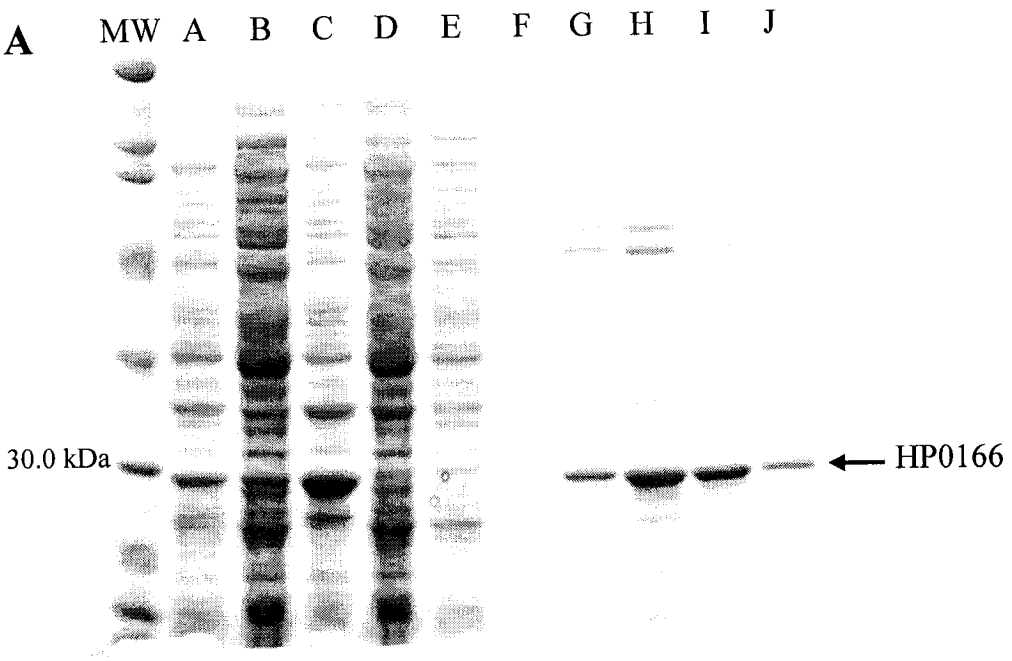


Figure 24. Over expression of *hp0166* and *hp1043* in *E. coli*. Three random clones of *E. coli* BL21 CodonPlus®(DE3)-RIL containing either pET-16b::*hp0166* or pET-16b::*hp1043* were grown to mid-log phase (OD_{600} ~0.6) and induced with 1 mM IPTG for 2 hours. The cultures were then lysed in LDS and boiled before run on an SDS-PAGE gel. The absence and presence of IPTG is indicated in each column with a – or + respectively. MW, molecular mass standard. HP0166 or HP1043 over expression is represented by an arrow. The asterisks represent the clones that were used for protein purification.

Figure 25. Purification of HP0166 and HP1043. After induction and over expression of (A) *hp0166* and (B) *hp1043* in *E. coli* BL21 CodonPlus®(DE3)-RIL with 1 mM IPTG the culture lysates were put through the Qiagen Ni^{2+}-NTA kit. MW, molecular weight standard; lane A, induced cell culture; lane B, bacterial pellet after lysis; lane C, bacterial supernatant after lysis; lane D, filtrate passed through Ni^{2+}-NTA column; lane E, column wash #1; lane F, column wash #2; lane G, wash #3; lane H, elution fraction #1; lane I; elution fraction #2; lane J, elution fraction #3. HP0166 or HP1043 are indicated by an arrow. Since there was a presence of high and low molecular weight contamination in the eluted fractions, the eluted fractions were pooled and dialyzed to remove the imidazole. Following dialysis, the fractions were subjected to the Ni^{2+}-NTA column again except that the three washes used 20, 40 and 60 mM imidazole respectively (data not shown).


A
MW A B C D E F G H I J
30.0 kDa
HP0166

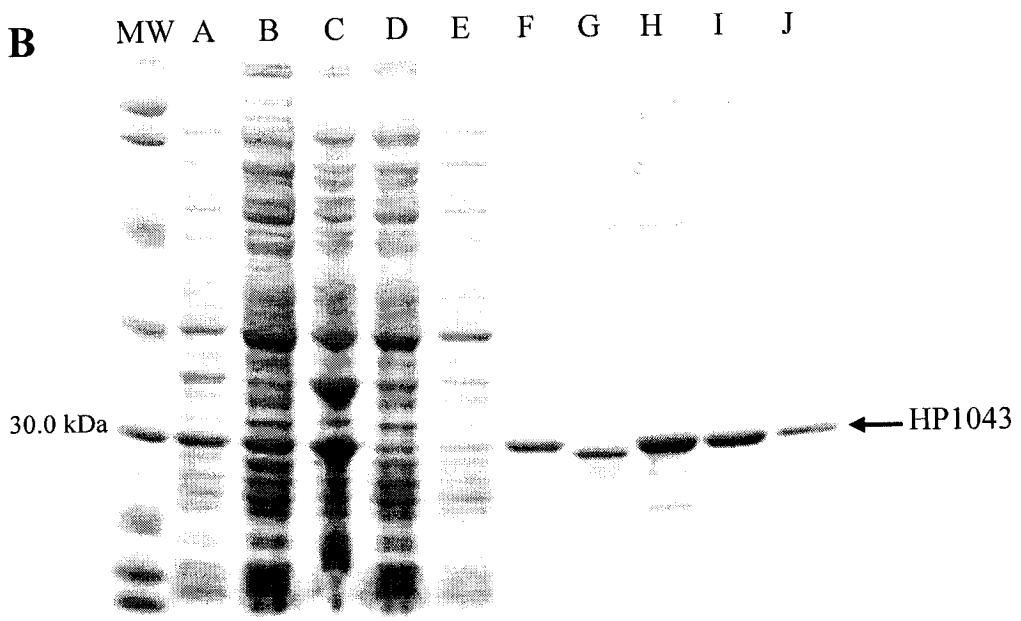
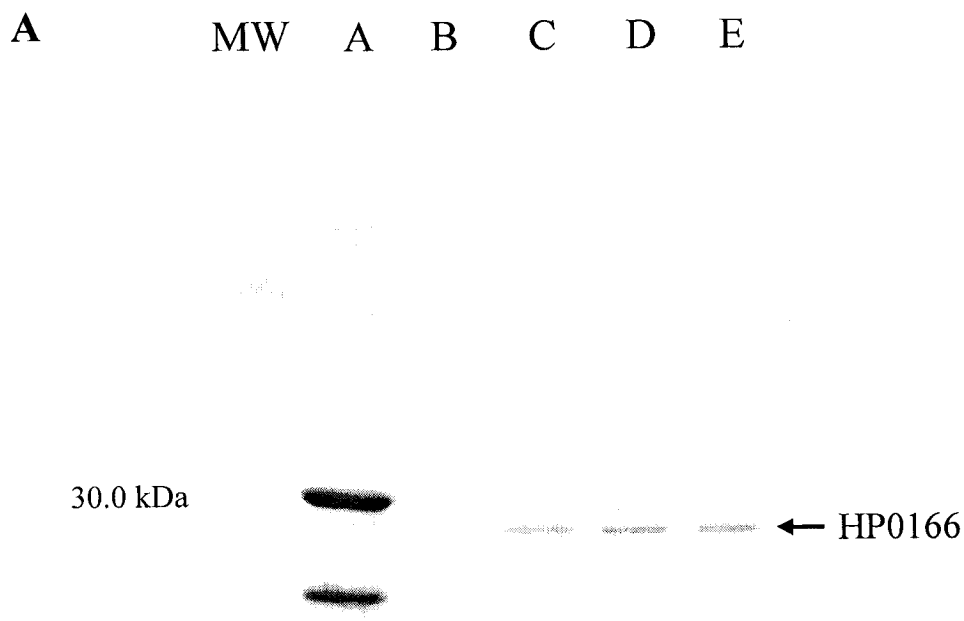
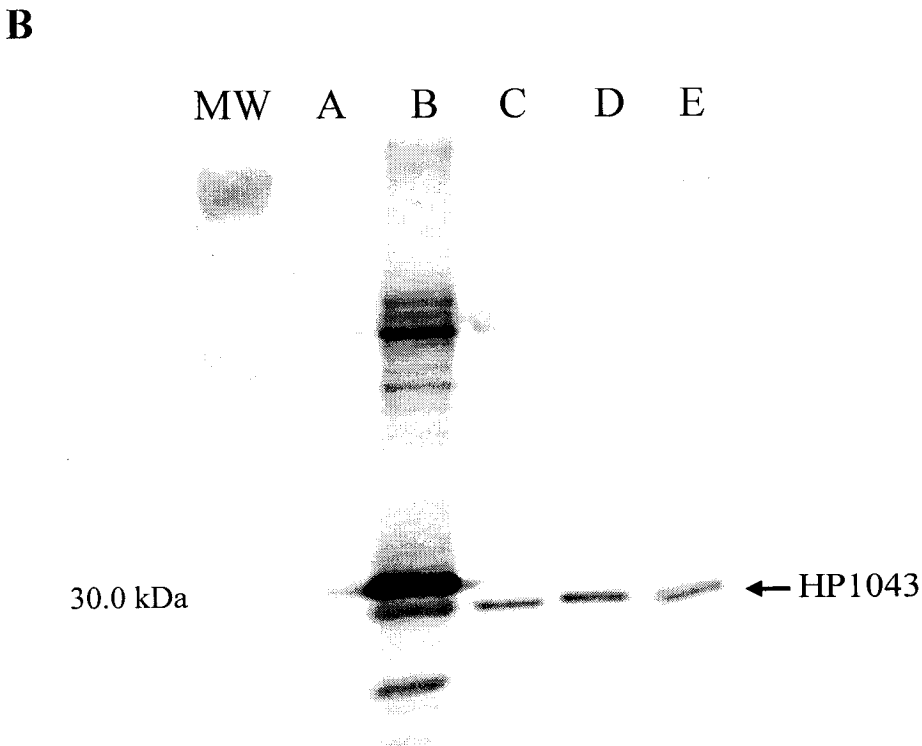

B
MW A B C D E F G H I J
30.0 kDa
HP1043

Figure 26. Polyclonal Antisera Against HP0166 and HP1043. Polyclonal antisera was raised in mice towards (A) HP0166 and (B) HP1043 and were tested with immunoblots. MW, molecular mass standard; lane A, purified HP0166; lane B, purified HP1043; lane C, *H. pylori* G27 wild type lysate; lane D, *H. pylori* SS1 wild type lysate; lane E, *H. pylori* X47 wild type lysate. HP0166 and HP1043 are indicated by arrows. Antisera towards HP0166 developed a single band in the three *H. pylori* lysates, the purified protein but not purified HP1043. Conversely, antisera towards HP1043 developed a single band in the three *H. pylori* lysates, purified HP1043 but not HP0166.


A
MW A B C D E
30.0 kDa
HP0166


B
MW A B C D E
30.0 kDa
HP1043

subjected to SDS-PAGE and immunoblotting with the polyclonal HP0166 antiserum (Figure 27). The upper panel demonstrates protein loading levels of each extract after Ponceau S staining. The lower panel shows the protein levels of HP0166 for the given colony extract. Although based on densitometry measurements, all clones had a knocked down level of HP0166, *H. pylori* SS1 *rdxA*::AS0166 clone #4 (indicated with an asterisks; herein referred to as *H. pylori* SS1 *rdxA*::AS0166) had the greatest knockdown (~40%) compared to *H. pylori* SS1 wild type (lane A) and the *H. pylori* SS1 *rdxA*::*aphA3* control (lane B).

