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Oxidative Stress Resistance in *Helicobacter pylori*.
(Under the Direction of Dr. ROBERT J. MAIER)

Helicobacter pylori is a gastric pathogen that infects the human gastric mucosa resulting in a number of inflammatory responses including gastritis and peptic ulcer disease. As a pathogen, *H. pylori* must find a way to overcome the harsh environment within a human stomach. Upon colonization with *H. pylori*, the host's phagocytic cells respond, releasing reactive oxygen species (ROS) in an attempt to eradicate the pathogen. Toxic oxygen products encountered by *H. pylori* must be neutralized by the bacterium in order to maintain a persistent infection. *H. pylori* is equipped with a number of oxidative stress resistant enzymes and antioxidants. One of those enzymes is the alkyl hydroperoxide reductase (AhpC) which belongs to the family of enzymes catalyzing the reduction of organic peroxides to alcohols. In an attempt to determine its role in oxidative stress, gene disruption mutations in the gene (*ahpC*) encoding AhpC were isolated. I describe two types of mutants which were obtained. Although both mutants were lacking AhpC protein, the most predominant (type I) mutant synthesized an increased level of another antioxidant protein – NapA (encoding neutrophile activating protein). The second (less common, termed type II) mutant showed wild-type levels of NapA. Both types of mutants were more sensitive to ROS than the wild-type, and they had higher spontaneous mutation freqencies. In all cases, the type II mutant's phenotype was more severe (more sensitive to stress) than the type I strains. In addition, neither the type I nor type II AhpC mutants were able to colonize mice, indicating AhpC is required for virulence. Two other oxidative stress mutants (*napA*:cm and double mutant *ahpCnapA*) were created and differentiated from the wild-type in their sensitivity to oxygen as well as to other

oxidative stress generating products. The highest level of sensitivity to oxidative stress imposing factors was observed for the mutant lacking both alkyl hydroperoxide reductase and neutrophile activating protein.

INDEX WORDS: Oxidative stress; Reactive oxygen species; Alkyl hydroperoxide reductase; Neutrophile activating protein.

OXIDATIVE STRESS RESISTANCE IN *HELICOBACTER PYLORI*.

By

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INTRODUCTION AND LITERATURE REVIEW

Helicobacter pylori

Background

The microorganism initially named as *Campylobacter pyloridis*, then *Campylobacter pylori*, and finally as *Helicobacter pylori* was first cultured from gastric biopsy of ulcer patients in 1982 by Marshall and Warren (54). It is a gram negative helical shaped bacterium which is now recognized to be the primary cause of gastric ulcer and is associated with the development of carcinoma. From one-third to one-half of the world's population is infected with *H. pylori* (62). *H. pylori* causes chronic inflammation of the gastric mucosa which may develop into a gastric cancer. More than half of the gastric adenocarcinomas in the world are related to *H. pylori* infections (78), and usually occur after the age of 40. Usually transmission of *H. pylori* is mediated by direct oral-oral contact or fecal-oral route through the consumption of contaminated food or water (44) with saliva (30), gastric content, or feces of the infected individual (73), (27). *Helicobacter* genus members colonize the gastric and intestinal mucosae of mammals. *H. pylori* is seldom associated with natural infections in other animal hosts. According to the experimental data, *H. pylori* infections are able to be established in both large and small mammals like gnotobiotic piglets, mice, and gerbils (23, 24).

Upon the entrance to the stomach, *H. pylori* has to survive low pH, proteolytic enzymes, and peristalsis. Urease enables the bacterium to overcome acidity of the stomach. *H. pylori* is motile via 2-7 sheathed flagella which it uses to penetrate the gastric mucus layer and colonize the gastric mucosa (26). Over 90 % of the bacteria are known to colonize the mucous layer and a much smaller proportion remains in the gastric surface. *H. pylori* mostly adheres to the less acidic antral region of the stomach; however under conditions of low acid secretions, it can also colonize the corpus (body region) of the stomach (46). Bacterial adhesins like proteins, glycoconjugates, and lipids mediate the interaction between the bacterium and the host. These include AlpAB, BabA, HopZ, HpaA, Hsp60, Hsp70 and LPS. *H. pylori* chemotactically moves toward the mucin and binds to it. The bacterium obtains access to receptors on the host cell by degrading mucin. Successful colonization by *H. pylori* can be achieved even when the host exhibits an inflammatory response. These, for example, include the presence of PMNs and other immune cells (B and T cells) in the submucosal tissue. However, an excessive host response can kill the invading bacteria or damage the host's normal gastric epithelial tissue; such tissue provides an environment needed for the survival of this pathogen (9).