4.3.1.2 Knockdown of HP0166 Does Not Enfeeble Growth

To ensure that knocked down levels of HP0166 does not alter in vitro growth of *H. pylori* we measured the optical density of a culture over a 32 hour period. As seen in Figure 28, there are no obvious differences in the growth of *H. pylori* SS1 wild type, *H. pylori* SS1 *rdxA*::*aphA3* or *H. pylori* SS1 *rdxA*::AS0166. It was reported that HP0165-null (ArsS) mutants were impaired for growth at pH 5.0 when compared to the wild type strain (Loh and Cover, 2006). However, attempts to compare growth rates of *H. pylori* SS1 wild type, *H. pylori* SS1 *rdxA*::*aphA3* and *H. pylori* SS1 *rdxA*::AS0166 at pH 5.0 were unsuccessful because the growth could not be supported for any of the strains (data not shown). Therefore, neither knocked down levels of HP0166 by antisense nor the kanamycin cassette (*aphA3*) altered in vitro growth of *H. pylori* under normal culture pH.

4.3.1.3 Knockdown of HP0166 Does Not Make *H. pylori* More Sensitive to Acid

Since we saw a knockdown of approximately 40% of HP0166 in *H. pylori* SS1 *rdxA*::AS0166, we wished to examine if there was any phenotype associated with the

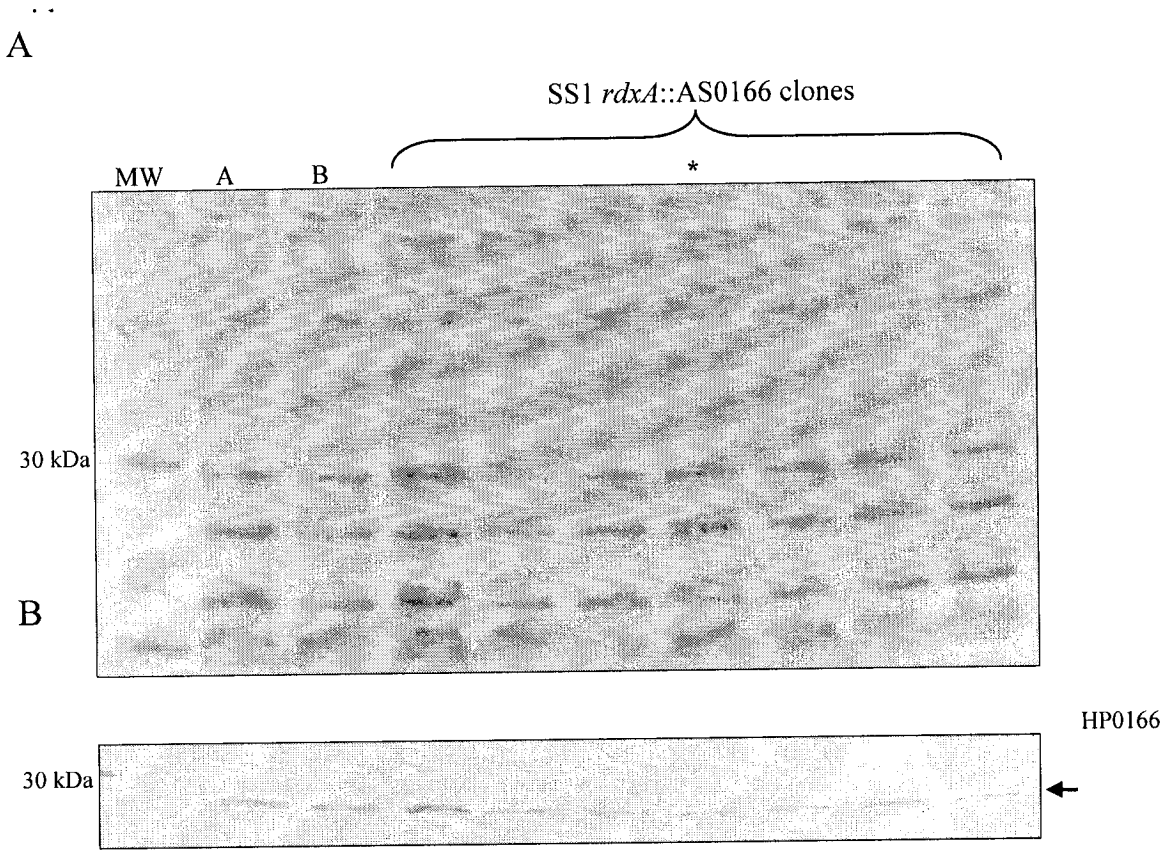


Figure 27. AS0166 in *H. pylori* SS1. MW, molecular weight standard; lane A, *H. pylori* SS1 wild type; lane B, *H. pylori* SS1 *rdxA::aphA3* control; remaining seven lanes; various *H. pylori* SS1 *rdxA*::AS0166 colonies. (A) Ponceau S staining of the nitrocellulose membrane indicating protein load. (B) Nitrocellulose membrane immunostained with antisera towards HP0166. *H. pylori* SS1 *rdxA*::AS0166 clone #4 (represented by an asterisk) was used for further studies. HP0166 is indicated by an arrow.

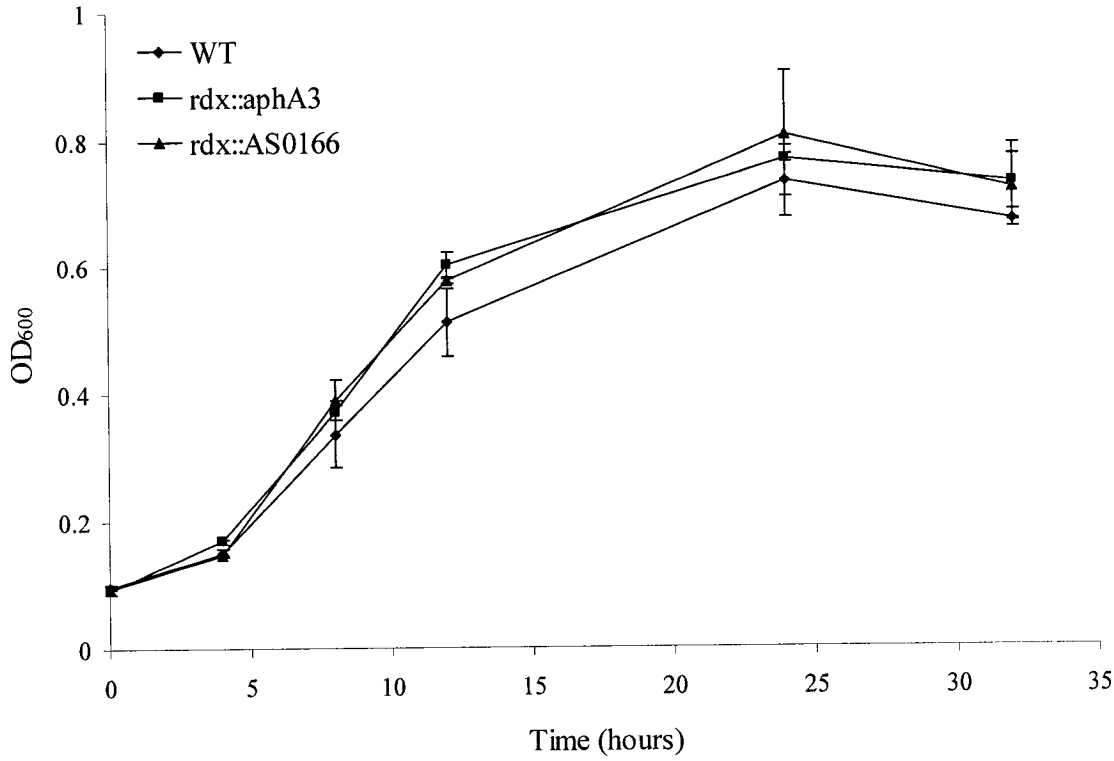


Figure 28. In Vitro Growth of *H. pylori* SS1. *H. pylori* SS1 wild type (♦), *H. pylori* SS1 *rdxA::aphA3* (■) and *H. pylori* SS1 *rdxA*::AS0166 (▲) was seeded with an OD_{600} ~ 0.1 into fresh brucella broth and OD_{600} was measured at 4, 8, 12, 24 and 32 hours. The averages of three growth curves are shown with ± standard deviations.

reduced levels of HP0166. We wondered if *H. pylori* survival during exposure to acid would be altered in the presence of urea. *H. pylori* SS1 wild type, *H. pylori* SS1 *rdxA*::*aphA3* and *H. pylori* SS1 *rdxA*::AS0166 were exposed to sterile PBS with a pH adjusted to 7.0 or 3.0 in the absence or presence of 5 mM urea for 1 hour (Table 8). There was no change in acid sensitivity between the antisense harboring strain when compared to the wild type and control strains. This data suggest that knockdown levels of HP0166 do not affect the ability of *H. pylori* to protect itself from acid in the presence of urea.

4.3.1.4 Knockdown of HP0166 Does Not Alter Protein Profiles

Although we lacked any phenotype for the knocked down levels of HP0166, we asked if there were any major changes in the protein profiles. *H. pylori* SS1 wild type, *H. pylori* SS1 *rdxA*::*aphA3* and *H. pylori* SS1 *rdxA*::AS0166 was exposed to brucella medium with a pH of 7.0 and 5.0 for 1 hour before being lysed and run on a two-dimensional gel (Figure 29). No obvious changes were noted when comparing the protein profiles of the wild type and control to the antisense-*hp0166* harboring strain. It was however observed that one spot (spot #2) that was absent in both treatments of *H. pylori* SS1 *rdxA*::*aphA3* control and *H. pylori* SS1 *rdxA*::AS0166 but present in *H. pylori* SS1 wild type. The circled spots were subjected to mass spectrometry. Spot #1, which is present in all strains under all treatment conditions and close to the spot of interest (spot #2), was used as a control to ensure that the proper the proper location of the spots were excised to be analyzed. Mass spectrometry analysis identified two major proteins in spot #1 for all treatments as a putative outer membrane protein, Omp11 (*hp0472*), an unknown hypothetical (*hp0596*) and the adhesin-thiol peroxidase, TagD (*hp0390*).

Table 8. Acid Sensitivity of *H. pylori* SS1 and its AS0166 Harboring Derivative

Strain	**pH 7.0**	**pH 7.0 + 5 mM urea**	**pH 3.0**	**pH 3.0 + 5 mM urea**
SS1 WT	$2.61 \times 10^8 \pm 0.58 \times 10^8$	$2.55 \times 10^8 \pm 0.16 \times 10^8$	$1.96 \times 10^8 \pm 0.22 \times 10^8$	$2.31 \times 10^8 \pm 0.28 \times 10^8$
SS1 *rdxA*::*aphA3*	$3.01 \times 10^8 \pm 0.66 \times 10^8$	$2.93 \times 10^8 \pm 0.07 \times 10^8$	$1.73 \times 10^8 \pm 0.15 \times 10^8$	$2.41 \times 10^8 \pm 0.11 \times 10^8$
SS1 *rdxA*::AS0166K	$2.90 \times 10^8 \pm 0.24 \times 10^8$	$2.61 \times 10^8 \pm 0.19 \times 10^8$	$1.89 \times 10^8 \pm 0.09 \times 10^8$	$2.43 \times 10^8 \pm 0.1 \times 10^8$

H. pylori SS1 wild type, *H. pylori* SS1 *rdxA*::*aphA3* and *H. pylori* SS1 *rdxA*::AS0166 were challenged in PBS (pH 7.0 and pH 3.0) in the presence and absence of 5 mM urea for 1 hour.

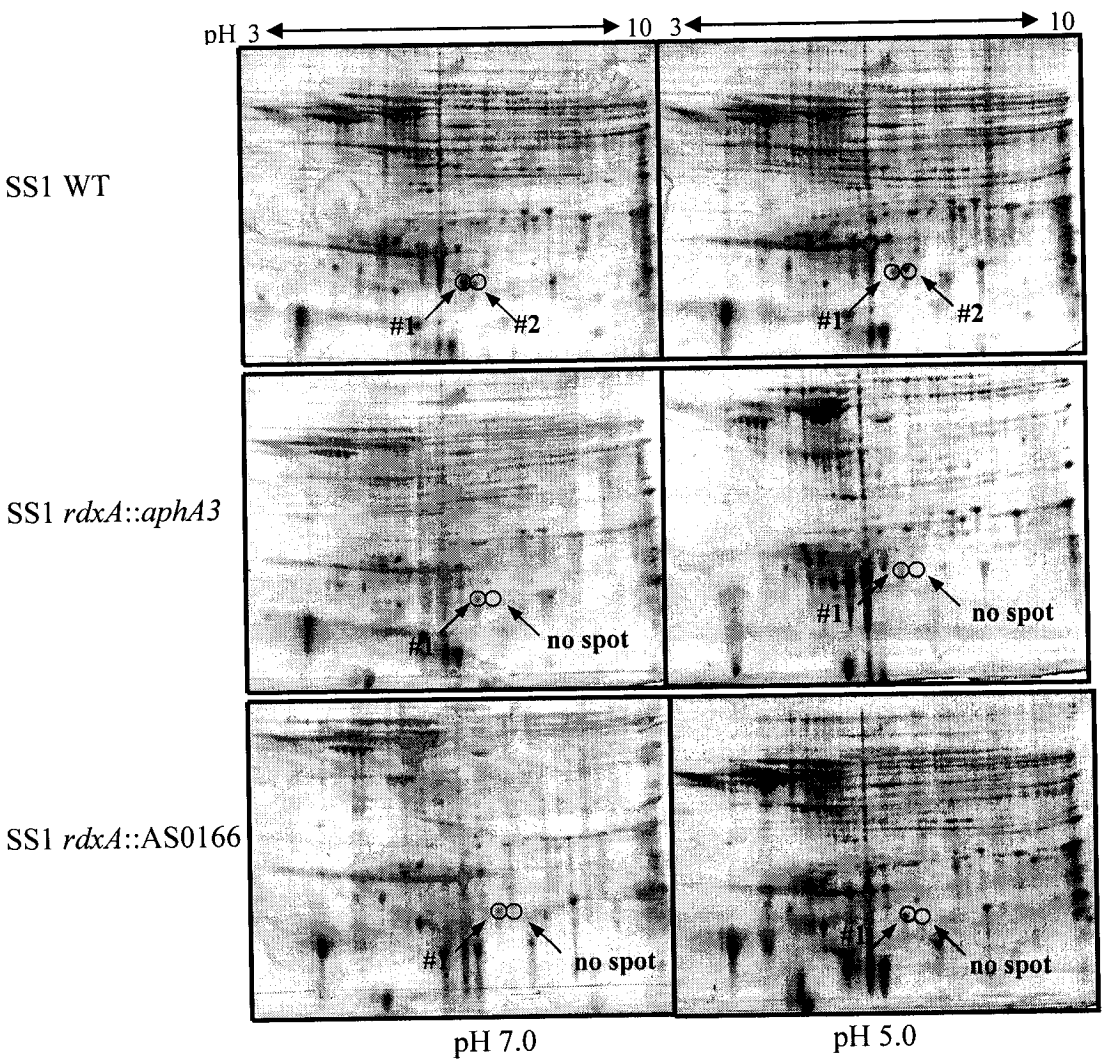
Unfortunately, the mass spectrometry results of spot #2 (only found on the wild type gels) identified only TagD suggesting that there may be some cross contamination during excision as TagD was also identified from spot #1. Originally, since the kanamycin cassette (*aphA3*) or the AS0166 antisense replaced the *rdxA*, the missing spot (#2) was predicted to be *rdxA*. Additionally, *rdxA* has a molecular weight of ~23 kDa and a pI of 7.8. All of the spots identified have molecular weights of 18 kDa (TagD; pI = 7.8), 21 kDa (Omp11; pI = 9.3) or 22 kDa (HP0596; pI = 7.9).

4.3.2 Attempts at Antisense-*hp1043* (AS1043) in *H. pylori*

An antisense construct similar to AS0166 was designed to knockdown HP1043 levels. The P_{ureA} promoter was used to drive expression of a 350 bp (~60% of the length of *hp1043*) antisense terminated by the *fd*-terminator and designated AS1043. The final construct was introduced into the *rdxA* gene of *H. pylori* SS1 via the pRDX-K+ suicide vector. *H. pylori* SS1 was chosen because it was widely used in our studies of the MCPs and pH taxis. Resulting colonies were expanded and crude lysates were run on an SDS-PAGE and subjected to immunoblotting with the polyclonal HP1043 antisera (Figure 30). The top panel shows loading of each extract after staining with Ponceau S. The lower panel is the immunoblot showing the individual HP1043 protein levels. It is evident that *H. pylori* SS1 wild type (lane A), *H. pylori* SS1 *rdxA*::*aphA3* control (lane B) and the four *H. pylori* SS1 *rdxA*::AS1043 clones have equal levels of HP1043. Several more colonies were obtained with subsequent transformations resulting in the same result.

Reports from Dr. Scarlato's group had attempted to over express *hp1043* in *H. pylori* could show an increase in *hp1043* mRNA but no alterations in HP1043 protein levels (Delany *et al.*, 2002). We thought that perhaps using the native P_{hp1043} promoter

Figure 29. Two-Dimensional Gel Electrophoresis of *H. pylori* SS1 *rdxA*::AS0166. *H. pylori* SS1 wild type, *H. pylori* SS1 *rdxA*::*aphA3* and *H. pylori* SS1 *rdxA*::AS0166 were exposed to brucella broth at pH 7.0 and pH 5.0 for one hour. Following lysis, protein concentratins were determined and equal amounts of protein were loaded for each lysate. Left column is the extracts treated at pH 7.0 and the right column the extracts treated at pH 5.0. The top row is *H. pylori* SS1 wild type; middle row *H. pylori* SS1 *rdxA*::*aphA3* and; bottom row *H. pylori* SS1 *rdxA*::AS0166. Spot #1 is indicated on all gels and was used as a marker/control for mass spectrometery. Spot #2 is only found in *H. pylori* SS1 wild type under both conditions but is missing in all conditions with *H. pylori* SS1 *rdxA*::*aphA3* or *H. pylori* SS1 *rdxA*::AS0166. Spot #1 and Spot #2 were excised and subjected to mass spectrometery. No other changes in protein profile were obvious.


pH 3
10
3
10
SS1 WT
#1
#2
#1
#2
SS1 rdxA::aphA3
#1
no spot
#1
no spot
SS1 rdxA::AS0166
#1
no spot
#1
no spot
pH 7.0
pH 5.0

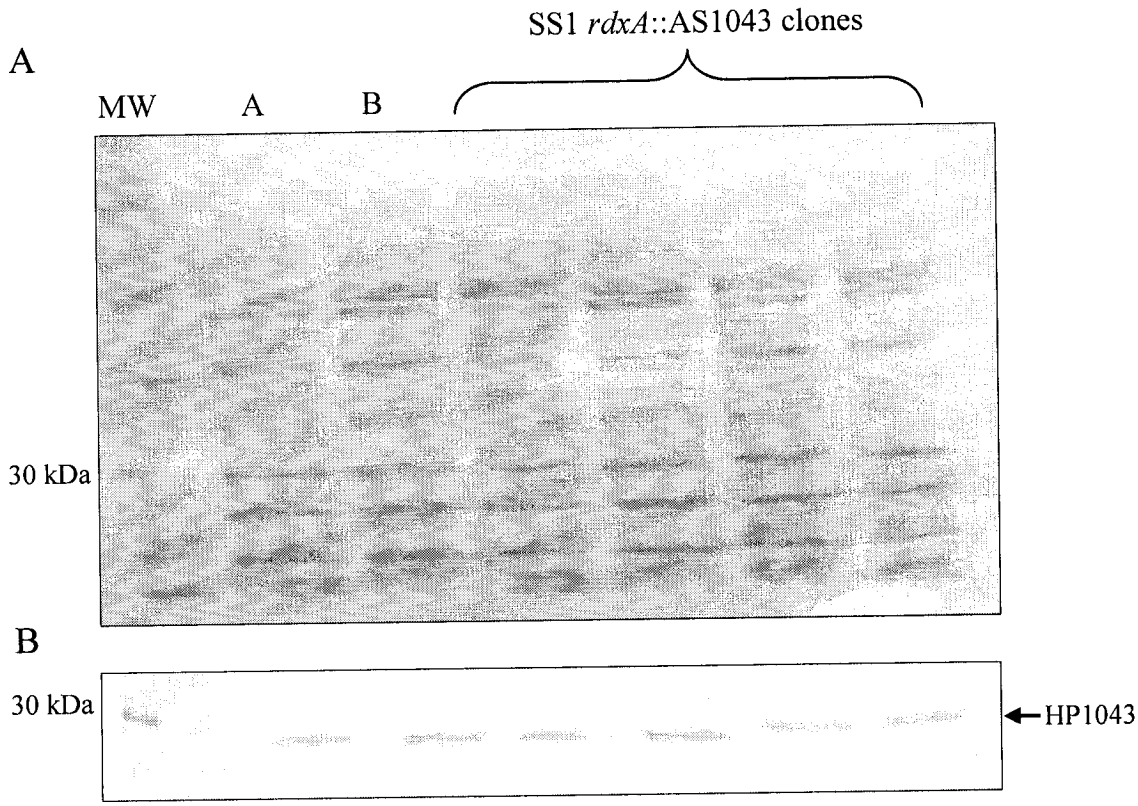


Figure 30. AS0143 in *H. pylori* SS1. Representative immunoblot of various attempts at incorporating antisense-*hp1043* into *H. pylori* SS1 (AS1043, AS1043-2, FAS1043 and FAS1043-2). MW, molecular weight standard; lane A, *H. pylori* SS1 wild type; lane B, *H. pylori* SS1 *rdxA*::*aphA3* control; remaining four lanes; various *H. pylori* SS1 *rdxA*::AS1043 colonies. (A) Ponceau S staining of the nitrocellulose membrane indicating protein load. (B) Nitrocellulose membrane immunostained with antisera towards HP1043. HP1043 is indicated by an arrow.

would allow for similar regulation of the sense *hp1043* message as well as the antisense-*hp1043* message and therefore have an overall knockdown of the protein. The P_{ureA} promoter was switched with the P_{hp1043} promoter, designated AS1043-2, and reintroduced into the chromosome of *H. pylori* SS1. Protein levels of HP1043 as judged by immunoblot were not altered (data not shown). We then created a new set of AS1043 antisenses with a full length ~600 bp antisense with either the P_{ureA} promoter (FAS1043) or P_{hp1043} promoter (FAS1043-2) and replaced the chromosomal copy of *rdxA* with the new constructs. Again, immunoblots showed that protein levels of HP1043 were not altered (data not shown).