The response of the infected host differs among the colonized subjects. Initial colonization is not always easily apparent especially when the symptoms are normally developed after decades of the chronic infection. The majority of patients usually experience suppression of acid output, which may attract the growth of other bacteria. This type of microbial competition may sometimes eliminate *H. pylori* from the gastric tissue. However, when successful and persistent stomach colonization is achieved, *H.*

pylori may cause modifications to the gastric mucosa. The host tissue response, namely gastritis (inflammation of the gastric mucosa), is normally associated with the persistent colonization by *H. pylori*. A number of factors may be involved in the process of inflammation of gastric mucosa. These include the formation of vacuoles in epithelial cells (34), CagA (cytotoxin associated gene) directed induction of the Interleukin – 8 by *H. pylori* (18), and interleukin induced collection of neutrophils (28). Lesions affecting glandular tissue may develop leading to formation of atrophic gastritis and gastric hyperplasia (55, 31). The end result is destruction of the mucosa barrier and ulcer formation may then occur. Unfortunately, the tissue damage cannot be reversed and therefore the risk of gastric and duodenal ulcers evolving into more severe manifestation like gastric adenocarcinoma increases significantly.

Once gastritis is suspected, *H. pylori* can be analyzed by invasive or noninvasive diagnostic methods. These include harvesting of gastric-biopsy specimens, detection of plasma or salivary immunoglobin G (IgG), and the urea breathe test (UBT).

H. pylori can be grown on complex solid media (such as Brucella agar) or in Mueller Hinton broth. Media have to be supplemented with 10% blood or 5% fetal bovine serum. *H. pylori* requires CO_2 and microaerophilic concentrations of oxygen (2-15%) for healthy growth. The colonies grown on the solid blood agar plates appear translucent and can reach 0.5 to 1 mm in size. Normally, a period of 3 to 5 days and incubation at 37˚C are required for the colonies to become clearly visible. Usually the individual bacteria in young cultures of *H. pylori* appear in the shape of curved rods. This shape will change to coccoid as the culture becomes older (5 days). The re-growth of *H. pylori* from the coccoid structures is not possible even though those structures

remain metabolically active for up to 3 months (10). *H. pylori* can be transformed via allelic exchange mutagenesis either by natural transformation or by electroporation the latter of which increases transformation frequency. The transformation rates depend on the recipient strain and the resistant marker used for screening. Actively growing *H. pylori* is normally more competent (37). Also, a central component of homologous recombination processes (*recA* gene) as well as three genes carrying homology to transformation competence have been identified in *H. pylori* (HP 333, HP 1378, and HP 1361) (65). These undoubtedly influence transformation efficiencies.

Two *H. pylori* strains 26695 and J99 have had their genomes sequenced. Both strains carry a similar number of open reading frames (ORFs), 1496 and 1553 for J99 and 26695 respectively (1, 75). The availability of the complete genomic sequence has helped scientists to identify cellular components of *H. pylori* and experimentally prove their involvement in cellular processes. Almost 60% of the ORFs have now been assigned the functions to proteins they encode.

Respiratory Chains in *H. pylori*

The genomic analysis unraveled not only the sequence of events taking place during the respiration but also the actual components of the respiratory chain. Microaerophiles like *H. pylori* can survive only in sub-ambient concentrations of oxygen, even though they use oxygen as a final electron acceptor and are obligate aerobes. Respiration in *H. pylori* involves a series of oxidation and reduction reactions whose main purpose is to transfer the reducing equivalents from electron donor to electron acceptor resulting in the production of a proton motive force. Although this process leads

to a conservation of energy in the form of ATP (58), it can become a source of oxidative stress from electron leakage. For instance, autoxidation of NADH dehydrogenase, succinate dehydrogenase, and D-lactate dehydrogenase may produce superoxides. Also, autoxidation of cellular components like ubiquinols, catechols, thiols, and flavins may result in nonenzymatic generation of superoxides.