Previous work showed that best antisense knockdowns of AhpC were obtained when introduced on a multicopy and in full length. Since single copy antisense was not able to knockdown HP1043 levels, we subcloned the FAS1043 and FAS1043-2 antisense constructs into the multicopy shuttle vector pHel3 (gift from Dr. Rainer Haas; Heuermann and Haas, 1998) yielding pHel3FAS1043 and pHel3FAS1043-2. These were introduced into *H. pylori* 1061, a strain permissive to the plasmids. Immunoblots on the crude lysates are shown in Figure 31 using pHel3FAS1043-2 as an example. The upper panel shows the loading levels of each extract by Ponceau S staining. The lower panel shows HP1043 following immunoblotting with polyclonal antiserum raised against HP1043. There are no differences in HP1043 protein levels between *H. pylori* 1061 WT (lane A), *H. pylori* 1061 pHel3 control (lane B) and the *H. pylori* 1061 pHel3FAS1043-2 clones. The same results were seen with the pHel3FAS1043 clones.

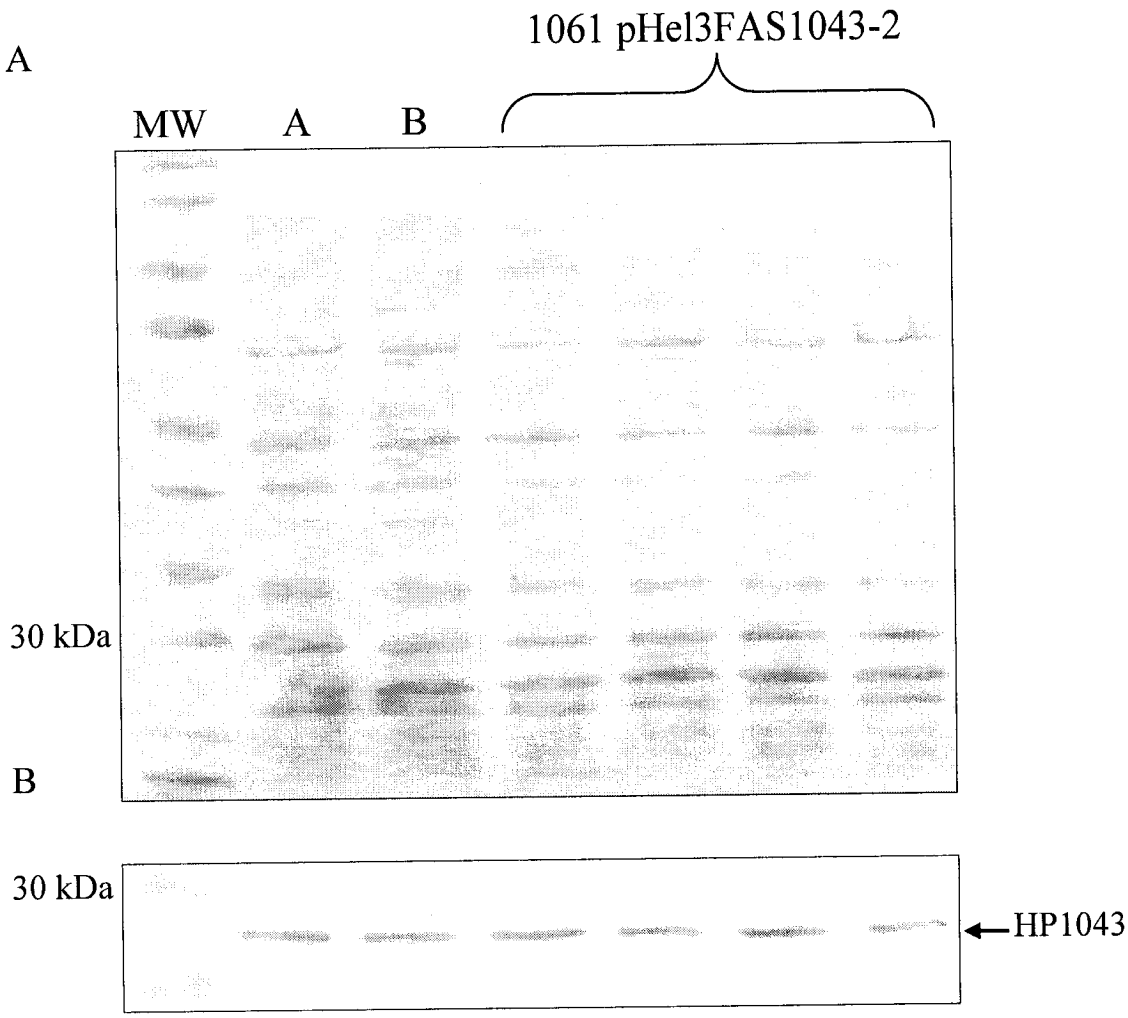


Figure 31. FAS0143-2 in *H. pylori* 1061. Representative immunoblot of plasmid based attempts of antisense-*hp1043* into *H. pylori* 1061 (pHel3FAS1043 and pHel3FAS1043-2). MW, molecular weight standard; lane A, *H. pylori* 1061 wild type; lane B, *H. pylori* 1061 pHel3 control; remaining four lanes; various *H. pylori* 1061 pHel3FAS1043-2 colonies. (A) Ponceau S staining of the nitrocellulose membrane indicating protein load. (B) Nitrocellulose membrane immunostained with antisera towards HP1043. HP1043 is indicated by an arrow.

Attempts in knocking down protein levels of HP1043 were unsuccessful. The use of four different constructs; two with a shorter (60%) length and two full length both driven by either the strong P_{ureA} promoter or the native P_{hp1043} promoter, showed no difference in wild type and *rdxA*::*aphA3* control in HP1043 levels when in single copy. Additionally, two multicopy plasmids carrying both full length antisense-*hp1043*s were unable to alter HP1043 levels.

Chapter 5: Discussion

In order to establish a chronic infection, *H. pylori* must first survive the acidic lumen of the stomach, swim through the gastric mucus to the underlying epithelium and evoke an inflammatory response in order to obtain growth promoting nutrients (Rautelin *et al.*, 1994). *H. pylori* is a remarkable organism because with such a small genome it is able to survive in a niche that is inhabitable by any other organism. As such, *H. pylori* must employ unique mechanisms to cope with both the stomach acidity and exposure to oxidative stress conferred by chronic inflammation (Pflock *et al.*, 2004; Pflock *et al.*, 2006a; Sachs *et al.*, 2005; Wang *et al.*, 2006a). We initiated this study to address both the role of pH sensing and the importance of the oxidative stress response in the colonization of the gastric mucosa by *H. pylori.*

Herein, we demonstrated that *H. pylori* has the ability to sense acid and swim away from acid in vitro. We further identified a chemoreceptor involved in this pH tactic behavior and showed its importance for colonization of mice. The second chapter of this dissertation dealt with development of molecular technologies to assess the relative role of AhpC in protecting bacteria from peroxides generated by oxidative stress. We developed an antisense RNA interference method that can be used as a way forward in the study of essential genes in *H. pylori.* Varying levels of AhpC knockdowns correlated with the degree of sensitivity to organic hydroperoxides. Moreover, in vivo mouse studies suggest that AhpC may not be an important factor for colonization suggesting that oxidative stress may not be a major barrier for initial colonization. The antisense RNA application was broadened in an attempt to knockdown levels of ArsR (HP0166) and HP1043, two essential two-component regulators thought to be involved in acid

resistance and potentially pH taxis, respectively. Although knockdowns were achieved for ArsR, no phenotype could be associated with decrease in protein level. Despite several constructs and attempts, no change in HP1043 levels could be detected.

5.1 pH Tactic Responses in *H. pylori*

H. pylori can be found within the first 25 μm of the mucus layer. The majority are swimming around the mucus while the rest are adhered to the gastric epithelium (Schreiber *et al.*, 2004). Within the mucus layer, three chemical gradients are believed to exist: (i) a urea/ammonia gradient; (ii) a bicarbonate/CO_2 gradient and; (iii) a pH gradient (Schreiber *et al.*, 2005). Disruptions of the first two gradients using peritoneal diffusion had no affect on the spatial orientation of *H. pylori* in the mucus layer of gerbils. However, disruption of pH gradient resulted in a random distribution of *H. pylori* throughout the whole mucus layer (predicted to be ~100 μm thick). These findings suggested that *H. pylori* senses pH gradients for proper orientation and establishment of infection in the gastric mucosa.

We further expanded this hypothesis by showing that motile wild type *H. pylori* was able to increase its motility in response to acid and collectively move away to a less acidic area. This establishes an important behavior during the colonization process. It can therefore be envisioned that the pH tactic behavior of *H. pylori* is utilized to swim away from the low pH of the gastric lumen to colonize the mucosa, where the pH is thought to be neutral. Moreover, pH tactic responses may also prevent *H. pylori* from randomly swimming back into the acidic lumen and therefore remains consistent with the spatial orientation found by Schreiber *et al.* (Schreiber *et al.*, 2005). Our findings that *H. pylori* forms a wall may just be an in vitro artifact. However, the sedentary state of the *H. pylori*

once it has escaped the pH may provide time for other functions required for establishment of infection such as adhesion (e.g. BabA) which may be important in initial colonization and can evoke an inflammatory response (Ilver *et al.*, 1998; Rad *et al.*, 2002) or the delivery of effector molecules such as VacA or CagA. From here, the incurred inflammation provides *H. pylori* with essential nutrients for growth.

Tactic responses in bacteria are sensed by MCPs. *H. pylori* contains four MCP orthologs; three of which are classical transmembrane proteins (*tlpA, tlpB* and *tlpC*) and a fourth that lacks a transmembrane domain (*tlpB*) (Tomb *et al.*, 1997). All four were systematically and successfully knocked out with a PCR based allelic replacement strategy (Chalker *et al.*, 2001). All mutants were motile and chemotactic as based on a brucella-based soft agar assay, although Δ*tlpB* and Δ*tlpD* demonstrated a reduced swarming phenotype. The swimming behaviors of each mutant, except Δ*tlpA* and Δ*tlpB,* differed from wild type as hypermotility was observed in Δ*tlpC* while excessive tumbling in Δ*tlpD*. Increased motility rates were observed when wild type *H. pylori* was exposed to acid as previously reported (Merrell *et al.*, 2003a); however, our calculated rates were still 4-fold less than Δ*tlpC*. To our knowledge, a hypermotile phenotype has not been observed in any of the MCP mutants in any other bacteria. Similarly, the observations that Δ*tlpD* excessively tumbles has not been reported for any other MCP. A tumbling phenotype is typically seen in *cheZ* and *cheB* mutants of *E. coli* and *B. subtilis* as the former is unable to enhance CheY~P dephosphorylation and the latter can not demethylate MCPs (adaptive response) (Parkinson, 1978; Stock and Koshland, 1978). Soluble MCPs have been identified in *Caulobacter crescentus, Rhodobacter sphaeroides* and *Pseudomonas aeruginosa* (Bardy and Maddock, 2005; Potocka *et al.*, 2002;

Wadhams *et al.*, 2002). Although no function has been assigned to any of the studied soluble MCPs, it was suggested that McpB of *C. crescentus* may be involved in aerotaxis as it displays some similarity to HemAT of *B. subtilis* (Potocka *et al.*, 2002). We could not demonstrate aerotaxis in *H. pylori.* Unlike *C. jejuni* which displays aerotaxis (Hazeleger *et al.*, 1998), thought to be mediated through CetAB (Hendrixson *et al.*, 2001), *H. pylori* does not have a homolog of the transmembrane receptor, CetA, or the soluble, PAS containing CetB.

During exposure to acid, Δ*tlpA* did not show any differences than wild type (increased motility with arcing). Although the behavior of Δ*tlpC* motility changed from random to arcing (like wild type), no change in the hypermotility was observed. Conversely, Δ*tlpD* demonstrated a decrease in motility, but still retained the arcing phenotype. The Δ*tlpA*, Δ*tlpC* and Δ*tlpD* mutants all demonstrated pH tactic behavior and wall formation was observed (albeit at a slower rate with Δ*tlpD*). Tactic behavior was; however, abrogated in a Δ*tlpB* mutant. During exposure to acid, no changes in swimming behavior were observed (random) and there was no collective movement of the *H. pylori* population away from the acid. After time, this resulted in coccoid bodies. Moreover, mouse colonization studies demonstrated that only Δ*tlpB* was defective in colonizing mouse stomachs. Since complementation studies of *tlpB* restored pH taxis, wild type soft agar chemotaxis and mouse colonization, it is therefore suggested that TlpB may be an important factor in pH taxis and colonization.

We attempted to express *tlpB* in *P. mirabilis,* an organism that does not display any tactic behavior (positive or negative) towards pH (Williams *et al.*, 1976). During a pH taxis assay, we could not demonstrate the ability to sense and respond to acid. It is

possible that the chemotaxis network found in *P. mirabilis* differs from that found in *H. pylori*. Secondly, since *tlpB* was placed under the P_{lac} promoter of the high copy pUC18, it is possible that the over expression of the MCP may have altered all tactic behavior in this organisms, a phenotype that could have been checked in a soft agar chemotaxis assay. This phenomenon was reported in *E. coli* as over expression of *tsr* and *tar* inhibited chemotactic responses (Liu and Parkinson, 1989). It is thought that over expression shifts the stoichiometric balance between receptors and trandsducers (CheW and CheA) and nullifies signal transduction. Therefore, expression levels of *tlpB* may need to be optimized (e.g. single-copy plasmid). In addition, we could not demonstrate pH tactic behavior in *C. jejuni* or *H. hepaticus*, two non-gastric colonizers. Interestingly, a genome search of these two organisms reveals that there are no homlogs of *tlpB* (Parkhill *et al.*, 2000; Suerbaum *et al.*, 2003). Moreover, homologs of *tlpB* can be found in two other gastric *Helicobacters*: *H. mustelae* (The Wellcome Trust Sanger Institute; http://www.sanger.ac.uk/Projects/H_mustelae/) and *H. acinonychis* (Eppinger *et al.*, 2006). Given the particular niches of these organisms, it is possible that TlpB is important for pH taxis for gastric colonization. The ability of *H. mustelae* and *H. acinonychis* to be pH tactic has not been explored, but an avenue worth pursing. Additionally, it would be of interest to clone and express *tlpB* from *H. pylori* into *C. jejuni* and *H. hepaticus* to determine if they gain a pH tactic response and the ability to colonize the gastric mucosa of mice. The latter may be more difficult do demonstrate, particularly with *C. jejuni* which lacks urease (Parkhill *et al.*, 2000). Also, both *C. jejuni* and *H. hepaticus* contain 10 and 8 MCPs (Parkhill *et al.*, 2000; Suerbaum *et al.*, 2003), respectively that may interfere or complex TlpB function.

Although *H. pylori* has been reported to be chemotactic towards other stimulants such as bicarbonate and urea (Mizote *et al.*, 1997) we suspect that the pH tactic response may be the dominant response that permits survival of *H. pylori.* Chemotactic responses to urea and bicarbonate may be more relevant once *H. pylori* has established colonization in the gastric mucosa and may be involved in keeping *H. pylori* closer to sources of nutrients or substrates required for acid survival over a persistence. Evidence to support this theory is that pH taxis may be the dominant response is that mutants in *tlpA* (taxis towards arginine and bicarbonate; Cerda *et al.*, 2003) had no effect on colonization of mice or gerbils (Andermann *et al.*, 2002). Therefore, since Δ*tlpB* mutants are pH blind and defective for mouse colonization, it could be reasoned that pH taxis is an important function required for initial colonization.

Current literature suggests that acid regulated gene expression and acid acclimation (e.g. urease) are important factors in colonization of the stomach (Merrell *et al.*, 2003a; Pflock *et al.*, 2006a; Wen *et al.*, 2003). While the acid response is unquestionably important in acid survival (Loh and Cover, 2006; Panthel *et al.*, 2003), the pH tactic response is probably more important in the establishment of colonization. While the urease system enables pH stasis for *H. pylori,* it is unlikely that much transcriptional activation of gene expression would occur under these extreme acid conditions. Moreover, it is unlikely that biosynthetic activities, such as DNA replication, protein synthesis, or cell division would occur during periods of extreme acid exposure. During the early colonization period we suggest that motility, pH taxis, and pH stasis mechanisms are crucial. As a comparison, enteric bacteria, a species that a doubling time nearly 6-fold faster than *H. pylori,* are unable to survive an acid shock without an initial

adaptation period (Lin *et al.*, 1995). This adaptation period (one doubling time) is sufficient for upregulation of acid shock genes (Foster, 1991) and subsequent survival in acid. Moreover, the energy required to quickly transcribe and translate new proteins would seem better utilized for increased motility, a phenotype that we and others observed (Merrell *et al.*, 2003a). Since the time required to mount a response to acid in *H. pylori* seems unlikely during initial colonization, pH tactic responses may provide a mechanism in finding a favorable niche before *H. pylori* succumbs to the acidity of the gastric lumen. In addition, pH taxis is probably also important in keeping *H. pylori* in the crypts of the gastric mucosa. In such a view, this would serve to keep *H. pylori* away from the acidity of the stomach and also serve to avoid washout during mucus turnover.

Although TlpB is probably involved in pH taxis, it cannot be ruled out as the sole contributor. Our report that *tlpB* mutants do not colonize mice and are not chemotactic is in contrast with another report that showed that *tlpB* mutants could colonize the stomachs of gerbils (McGee *et al.*, 2005). Although pH taxis was not studied, we obtained their strain and determined it to be genetically altered at the *tlpB* locus. We demonstrated that it was pH tactic (albeit motility was lower than our strain) in our slide-based assay. In this regard, it is probable that an unidentified second site mutation, or a gene duplication, which is not uncommon in *H. pylori* (Chalker *et al.*, 2001) might produce these results. In our studies, we used a PCR-based method to create the mutant amplicon and used multiple occasions to independently produce *tlpB* mutants that exhibited the same phenotype. Furthermore, allelic complementation of *tlpB* in the *rdxA* gene restored the pH tactic behavior and infectivity for mice.

Further characterization of TlpB and the other Tlp proteins is required. It is now becoming evident that MCPs are clustered together at the cell poles form mixed trimers of dimers in vivo (Studdert and Parkinson, 2004) and therefore may act cooperatively in signal transduction. Therefore, the roles of TlpA, TlpC and TlpD in pH tactic responses should not be excluded. The overall abundance of each MCP will also need to be addressed and may provide insight on the importance of certain receptors over others. Double, triple and possibly quadruple systematic knockouts of each MCP may have to be performed. Typically the chloramphenicol (*cat*) and kanamycin (*aphA3*) cassettes are used to make mutants in *H. pylori.* There have, however, been reports with the use of erythromycin (Hofreuter *et al.*, 2001) and gentamicin (Bury-Mone *et al.*, 2003) thereby supplying the needed resistance markers. Although requiring a bit more work, it may be more desirable to create unmarked deletions, thereby circumventing the need for antibiotic resistance markers and allowing for complementation studies with a plasmid (typically chloramphenicol or kanamycin resistant) or in the *rdxA* locus. The *sacB* gene from *B. subtilis* has been used to create unmarked deletions in *vacA* (Copass *et al.*, 1997) while exploitation of the *rspL* phenotype was used as a counter selectable marker (Dailidiene *et al.*, 2006; Fischer *et al.*, 1999).

Site-direct point mutations in the N-terminal domain of TlpB might be used to identify what residues are important in pH sensing as no PAS domains are present as in other energy taxis sensors (Alexandre *et al.*, 2004). In *E. coli*, Tar and Tsr have been implicated in the sensing of internal pH with Tsr as the major sensor (Krikos *et al.*, 1985). Residues 258-280 of Tsr have been identified to be responsible for the pH sensing properties (Krikos *et al.*, 1985; Umemura *et al.*, 2002). Although the mechanism of pH

sensing is highly unknown it is postulated that protonation and deprotonation may alter interactions with the methyl-accepting domains and prevent transduction of signal (Umemura *et al.*, 2002). An alternative hypothesis presented by the same group proposed that pH may affect membrane integrity and alter receptor conformations to change the signaling cascades.

Here we show the importance of *H. pylori* in being able to sense pH changes and respond using pH tactic behavior. This response is probably one of the most important factors during initial colonization as it allows *H. pylori* to rapidly respond to different pH gradients and seek out an optimal niche to establish an infection. That a gene-expression based response (via ArsRS) would be unlikely to occur quickly enough during initial exposure to acid also supports our view that pH taxis is one of the most important factors during colonization. In addition, the ability of *H. pylori* to sense pH gradients also allows it to stay deep in the crypts of the gastric mucosa. This provides a mechanism to avoid being cleared from the stomach during mucus turnover and allows for a long-term, persistent infection. The identification of a chemoreceptor involved in this pH tactic response may provide a therapeutic target as *H. pylori* that is pH blind, hypothetically, would not be able to maintain a persistent infection and thus would be cleared due to the acidity of the stomach and mucus turnover.

5.2 Antisense RNA as a Molecular Tool in *H. pylori*

AhpC is one of the most abundant proteins synthesized by *H. pylori* and is a key component of the oxidative defense system (Olczak *et al.*, 2002). The absence of AhpC renders *H. pylori* acutely sensitive to oxidative stress as evidenced by an inability of an *ahpC* mutant to grow in oxygen tensions above 2% (Olczak *et al.*, 2002). Much like other

mutants of components of the oxidative defense system such as *sodB* and *katA* (Ramarao *et al.*, 2000; Seyler *et al.*, 2001), *ahpC* mutants were much more sensitive to oxidative stress (Olczak *et al.*, 2002). Moreover, *ahpC* mutants were unable to colonize the stomachs of mice (Olczak *et al.*, 2003) suggesting that AhpC plays an important role in colonization and virulence. More recently, it has been suggested that AhpC, during prolonged exposure to oxidative stress, forms a high molecular weight complex that can act as a chaperone (Chuang *et al.*, 2006).

Herein, we sought to determine how much AhpC is required for colonization in the murine model. To achieve this we developed and employed an antisense RNA interference mechanism to knockdown levels of AhpC. Although the exact mechanism of antisense RNA is unknown we hypothesize that it functions in one of, or a combination of, the following: (i) that binding of antisense to the Shine-Dalgarno sequesters sequences required for ribosomal binding; (ii) antisense binding downstream prevents translocation of the ribosomes on the mRNA and/or; (iii) the formation of antisense-target duplex is degraded by endogenous RNases. The final outcome of this interaction is the knockdown of protein levels of the targeted gene.