Dehydrogenases initially transfer electrons from electron donors to the quinone pool. In *H. pylori*, cells were capable of oxidizing D-glucose, formate, DL-lactate, succinate, and pyruvate (4). The rates of oxidation were significantly higher for lactate, succinate, and pyruvate. The genome sequence allowed identification of some of the dehydrogenases involved in the transfer of electrons to the membrane bound quinones. Genes encoding lactate dehydrogenase, glycerol-3-phosphate dehydrogenase, proline dehydrogenase, glycolate oxidase and amino-acid dehydrogenase have been found in *H. pylori*'s genome sequence. The involvement of malate:quinone oxidase was shown experimentally (43) which suggest malate is also an electron donor. The H_2-uptake hydrogenase of *H. pylori* is an electron donor (51), and can be coupled to respiration via a membrane bound chain. NADH normally acts as a major electron donor during respiration, but a membrane preparation of *H. pylori* showed rather low rates of NADH oxidation and high oxidation rates of NADPH (16). The gene cluster encoding the putative NAD(P)H quinone oxidoreductase is lacking the genes encoding the binding site for NADH (32). It is possible that some other protein is responsible for delivering electrons to this NDH-1 (NADH:quinone reductase) complex of the respiratory chain. The analysis of lipid extracts of *H. pylori* revealed that this bacterium uses menaquinones (MK) as its major quinones (52). Nevertheless, genes encoding biosynthesis of MK have

not been identified in the *H. pylori* genome, but genes responsible for the biosynthesis of ubiquinones have been identified (75). This implicates that possibly the microorganism uses the host's MK. From the quinol pool, electrons are passed to the bc_1 and 2Fe-2S protein complex and to cytochrome *c*. The latter heme-protein is either periplasmic (soluble) or membrane-anchored and takes electrons from the quinones and passes them to oxygen with the help of a type cbb_3 terminal oxidase. This oxidase was shown to be contained within *H. pylori* (56). The leakage of electrons at any point of this respiratory chain may easily result in the formation of reactive oxygen species, causing oxidative stress. Here is the sequence of reactions forming reactive oxygen species:

$$O_2 \xrightarrow{e^-} O_2^- \xrightarrow{e^-,\,2H^+} H_2O_2 \xrightarrow{e^-,\,H^+} OH^\bullet \xrightarrow{e^-,\,H^+} H_2O.$$

Oxidative stress

Background

Oxidative stress normally occurs when the concentration of reactive oxygen species (ROS) overrides the defense capacity of the cell. Molecular oxygen possesses two unpaired electrons in its molecular orbitals. This makes oxygen very reactive with other compounds containing unpaired electrons. Those may include, for instance, ferric iron or copper. In a complete four-electron reduction, molecular oxygen can be reduced to water. However, upon the acceptance of only one, two, or three electrons, oxygen will form superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) or hydroxyl radical (OH•) respectively. In less physiological pH (more acidic), O_2^- can be protonated to form even more reactive hydroperoxyl radical (HOO•) (29). Both O_2^- and HOO• have been shown to be involved in DNA strand breaks (21).

Free reactive oxygen species can frequently result from the normal biochemical processes occurring within a cell (38). These include respiration, metabolism and exposure to environmental levels of oxygen, metals or UV radiation (22). Oxygen is a very efficient terminal electron acceptor during aerobic respiration in the vast majority of electron transport chains. One of the paths leading to oxidative stress is a leakage of electrons from respiratory chains. The four-electron reduction of O_2 to H_2O is performed sequentially and is catalyzed enzymatically by a number of membrane-associated respiratory-chain enzymes. For example, redox agents carrying reducing equivalents from electron donors to O_2 may fail to reduce O_2 to water completely thus resulting in the one electron reduction product, highly reactive superoxide. Further, superoxide can contribute to the production of hydrogen peroxide resulting either from spontaneous or superoxide dismutase (Sod) -mediated dismutation reaction. Superoxide may also release iron from the iron-sulfur clusters. In this process, superoxide oxidizes the [4Fe-4S] center, causing release of iron. Cellular reductants keep the released iron reduced, which can easily react with H_2O_2 (33). This process ($H^+ + Fe\ (II) + H_2O_2 = \bullet OH + H_2O + Fe(III)$), termed the Fenton reaction, generates extremely reactive hydroxyl radicals (HO•). Hydrogen peroxide can also be decomposed into hydroxyl radicals by UV light. Finally, ROS may also evolve from macrophages stimulated upon an inflammation within the gastric mucosa (also referred to as oxidative burst).