Plasmid-borne copies of three varying sized antisense-*ahpC* constructs introduced into *H. pylori* displayed varying levels of AhpC knockdown which correlated with sensitivities to organic hydroperoxides. With a 72% knockdown with the full length antisense-*ahpC* construct, we saw the most sensitivity to organic hydroperoxides; however, in vitro growth was not impaired. Two smaller antisense-*ahpC* constructs, 100 and 250 bp, showed a reduced knockdown of AhpC with 42 and 34%, respectively. The fact that these two constructs should anneal to the 3' end of the target suggests that

antisense may also function to prevent the translocation of ribosomes on the mRNA and thus abrogating transcription. Similar results were also seen in another study which proposed that efficient antisense based knockdown of a target could be achieved when targeted to both the 5' and 3' ends of the mRNA (Wang and Kuramitsu, 2005). Furthermore, this group demonstrated that only 4-5% of the target length is required for a significant knockdown of the target. We feel that a caution should be employed in this regard. Using only 5% of smaller genes (e.g. antisense of 20 bp) may be subject to non-specific knockdowns of other genes. This is an issue that has been reported with siRNA that employs 20-22 nt antisense to knockdown targeted genes (Racz and Hamar, 2006). In such cases, it may be more suitable to choose a longer antisense to circumvent these potential issues.

We further developed a single-copy chromosomal based antisense directed towards *ahpC*. Knockdown levels of AhpC were achieved; however, at much lower levels (e.g. 32% knockdown) than the plasmid-borne system. This is probably owing to expression of a single copy in the chromosome versus expression from multiple copies of the plasmid-borne constructs (Heuermann and Haas, 1998). In this regard, maximal knockdowns are probably more easily achieved in a plasmid-borne system; however, the strain to strain differences with plasmid permissiveness (Ando *et al.*, 2000) and instabilities of the plasmids in vivo (personal communication; Dr. Rainer Haas) are a major limitation of this system. In contrast, single-copy constructs can be chromosomally inserted into all strains and is more suitable for in vivo studies; however, higher knockdown levels may be more difficult to obtain.

As in vivo mouse colonization studies with our AS1563 and AS1563-2 harboring strains (15% and 32% decrease in AhpC, respectively) showed no difference in the ability to colonize the stomachs of mice, one could speculate that the oxidative burst defense system by the host is not a barrier for initial colonization or AhpC is not required for colonization. To further evaluate how much AhpC is required for colonization, greater knockdowns would need to be generated. This could possibly be achieved by altering or swapping the native *ahpC* promoter in the chromosome with a weaker promoter and then target *ahpC* with antisense. Our in vivo results also do not rule out the possibility that AhpC may be involved in the chronicity of infection. Since mice have been shown not to have a significant inflammatory response against *H. pylori* until 8-16 weeks post infection (Garhart *et al.*, 2002), further studies could include a longer infection or the use of the Mongolian gerbil model that has been shown to have severe inflammation early in infection with *H. pylori* (Matsumoto *et al.*, 1997). Alternatively, mice could be immunized with the supernatant of *H. pylori* sonicate as it was shown that after immunization with this sonicate significant inflammation could be measured after 3 days post infection (Garhart *et al.*, 2002). With such experiments, we may find that an AhpC level of 68% is not sufficient for colonization and it may suggest the importance of AhpC during persistent infections.

Although AhpC was determined to be a virulence factor in *Mycobacterium bovis* (Wilson *et al.*, 1998), this was not the case for *M. tuberculosis* (Springer *et al.*, 2001) or *M. marinum* (Pagan-Ramos *et al.*, 2006). They reported that *ahpC* mutants were fully virulent in their mouse and fish models (*M. tuberculosis* and *M. marinum*, respectively). The discrepancies between *M. bovis* and *M. tuberculosis* are unknown, but it may be due

to differences in the animal model chosen or regulation (personal communication; Dr. Fanny Ewann). Reports that AhpC is not a virulence factor were also demonstrated for *Salmonella enterica* serovar *typhimurium* as *ahpC* mutants were fully virulent in mice (Taylor *et al.*, 1998) and more recently in *Legionella pneumophilia* where mutations in two phylogenetically distinct *ahpC* displayed wild type growth in macrophages (Leblanc *et al.*, 2006). The latter study; however, was not tested in an animal model. Moreover, studies in *H. hepaticus*, have demonstrated that *ahpC* mutants were fully able to colonize the liver and cecum of mice and showed no differences in pathogenesis when compared to wild type (Mehta *et al.*, 2007). Unlike *H. hepaticus tsaA* (*ahpC*) mutants, we did not see compensatory increase in catalase which is also consistent with *ahpC* mutants of *H. pylori* (Olczak *et al.*, 2005). Though AhpC may not directly show a role in virulence, other factors may be involved that aid the organism in evading oxidative bursts. For example, *Salmonella* has been shown to exclude the cytochrome b_{558} from NADPH oxidase thereby preventing oxidative bursts and exposure of the organism to ROS (Gallois *et al.*, 2001). Therefore, despite the function of AhpC to protect against ROS, organisms also demonstrate a remarkable ability to prevent the releases of oxidative bursts. Also, unidentified or untested conditions that these organisms might face may demonstrate a role for AhpC. For example, although AhpC is not required for *M. tuberculosis* virulence in the mouse model, there is some evidence that AhpC may be involved in protection during host to host transmission or reactivation from dormancy (Master *et al.*, 2002).

In *M. bovis* it was shown that their strain harboring an antisense-*ahpC* greatly reduced virulence in the guinea pig model, however, several revertants (loss of antisense-

ahpC construct) were isolated 8 weeks post infection (Wilson *et al.*, 1998). This indicated that the host provided a selective pressure for the loss of the antisense. We have also encountered this in vitro, in our laboratory with antisense-*hsp60* in *L. pneumophilia* (unpublished data; Dr. Fanny Ewann). We, however, did not see this in our animal model as *H. pylori* harboring AS1563 or AS1563-2 was equally sensitive to tBOOH post-infection as they were pre-infection. It is still possible, given enough selective pressure (e.g. time and inflammation) that *H. pylori* may also alter expression levels of antisense-*ahpC* to properly adapt to its host as recombination and mutation rates are high (Falush *et al.*, 2001). Furthermore, a greater knockdown of AhpC (e.g. 72%) may also facilitate revertants in a shorter time as the selective pressures may be higher. Other in vivo studies have shown that antisense RNA interference to be stable; however, unlike *M. bovis*, the antisense was expressed on a plasmid and targeted the α-toxin of *S. aureus*, a gene that probably would not affect the fitness of the organism like AhpC (Ji *et al.*, 1999).

Antisense knockdown was also attempted against two essential two-component regulators: *hp0166* (ArsR) and *hp1043* (Beier and Frank, 2000). ArsR has been shown to be involved in the acid regulation of various genes (Pflock *et al.*, 2006a). A mutant in the response sensor (ArsS) was achieved; however, attempts at mutational inactivation of ArsR were unsuccessful (Beier and Frank, 2000). Therefore, to further demonstrate the usefulness of antisense RNA, an antisense-*hp0166* (AS0166) was designed to knockdown ArsR levels. The introduction of AS0166 into the *rdxA* of *H. pylori* SS1 showed a knockdown of ArsR by approximately 40% as judged by immunoblotting with polyclonal antisera and densitometry. In vitro growth rates demonstrated that the AS0166 construct did not enfeeble growth.

No phenotype for the 40% knockdown of ArsR was evident and preliminary acid challenge experiments did not reveal any differences in proteome. Another group's studies could not identifiy any changes in the proteome of an *arsS* mutant; however, these cells were not challenged with acid (Beier and Frank, 2000). Although our experiments should be repeated, there simply may be insufficient knockdown of ArsR to show any changes in the proteome during acid challenge. Moreover, identification of proteins may be complicated by limitations of two-dimensional gels (Wilkins *et al.*, 1998). The solubility of hydrophobic (or membrane-associated) proteins is often a problem and low copy numbers are not easily detected by silver stains. In addition, high abundance proteins that do not display changes may mask or hide changes of low abundance proteins that have the same pI and molecular mass. Transcriptome studies with *arsS* mutants (Forsyth *et al.*, 2002a; Pflock *et al.*, 2006a) have identified up to 109 genes to be regulated during exposure to pH 5.0, of those 75 were judged to be upregulated and 34 downregulated. However despite the changes in mRNA level, there has been no reports that this is reflective of a proteome change, which would provide function for *H. pylori* survival. Moreover, as the pH of the stomach can be as low as 1.0 (Sachs *et al.*, 2003), the orientation of *H. pylori* is normally close to the gastric epithelium which is close to a neutral pH (Schreiber *et al.*, 2004). Since *H. pylori* is likely to transiently see a variety of pH 5.0, predictably between 1.0 and 7.0, perhaps the regulation of acid response genes mediated by ArsSR should be more closely looked at over several pH values that are lower than 5.0.

Acid resistance studies showed that the strain harboring AS0166 was just as resistant to pH 3.0 as wild type and controls even in the presence of urea. Since ArsR is

involved in the positive regulation of urease (Pflock *et al.*, 2005), we speculated that ArsR may be insufficient to increase urease and thus show that in the presence of urea a difference in resistance to acid. This was not the case and three possibilities may account for this. First, with 60% wild type ArsR still present in the cell, sufficient levels are available for phosphorylation by ArsS and to regulate genes during acid exposure. In addition, this may demonstrate a limitation of antisense in *H. pylori* as we are limited to using strong promoters and can not control how much antisense is made. Secondly, in an acidic environment (pH 3.0), it is possible that *H. pylori* is unable to actively transcribe transcripts or translate them into protein. Therefore, in such low pH, *H. pylori* would be unable to provide any changes to the proteome and must therefore use what is currently available for protection. Finally, the basal level of urease is thought to make up 10% of the protein weight of *H. pylori* (Bauerfeind *et al.*, 1997). This is a copious amount of protein and it begs the question why *H. pylori* requires so much urease present? But even further, why would it need to be upregulated in the presence of acid? These questions remain unanswered.

Improved knockdowns of AS0166 may be achieved by using the multicopy plasmids (e.g. pHel) instead of the single-copy in the chromosome. As demonstrated with antisense-*ahpC*, best AhpC knockdown was achieved when present on the pDH37 *E. coli* / *H. pylori* shuttle plasmid. Therefore it is conceivable that movement into the plasmid may allow for a more dramatic knockdown level of ArsR. One drawback of this system would be in vivo studies. Our plasmid-harboring strain (*H. pylori* 1061) was unable to colonize an IL-12 deficient mouse, a mouse strain that is highly permissive to *H. pylori* colonization (Hoffman *et al.*, 2003) and even mouse colonizing strains that can harbor a

plasmid can not stably maintain them in vivo. Therefore, in the absence of controllable promoters and for in vitro studies, a plasmid-based antisense may be the best option for achieving optimal knockdowns of the target in *H. pylori.*

Recently, the phosphorylation status of ArsR (D52N mutant) has been shown to be dispensable for cell viability (Schar *et al.*, 2005). This finding suggests that ArsR has two roles: an essential role in the non-phosphorylated state and a non-essential role in the phosphorylated state. Since phosphorylation of ArsR is through ArsS (Beier and Frank, 2000) in response to acid (Pflock *et al.*, 2004) the control of acid regulation genes are not required for cell viability. In vivo, an *arsS* mutant was unable to colonize the stomachs of mice (Panthel *et al.*, 2003) and showed enfeebled growth in medium adjusted to pH 5.0 (Loh and Cover, 2006). Therefore, the inability to display an acid response (mediated through ArsS) is probably an important factor during initial colonization of *H. pylori.* Further studies should include the ArsR D52N mutant (or if a strong antisense-directed knockdown could be achieved) to further demonstrate its importance in colonization. It may also be interesting to compare colonization in the mouse model where the gastric acidity is raised by treatment with a PPI. This would also provide evidence that the acid response of ArsS and ArsR D52N mutant are important factors for colonization. Furthermore, if colonization of the ArsS or ArsR D52N mutants could be demonstrated with a PPI, it would also be interesting to allow colonization, and then remove the PPI to identify if the acid response is greatest during transmission / initial colonization or during establishment of a chronic infection.