For organisms grown in oxygen rich environment, superoxide, hydroxyl radicals, and organic and non-organic peroxides become a serious threat. Reactive oxygen species can cause damage to lipids, DNA, and proteins. Toxic and reactive oxygen-originated intermediates react rapidly with the components within the cell. Many organic and

unsaturated fatty acids like linoleic acid, for instance, are present in membrane lipids. Those lipids may be non-enzymatically converted to lipid hydroperoxides in the presence of ROS (53). Fatty acid chains may become shorter which makes them not capable of properly rotating within the membranes. This loss of structural integrity causes membrane permabilization, loss of proton gradient, inability to transport nutrients and the loss of other membrane associated functions. Lipid hydroperoxides may also undergo metal ion (such as ferric iron) mediated decomposition during which they will form an alkoxy radical and become unsaturated aldehyde genotoxin (47). Lipid hydroperoxide-derived genotoxins can then react with DNA bases converting DNA to adducts. These radical intermediates are the immediate precursors to cause DNA base damage. As a result, mutations are formed more frequently and they may eventually lead toward the development of cancer (15). DNA damage may also be caused by the action of hydroxyl radicals originated from H_2O_2 via the Fenton reaction near DNA-bound iron. Exposure to hydrogen peroxide tends to have a number of harmful consequences. These include chromosomal deletions, mutations, breaks in DNA or formation of DNA crosslinks (40). Damaged bases interfere with replication by DNA polymerase, transcription by stopping RNA polymerase or may cause misreading resulting in synthesis of truncated or abnormal proteins. ROS are known for being involved in aging, and disorders like arthritis or cancer (70).

Microorganisms must adopt strategies for managing their exposure to ROS. Cellular components damaged by oxidative stress must later be detoxified. The response to an oxidative stress can be either preventive (also referred to as a primary defense) or reparative (secondary defense). The preventive type of response includes enzymes

responsible for destruction of oxidants and synthesis of the proteins limiting the formation of oxidants. The enzymatic prevention by destruction of toxic oxidants includes actions of superoxide dismutase (Sod), catalase, and a bicomponent peroxidase system of alkyl hydroperoxidase (AhpCAhpF). Sod converts superoxide into hydrogen peroxide ($2H^+ + O_2^- = H_2O_2$), catalase converts H_2O_2 into water and oxygen ($2H_2O_2 = 2H_2O + O_2$) while AhpC and AhpF are responsible for converting harmful organic peroxides to their corresponding alcohols. In *Escherichia coli* and *Salmonella enterica* serovar Typhimurium the *ahpC* gene is located and transcribed from the same operon as *ahpF*. The smaller, substrate binding AhpC subunit (22kDa) is responsible for peroxidase activity while the larger, flavoprotein subunit AhpF (52kDa), uses NADPH as an electron donor for AhpC (71). AhpC reduces physiological peroxides such as linoleic acid hydroperoxide or thymine hydroperoxide and nonphysiological alkyl hydroperoxides to their respective nontoxic alcohol forms (41). AhpF then recycles the oxidized AhpC to its original active (reduced) form.

Another class of protein involved in the primary defense are those which play a protective role, but do not detoxify ROS. The proteins of this category belong to Dps (DNA – binding protein from starving cells) family of proteins. Dps is a 19 kDa nonspecific DNA – binding protein that accumulates during the stationary-phase growth of *E. coli* cells. The phenotype of Dps deficient cells implicated a role of Dps in protection during starvation (2). Like many other starvation induced proteins, Dps induction depends on σ^S transcription factor (45). The protein is expressed not only during stationary phase but also during exponential growth after the treatment of the cells with low doses of H_2O_2. Dps protects DNA from oxidative damage. Hydroxyl radicals

generated during Fenton reaction are often formed in proximity to the DNA, since ferrous atoms can be complexed with the phophodiester backbone of DNA. Dps of *Streptococcus mutans* protects DNA by sequestering it through forming an extensive crystalline lattice *in vitro* and *in vivo* (79). According to the proposed model, this histone-like protein forms complexes with DNA, thereby protecting the DNA against being attacked by H_2O_2 (22). Other Dps family proteins include the nonheme ferritin of *Listeria innocua* (11), the pilus major subunit of *Haemophilus ducreyi* (12), Dpr (dps like peroxide resistance protein) from *S. mutans* (80), and the neutrophil-ativating protein (NapA) of *H. pylori* (76).

In *S. mutans* the Dpr antioxidant system was discovered when Nox1 and AhpC (alkyl hydroperoxide reductase) were deleted. The crystal structure of the protein suggests it contains a ferritin like homologue thus it possibly protects by sequestering iron (35). Dpr also has iron-binding ability (80). It may therefore indirectly be involved in protecting the cells against an oxidative stress by keeping the intracellular level of free iron low; in this hypothesis it would prevent the Fe catalyzed production of hydroxyl radicals (see above).

In *L. innocua* and *H. pylori*, members of Dpr proteins function only as ferritin and are not able to bind DNA (11, 76). A homologue of Dpr in *H. pylori* is the NapA protein, which promotes adhesion of neutrophils to endothelial cells with a subsequent production of reactive oxygen radicals (28). NapA is encoded in the *H. pylori* chromosome by the *napA* gene. NapA is structurally similar to Dps type of proteins and functionally similar to ferritins. It shares sequence homology with bacterioferritins of *Anabaena variabilis, Bacillis subtilis,* and *Treponema pallidum* (28). In solution, NapA forms a decamer

composed of ten 15 kDa subunits that can bind 40 iron atoms per monomer (76). NapA accumulates in the stationary phase of bacterial growth. Unlike Pfr (ferritin) of *H. pylori*, the synthesis of NapA is not regulated by the presence or absence of iron in the growth medium (25). *H. pylori* is able to activate monocytes and polymorphonuclear leukocytes (PMN) like neutrophils. Activation of PMN has been associated with *H. pylori* or cell-bound compounds released from the bacterium (19, 50). It was suggested that NapA protein is released into the medium (most likely after cell lysis) and binds to the bacterial surface where it acts as an adhesin, mediating binding to mucin (57). Activation of neutrophils by a water extract of *H. pylori* promotes upregulation of CD11b/CD18 integrin (28, 81). It is a chemotactic response which increases adhesion of neutrophils to endothelial cells (59, 64). The basis for the proadhesive activity of NapA remains to be revealed. Possibly NapA may contain the amino acid motif within its subunit which can be recognized by a receptor on the surface of neutrophils (28). Recently, a selective binding of the neutrophil activating protein (NapA) to four compounds in the acid glycosphingolipid fraction of human neutrophils was identified with the sequence of NeuAcα3Galß4GlcNAcß3Galß(4GlcNAcß) in the fraction of human neutrophils (72). NapA isolated from bacterial extracts and the OMPs of *H. pylori* were shown to bind specifically to the MG1 (sample taken from a male suffering from gastritis) fraction of salivary mucin which contains a wide diversity of carbohydrate structures. NapA adheres to sulfated carbohydrate structures such as sulfo-Lewis A on MG1 (57). NapA displays numerous functions: these include binding to (nonglycolipid) sulfated carbohydrate structures such as the sulfo-Lewis A fraction of salivary mucin and binding to the glycolipid fraction of neutrophils. Upon the activation of neutrophils, reactive oxygen

radicals result as products of activation of membrane NADPH-oxidase. This results in oxidation of cellular membrane proteins, lipids, nucleic acids, ultimately increasing the incidence of ulcer formation.

The secondary defenses (reparative means of action against oxidative stress) repair damaged components like DNA and enzymes. This may be accomplished by DNA repair enzymes like exonuclease III (*xth*A), DNA polymerase I (*pol*A), and RecA (48). Peptide methionine sulfoxide reductase (MsrA) has recently been considered as one of the repair enzymes (13). MsrA can reverse the oxidation of essential Met containing proteins inactivated by oxidation in the presence of H_2O_2. Alkyl hydroperoxide reductase is also part of the reparative responses against oxidative damage. By reducing fatty acids hydroperoxides, it permits the repair of membranes (29).