Acid resistance between *H. pylori* and enterobacteria seem to be fundamentally different in mechanism. Where *H. pylori* seems to survive low pH in the presence of urea,

Salmonella, *E. coli* and *Shigella* demonstrate an adaptive tolerance response (Lin *et al.*, 1995). If these organisms are shifted from neutral pH to a mild acid shock (pH of 4.0 to 4.8) for one generation, then shifted to an acid challenge (pH of 2.0-3.0), they are able to partially survive the acid challenge. However, a direct shift from a neutral pH to 3.3 results in death (Foster and Hall, 1991). It was further shown that a series of acid shock proteins are induced during the mild acid shock that allows the organisms to adapt and tolerate lower pH (Foster, 1991). Moreover, it was also shown that the acid shock proteins are upregulated during stationary phase and thus cells in stationary phase can tolerate acid shocks (Ma *et al.*, 2003). It is thought that these organisms are subjected to a lower pH in non-host environments or in fecal matter or found in stationary phase and therefore prepared for subsequent acid shocks (e.g. during passage through the stomach of a new host). As a point of interest, it was shown that the *E. coli* O157:H7 was able to remain adapted for acid resistance for at least one month in refrigerated hamburger (Lin *et al.*, 1996). Currently, there are no models of *H. pylori* needing to become adapted for acid survival. Acid resistance seems to be largely due to urease (Sachs *et al.*, 2005) and survival in it's ability to sense pH and seek a favorable niche. Additionally, the ArsSR regulon is becoming defined and in time, more insights and mechanisms during the acid response may appear.

The second two-component orphan regulator, HP1043 was also shown to be essential for viability in *H. pylori* (Beier and Frank, 2000). Six different antisense constructs, two of which were plasmid-borne, were designed to knockdown HP1043 levels but no knockdown could be obtained. Recent studies on HP1043 may give some insight as to why the antisense strategy was unsuccessful. Over expression of *hp1043* in

merodiploid strains proved to be unsuccessful in two different attempts (Delany *et al.*, 2002; Muller *et al.*, 2006). The addition of a heterologous promoter (P_{flaA}, P_{cagA} and P_{pfr}) to drive expression of a second copy of *hp1043* was integrated in the chromosome of *H. pylori.* Although they could demonstrate an increase in message up to 11x (Delany *et al.*, 2002), no changes in protein levels were observed. This suggests that *hp1043* is strongly regulated, possibly at the post-transcriptional and post-translational levels. To date, no factors have been identified in regards to this regulation. It is possible that secondary structure in the leader sequence could provide translation attenuation (sequestering of Shine-Dalgarno); however, the heterologous promoter constructs made by (Delany *et al.*, 2002) only included the native *hp1043* Shine-Dalgarno (17 nt upstream of ATG) and no other upstream regions thereby making this scenario unlikely. This does not rule out the possibility of small antisense RNAs or protease activity that may be involved. Based on the fact that over expression (11x mRNA) and the inability for 6 different antisense-*hp1043* constructs to alter HP1043 levels, it may reflect a remarkable mechanism in *H. pylori* to maintain a consistent protein level and may be reasoned that it is of particular importance to the cell.

No cognate histidine kinase has been demonstrated for HP1043 and phosphorylation of HP1043 by acetyl phosphate could not be demonstrated (Beier and Frank, 2000; Schar *et al.*, 2005). In the receiver domain of HP1043 is a lysine residue followed by asparagine that is normally an aspartic acid moiety followed by an acidic amino acid or histidine such as the case with CheY of *E. coli* (Hong *et al.*, 2004; Lee *et al.*, 2006). A site-directed mutation of the aspartic acid residue to a lysine residue of CheY is believed to alter the confirmation of CheY and allow it to act similarly as

CheY~P (always activated) (Bourret *et al.*, 1993). Therefore HP1043 function may be independent of phosphorylation events as site-directed changes of this lysine could be added as a second copy in the chromosome of *H. pylori* while the wild type copy could be disrupted with a kanamycin cassette (Schar *et al.*, 2005). Additionally, these authors performed site-directed mutagenesis on a potential secondary phosphorylation site and demonstrated that it could be used in place of the wild type gene. Despite the fact that HP1043 could not be phosphorylated does not rule out the fact that it still may be in vivo under unknown conditions. Obviously the phosphorylation status of HP1043 is not important for cell viability as described above (Schar *et al.*, 2005) but just as it has been proposed with ArsR, HP1043 may serve two roles: essential non-phosphorylated regulation and non-essential phosphorylated regulation of genes.

Other than HP1043 being able to bind its own promoter and the promoter of the MCP *tlpB* (Delany *et al.*, 2002) no other genes have been identified. Since *tlpB* is not essential for *H. pylori* viability (Croxen *et al.*, 2006; McGee *et al.*, 2005) it is reasonable to assume that HP1043 must control some other essential factor for *H. pylori.* To date, no reports on the identification of a regulon have been made. Since antisense directed towards *hp1043* has been unsuccessful and the relative difficulty in altering HP1043 levels, immunoprecipitation / macroarray experiments as recently described for Fur (Danielli *et al.*, 2006) may provide a way forward in identifying factors in the HP1043 regulon and clues towards its role in the cell.

In organisms that lack basic genetic tools to permit their studies, antisense RNA interference may provide a way forward in the study of essential genes. Although it is desirable to control antisense expression with an inducible promoter (e.g. P_{tac} or P_{tet}), this

is simply not a luxury found in organisms such as *H. pylori.* Therefore, different promoters of varying strengths can be utilized to obtain intermediate levels of the target protein. In addition, if in vivo studies are not required, a plasmid-borne antisense may offer improved knockdowns, presumably due to multiple copies found in the cell (Heuermann and Haas, 1998). However, single-copy antisense can also be used, although further improvements may be needed (see section 5.2.1). Antisense could also be used to answer the obvious questions about non-essential genes. For example: why does *H. pylori* make so much urease? Intermediate knockdowns of urease could be achieved and investigated to see that *H. pylori* may only require 50% or as little as 25% for colonization of the mouse stomach.

There are however some drawbacks of antisense. As aforementioned, in *H. pylori,* chromosomal copies of antisense could not achieve the same level of knockdown as plasmid-borne constructs and sometimes the knockdown is not efficient enough to observe any phenotypes (e.g. AS0166). However, achievable knockdowns may be limited to thresholds of protein that are required by *H. pylori* to survive. For example, a maximal knockdown of AhpC may be ~ 72% as a greater knockdown may not permit growth under normal growth conditions. To this end, it is then foreseeable that nearly 100% knockdown of non-essential genes should be achieved with antisense. A second caveat is that antisense which targets operons may have a polar effect in preventing the translation of downstream genes and therefore it would be difficult to attribute a phenotype to the targeted gene unless the other genes in the operon are complemented (without the target, elsewhere). Although plausible, this could be a daunting task. Finally, although the stability of antisense-*ahpC* was stable in vivo, a stronger selective pressure (e.g.

increased knockdown, oxidative burst or time) may force *H. pylori* to revert and lose the antisense construct as a deletion. Nonetheless, antisense RNA technology should provide a way forward in organisms such as *H. pylori* in studying genes of essential function.

5.2.1 Enhancing Antisense Stability

With the lack of controllable promoters in *H. pylori,* we are limited to the use of an uncontrolled promoter. Although strong knockdowns of AhpC could be achieved from a plasmid-borne antisense-*ahpC*, this application can not apply to the majority of strains as they can not stably maintain a plasmid. Therefore, there needed to be more consideration in the design of the antisense-*ahpC* for a chromosomal based copy as the same constructs that were plasmid-borne could not efficiently knockdown AhpC. We improved the antisense-*ahpC* by adding an antisense towards the 5' untranslated region (containing the Shine-Dalgarno) and by adding a transcriptional terminator at the 3' which has been shown to stabilize mRNA though prevention of 3' → 5' exoribonucleic degradation (Wong and Chang, 1986). This was also shown to be required for efficient knockdown in *Lactococcus* as antisense constructs lacking the T7 terminator were unable to knockdown of the *orf2* gene of a bacteriophage (Walker and Klaenhammer, 1998). Knockdowns of AhpC could be achieved at 15% with the P_{ahpC} promoter that was used in the plasmid-borne constructs. Conversely, 32% knockdown was achieved with the P_{ureA} promoter. Both are considered strong promoters due to their predicted total protein weight being 2-3% for AhpC (Wang *et al.*, 2004) and 10% for urease (Bauerfeind *et al.*, 1997). However, the stability of the mRNA transcript may also play a factor with the above numbers. Our promoter-GFP3 constructs also suggested that P_{ureA} was a stronger promoter than P_{ahpC}, but not by 2 to 3-fold as reported for the total protein weight.

Little is known about mRNA stability or folding in *H. pylori.* The 100 bp antisense-*ahpC* construct proved to be better at knocking down AhpC in *H. pylori* 1061 (42%) than the larger 250 bp construct (34%). In the *E. coli* model, the 250 bp construct was unable to alter *H. pylori* AhpC levels at all while the 100 bp construct showed nearly 100% knockdown. While other groups have shown that an antisense length of 4-5% of the target gene is required for efficient knockdown (Wang and Kuramitsu, 2005) the actual efficiency may be more reflective of the secondary structure of both the target and antisense construct. In this regard, it is possible that the secondary structure of the 250 bp antisense-*ahpC* may be more susceptible to degradation or the folding is too extensive to provide sufficient duplex formation with the target mRNA. With this in mind, it may be best to look for regions that have little secondary structure using developed software modeling. Additionally, imperfect basepairing may also provide a way to reduce secondary structure in an antisense construct. Many of the small antisense RNAs do not bind to their target with perfect basepairing, however the factors that constitute efficient basepairing remain unknown and are currently under investigation (Storz *et al.*, 2005).

Further improvements however can be proposed. Stability of long-lived mRNAs has been attributed to the 5' untranslated regions of both Gram-negative (Emory *et al.*, 1992) and Gram-positive (Hambraeus *et al.*, 2000) bacteria. In particular, the formation of a stem-looped structure at the 5' end has been shown to increase the half-life of the mRNA 4 to 5-fold. In addition to its native mRNA, these stem-loop structures were also able to increase the half-life of heterologous mRNAs (Arnold *et al.*, 1998) and in *E. coli* must be no further than 2-4 nt from the extreme 5' terminus otherwise it was insufficient for increasing stability (Emory *et al.*, 1992). It is believed that these stem-loop structures

protect the mRNA against RNA cleavage by endonucleases (e.g. RNase E in *E. coli*) which are thought to be the first step in mRNA decay pathway (Bouvet and Belasco, 1992; Emory *et al.*, 1992). It is therefore conceivable that the addition of a 5' stem-loop to the antisense construct might provide further mRNA stability.