Microorganisms have developed means of controlling the expression or synthesis of oxidative stress relief proteins. Although *H. pylori* 's regulatory systems remain undetermined, other organisms have developed a number of genes encoding proteins which are involved in overcoming the oxidative stress. Expression of these proteins is controlled by global regulators such as OxyR, SoxRS, PerR, or RsrA. In both *S. typhimurium* and *E. coli*, upon exposure to H_2O_2, regulator-dependent synthesis of approximately 30 new proteins was induced (17). Although not all the H_2O_2 -induced proteins fall into the oxidative stress category, the first five proteins are clearly controlled by the *oxy*R regulon. These proteins included 2 catalases, the 2 subunits of alkylhydroperoxide reductase, and glutathione reductase (69). All five proteins are known to be actively involved in oxidative stress and regulated by *OxyR* in *S. typhimurium* (17).

The 2D-gel analysis of *E. coli* cells pretreated with low doses of O_2^- generating components (plumbagin) revealed that approximately 40 new proteins are induced. The genes encoding these proteins are part of a *soxRS* regulon. They include manganese superoxide dismutase (*sod*A), the DNA repair enzyme endonuclease IV (*nfo*), and glucose-6-phosphate dehydrogenase (*zwf*) which helps to maintain a high reducing power of the cell (36).

When gram positive bacteria such as *B. subtilis* are treated with H_2O_2, they increase expression of catalase, AhpC/AhpF, Dps homologue (*mrg*A), and the heme biosynthesis operon. Sequences overlapping the promoters of the genes encoding these proteins revealed considerable homology to the Fur (ferric uptake regulator) box. Like other Fur family proteins, the PerR protein has a helix-turn-helix DNA binding motif and it also contains two CXXC motifs in a presumed metal-binding domain. The metal bound to PerR may play an important role in sensing peroxides thus contributing to oxidation (activation) of the PerR peptide in *B. subtilis*. Another microaerophilic pathogen, *Campylobacter jejuni*, contains a PerR regulon which is responsible for regulating expression of the *katA* and *ahpC* genes (77). *Helicobacter pylori*, however, does not show any homologous protein to OxyR, SoxRS or PerR (1).

RsrA is a system used by *Streptomyces* species like *Streptomyces coelicolor.* The thiol-based thioredoxin system is responsible for maintaining "reducing status" within the cell during oxidative stress. RsrA system is responsible for induction and shutting off the transcription of thioredoxin and thioredoxin reductase (*trxBA*) under oxidized versus reduced cellular conditions, respectively.

Microaerophilic organisms are very sensitive to lethal effects of oxygen and oxidative stress. They have to be able to minimize toxic oxygen-derived products. Besides the internally generated by-products of oxygen metabolism, the successful pathogen must be able to overcome reactive oxygen species originated from the phagocytic cells of the host immune response. Various enzymatic means to dissipate reactive oxygen species in bacteria are known, but the roles of these proteins in bacterial virulence for host infection have been determined for only few of these. In another microaerophile like *Campylobacter coli*, for instance, superoxide dismutase mutants have been shown to have impaired ability to colonize the chicken gut and they are more sensitive to air exposure (in their survival) (63). In *H. pylori*, catalase mutants have been characterized (61), however, they have shown no difference in the binding ability to epithelial cells when compared with the parent strain. Recently, *sodB* mutants of *H. pylori* have been isolated and it was concluded that SodB is necessary to obtain successful colonization by the pathogen within the mouse stomach.

Oxidative stress in H. pylori

The microaerophilic lifestyle of *H. pylori* is one of its most distinctive features, and is undoubtedly related to its persistence in the gastric mucosa environment. The organism is also often exposed to reactive products of partial oxygen reduction, either during aerobic respiration or when residing in the inflamed tissue. Upon infection with *H. pylori*, the host immune response elevates the amount of ROS (reactive oxygen species) within the gastric mucosa (5). In order for *H. pylori* to establish a persistent infection under these increased levels of ROS within the gastric environment, it must utilize protective systems designed to minimize oxidative stress. These systems include

preventive response by enzymes like superoxide dismutase, catalase, and alkyl hydroperoxide reductase. These three proteins play a very important role in enzymatic destruction of toxic oxidants. It has been determined that Sod is actively involved in overcoming oxidative stress by preventing the accumulation of lethal superoxide (67). Sod catalyzes the reduction of superoxide to hydrogen peroxide. Unlike most bacterial catalases that are located in the cytoplasm, *H. pylori* catalase is localized on the cell surface (61). Here it will fulfill its primary function against phagocytes and macrophages infiltrated to the infection site of the host. Catalase detoxifies hydrogen peroxide generated by Sod by breaking it down to water and oxygen. AhpC is a component of thiol-specific antioxidant (TSA) family of proteins. It is the third most abundant protein of *H. pylori* (42) based on 2-D gel electrophoresis analysis. The AhpC subunit was originally identified in *H. pylori* as a 26 kDa antigen (60). The gene encoding AhpC of *H. pylori* is transcribed as a mono-cistronic mRNA (49). *H. pylori,* however, lacks a homologue to AhpF. Analysis of the genome yielded a homologue of *E. coli trx* encoding a predicted Trx1 (thioredoxin) in *H. pylori* (HP0824) along with *trxB* homologue encoding a predicted TrxR (thioredoxin reductase) (HP 0825) (7). These two proteins are responsible in transferring electrons (in *H. pylori*) from NADPH to oxidized AhpC, suggesting that they fulfill the role of AhpF in *H. pylori* (7). Generally, there are 2 conserved cysteine residues necessary for antioxidant activity in the peroxiredoxin family of proteins. The cysteine residues Cys-49 and Cys-168 of *H. pylori* are considered to be important for the antioxidant activity. During oxidative stress, the N-terminal cysteine of AhpC initially becomes oxidized to sulfenic acid. At this point, the reduction of harmful peroxides to alcohol occurs with the subsequent release of H_2O (66). Later,

AhpC is regenerated to its original (reduced) form by Trx, and the reductase (TrxR) with the reducing equivalent provided by NADPH. Previously, insertional mutagenesis of the *ahpC* gene of human pathogen *Campylobacter jejuni* has been obtained (6). Here, AhpC deficient cells were less resistant to killing by cumene hydroperoxide reductase and surviving exposure to atmospheric oxygen than the wild-type.

NADH oxidases regulate the oxygen concentration in microaerobic organisms by scavenging oxygen. *H. pylori* was confirmed to possess NADH and NADPH oxidase activity (68). Both enzymes are capable of reducing oxygen to hydrogen peroxide and water. Unlike *E. coli* in which NADH plays an important role in bioenergetics, NADH oxidase of *H. pylori* was suggested to be involved in oxygen scavenging in order to keep the cytoplasmic oxygen concentration low (68) or for regenerating NAD^+ (3).

The redox status can influence the metabolic processes within *H. pylori*. Genome analysis did not reveal a homologue of the gene coding for glutathione reductase (1). The only free thiol compound within *H. pylori* is probably cysteine which is also used in maintaining reduced environment within the cell by microaerobic protozoan species lacking glutathione (14). It is possible that a reduced state of cysteine is maintained by the reductant thioredoxin in *H. pylori*. Thioredoxin and thioredoxin reductase provide NADPH – linked thiol – dependent redox system helpful in keeping proteins in their reduced forms (39). *H. pylori* contains two ORF's encoding thioredoxin reductases and two ORF's encoding thioredoxins (1, 75). It is possible that the thioredoxin system composed of these proteins is a response to oxidative stress.

The reparative response of *H. pylori* to oxidative stress are possibly performed mainly by RecA since mutants in *recA* showed high sensitivity to UV light (74). From earlier studies, it was shown that Msr acts as a reductase. When proteins containing methionine residue become oxidized, Msr repairs oxidized proteins by reducing them back to their original form (20). A *H. pylori* mutant in *msrA* has recently been isolated and is more sensitive than wild-type in response to oxidative stress (Praveen Alamuri, personal communication). It is not yet known, however, if this phenotype is due to excessive production of oxidized proteins.