In addition to the stem-loop structure found in the 5' untranslated region, it is also thought that ribosomal loading may also contribute to mRNA stability. In *B. subtilis* it was found that weakening the Shine-Dalgarno sequence of the *aprE* mRNA reduced mRNA stability 4-fold. Moreover, removal of the initiation codon did not alter mRNA stability suggesting that ribosomal protection is independent of translation (Hambraeus *et al.*, 2002). Therefore, it is possible that the incorporation of a pseudo Shine-Dalgarno (i.e. a strong ribosomal binding site without an initiation start site) may provide further protection of endonucleolytic cleavages by RNase. However, a potential caveat is that in *E. coli*, protection from degradation provided by ribosomes is dependent on translation (Joyce and Dreyfus, 1998). This is probably due to differences in mRNA decay mechanisms. To this end, it is still possible that *H. pylori* may have a mRNA degradation pathway that is similar to *B. subtilis* as both lack a homolog of RNase E (Kunst *et al.*, 1997; Tomb *et al.*, 1997). However, in either view, the addition of a pseudo Shine-Dalgarno should only prove to be beneficial or ineffective in antisense RNA stability in *H. pylori* as there are no indications that the binding of ribosome enhance mRNA decay in other bacteria (Hambraeus *et al.*, 2002; Joyce and Dreyfus, 1998).

In summary, an optimized antisense RNA construct might include, but not limited to: (i) 5' stem-loop structure to protect against initial endoribonucleic cleavage; (ii) a pseudo Shine-Dalgarno for ribosome binding, but no translation of initiation (iii)

complementary sequences to the target Shine-Dalgarno to sequester ribosome binding to the target; (iv) a reasonably sized antisense region towards the target with little secondary structure and; (v) 3' transcriptional terminator to protect against 3' → 5' exoribonucleases.

5.2.2 Towards an Inducible *H. pylori* Promoter

To date there are no known controllable promoters in *H. pylori.* This is probably due to the incompatibility of the principle sigma factor, σ^{80}, of *H. pylori* to interact with *E. coli* σ^{70}-promoters to initiate transcription (Beier *et al.*, 1998). However, the design of an *H. pylori* inducible promoter may be plausible by using a *tet*-based construct. The Tet repressor (TetR) is a regulator of a tetracycline (Tet) efflux pump (TetA) from *Tn*10 (Hillen and Berens, 1994). In the absence of Tet, TetR is tightly bound to two *tet*-operators (*tetO1* and *tetO2*) to repress transcription of *tetA*. Once Tet enters the cell, it rapidly binds TetR and causes a conformational change so that transcription TetR can no longer repress *tetA* expression. It should be noted that anhydrotetracycline, a derivative of Tet, can also be as it has approximately 500-fold greater affinity for TetR and does not exhibit any antimicrobial acivity (Scholz *et al.*, 2000).

A synthetic *H. pylori* promoter could be designed by using a strong promoter and the *tetO* operators from the Tet system. One *tet*-operator could be strategically placed between the putative -35 and -10 while a second could be placed downstream of the -10 to perhaps provide further steric hindrance to prevent RNA polymerase binding during TetR repression. Such a mechanism has been proposed as the positioning of the *lac*-operator between the -10 and -35 of the P_{tac} reduced basal levels of expression by 50% (Ezaz-Nikpay *et al.*, 1994). Therefore it is possible that a second *tet*-operator downstream

of the -10 may provide tighter repression. This can be done by using a PCR based strategy that incorporates the operator sequences to the 5' end of several overlapping primers. Once the synthetic promoter is made it could be tested with GFP fluorescence within the cell. In order to incorporate *tetR* into *H. pylori, tetR* would need to be cloned behind an *H. pylori*-specific promoter so that after insertion into the chromosome of *H. pylori,* expression could occur. A potential pitfall might be codon-usage of TetR lowering expression levels; however, those could be changed with site-directed mutagenesis.

We have attempted one such construct and designated it P_{mur} (data not shown). One *tetO2* (stronger affinity for TetR than *tetO1*) was placed between the putative -35 and -10 of P_{ureA} while a second *tetO2* was placed downstream of the -10. Since it had been shown that a TGN sequence upstream of the -10 was important for P_{ureA} expression (Davies *et al.*, 2002), we insured that that motif was not altered. Unfortunately, the when P_{mur} was tested in the chromosome of *H. pylori* G27, no difference in GFPmut3 fluorescence could be detected when compared with the promoterless control. This problem is probably attributed to our lack of understanding of *H. pylori* promoters. There has been no defined consensus sequence beyond the -10 TATAA box (and the frequency of TGN immediately upstream of TATAA). Many groups have attempted bioinformatic analysis of *H. pylori* and *C. jejuni* promoters but with little success or agreement (Petersen *et al.*, 2003; Vanet *et al.*, 2000). Therefore, the addition of the *tetO2* sequences probably disrupted an unknown important element of the P_{ureA} promoter. Another candidate might be the P_{katA} promoter which has 55% identity to the *tetO1* operator between the putative -35 and -10. Perhaps there would be enough conserved to allow for expression and therefore be utilized with the Tet system. Until we have a better

understanding of how *H. pylori* promoters work, attempts at a synthetic promoter may be in vain.

5.3 Model of *H. pylori* Colonization and Persistence

A proposed model for colonization and persistence can be seen in Figure 32. It has been well established that urease and motility are key components for the colonization of *H. pylori* (Eaton *et al.*, 1996; Tsuda *et al.*, 1994b). We show that pH tactic responses are also important in colonization of the gastric mucosa (Croxen *et al.*, 2006). Once *H. pylori* reaches the gastric mucosa, it can attach to the epithelial cell lining (BabA) and evoke an inflammatory response resulting in the release of nutrients (Hessey *et al.*, 1990; Ilver *et al.*, 1998). Upregulation of acid response genes are mediated by ArsSR in maintaining resistance (Loh and Cover, 2006; Pflock *et al.*, 2006a) to acid while pH taxis may also keep *H. pylori* from venturing back into the gastric lumen. With the bombardment of ROS and RNS from infiltrating neutrophils *H. pylori* utilizes a diverse antioxidant system which includes AhpC to detoxify the oxidative burst, a barrier that does not seem to be involved in initial colonization.

5.4 Conclusions and Future Directions

These studies have demonstrated that *H. pylori* displays a tactic response to pH that may be an important mechanism for the initial colonization of the gastric mucosa. In addition, at least one key component of this pH sensing, TlpB, was identified. The mechanism of acid sensing is poorly understood in all bacteria and closer investigations of TlpB and other components will be needed to further elucidate the mechanism

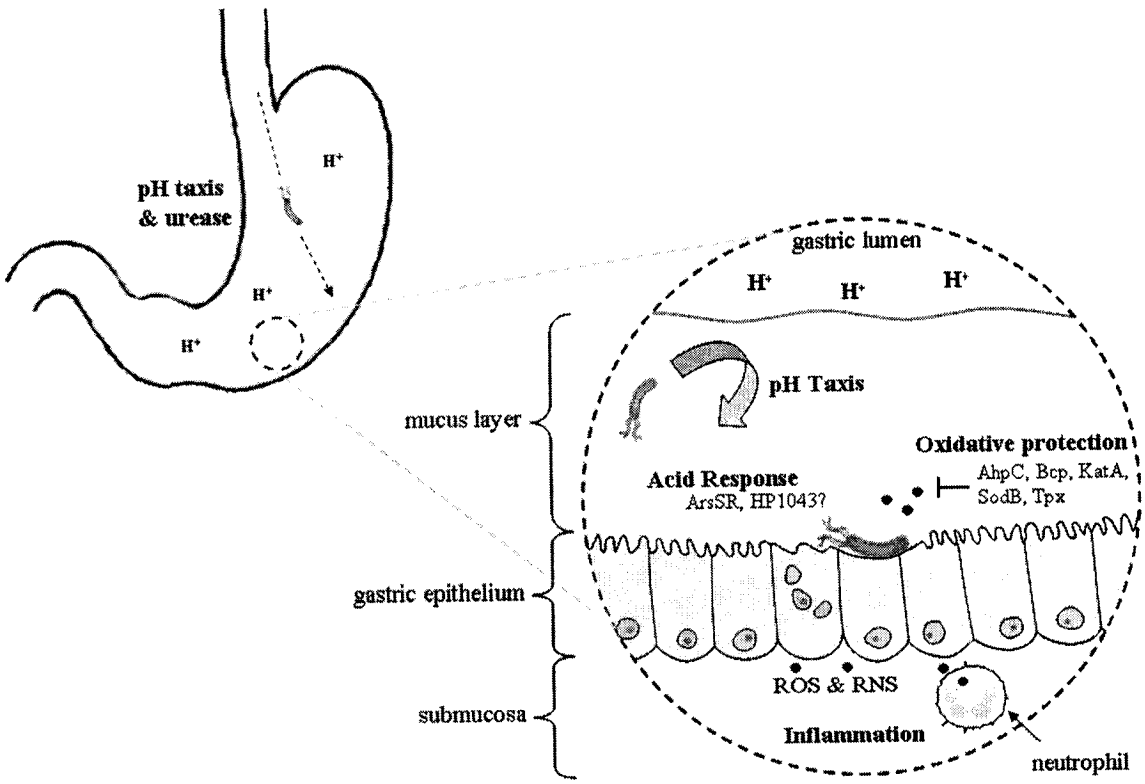


Figure 32. Model of *H. pylori* Colonization and Persistence. Following entry of *H. pylori* into the acidic gastric lumen, pH taxis allows *H. pylori* to sense pH gradient to the gastric mucosa and utilize these gradients to estabilish colonization near the gastric epithelial layer. Here, *H. pylori* must obtain nutrients by evoking an inflammatory response but, as a consequence, must deal with the burden of the accompanying oxidative bursts. AhpC and other detoxifying enzymes contribute to this defense while the acid response regulator ArsSR is probably involved in maintaining gene expression involved in resistance to acid. pH taxis may further provide a role in preventing *H. pylori* from returning to the acidity of the lumen. The role of HP1043 as its role in *H. pylori* survival and *tlpB* expression is unknown and denoted with a question mark.

Creating double, triple and quadruple mutants of the four MCPs will aid in isolating functions of not only TlpB, but the other three MCPs.

It was also shown that an antisense RNA strategy can be applied to the study of essential genes not only in *H. pylori,* but probably other bacterial backgrounds that are difficult to manipulate genetically. That a 32% knockdown of AhpC did not change the colonization efficiency of *H. pylori* in the mouse stomach suggests that the host's defense system (e.g. inflammation / oxidative bursts) might not be a major barrier of initial *H. pylori* colonization. Further studies should include further enhancements of antisense RNA, such as a stabile 5' untranslated region, for greater target knockdowns and extended in vivo studies with AhpC to determine how much AhpC is required for colonization and its role in persistence.

The antisense RNA application was expanded towards two essential transcriptional regulators: ArsS (HP0166) and HP1043. Although protein knockdowns of HP1043 could not be achieved, ArsS was knocked down approximately 40% with a chromosomal copy of the antisense-*hp0166*. However, changes in phenotypes or regulation of the proteome associated with knockdowns of ArsS could not be observed. Further studies should involve a plasmid-based antisense approach for ArsS to determine if defined phenotypes are observed with more efficient knockdown approaches.

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