H. pylori lacks the typical key regulatory proteins such as OxyR, PerR, and SoxRS (1). Surprisingly, *H. pylori* also lacks homologues of genes encoding for regulatory sigma factor σ^{32} (heat shock) and σ^{S} (stationary phase) (1, 75). Only the housekeeping σ^{70}-like sigma factor and the alternative sigma factors σ^{54}, and σ^{28} were identified (1, 75). *H. pylori* contains a Fur homologue, a regulator which uses not only iron but also other metals as cofactors (8). It remains to be determined how *H. pylori* regulates the enzymes involved in the destruction of oxidative stress related substrates. It is possible that this organism has adapted to an environment of constant oxidative stress (constitutive expression). Alternatively, it may contain other, unknown regulatory systems.

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CHAPTER 2

OXIDATIVE STRESS RESISTANCE MUTANTS OF *HELICOBACTER PYLORI* [1]

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Abstract

Within a large family of peroxidases, one member that catalyzes the reduction of organic peroxides to alcohols is known as alkyl hydroperoxide reductase, or AhpC. Gene-disruption mutations in the gene (*ahpC*) encoding AhpC of *H. pylori* were generated by screening transformants in low oxygen conditions. Two classes of mutants were obtained. Both types lack AhpC protein, but the major class (type I) isolated was found to synthesize increased levels (five-fold more than the wild type) of another proposed antioxidant protein, an iron binding neutrophil activating protein (NapA). The other, minor class (type II) produced wild type levels of NapA. The two types of AhpC mutants differed in their frequency of spontaneous mutation to rifampin resistance and in sensitivity to oxidative stress chemicals, with the type I exhibiting less sensitivity to organic hydroperoxides as well as having a lower mutation frequency. The *napA* promotor region of the two types of AhpC mutants were identical, and primer extension analysis revealed their transcription start site to be the same as for wild type. Gene disruption mutations were obtained in *napA* alone and a double mutant strain (*ahpC napA*) was also created. All four of the oxidative stress resistance mutants could be distinguished from the wild type in oxygen sensitivity or in some other oxidative stress resistance phenotype (i.e. in sensitivity to stress-related chemicals and spontaneous mutation frequency). For example, growth of the NapA mutant was more sensitive to oxygen than the wild type strain, and both of the AhpC-type mutants were highly sensitive to paraquat and to cumene hydroperoxide. Of the four types of mutants the double mutant was the most sensitive to growth inhibition by oxygen and by organic peroxides, and it had the highest spontaneous mutation frequency. Notably, two

dimensional gel electrophoresis combined with protein sequence analysis identified another possible oxidative stress resistance protein (HP 0630) that was up-regulated in the double mutant. However, the transcription start site of the HP0630 gene was the same for the double mutant as for the wild type. It appears that *H. pylori* can readily modulate the expression of other resistance factors as a compensatory response to loss of a major oxidative stress resistance component.

Introduction

Helicobacter pylori is a spiral bacterium that colonizes the gastric mucosa of humans, leading to a variety of gastric inflammatory responses, including peptic ulcer disease and chronic gastritis. Prolonged infections and inflammation due to persistent *H. pylori* colonization leads to severe tissue damage and sometimes to adenocarcinomas of the stomach (4). The pathogenesis of *H. pylori* depends on its persistence, which in turn depends on its surviving a harsh environment, including acidity, peristalsis, and attack by phagocyte cells and their released oxygen species (19, 25). These and other host defenses are undoubtedly counteracted by the successful pathogen, and some of these host responses are even beneficial to further colonization (5). Even outside of the host, it is clear that a microaerobic environment is important for survival of *H. pylori*. Consequently, there is a prevailing view (12, 19, 24) that O_2-mediated damage is commonly encountered by the bacterium, and that protective pathways to deal with toxic O_2 derived products may be key to the organisms ability to colonize the mucosal environment of the host.

Alkyl hydroperoxide reductase (AhpC) belongs to a family of peroxidases that are beginning to receive intense research focus due to their roles in dissipating damaging hydrogen peroxides (10) and related hydroperoxides. The specific role of AhpC in catalyzing the reduction of organic hydrogen peroxides to their respective alcohols, and the roles of accessory enzymes in these reductions are being determined (13, 33, 34). In addition, AhpC of *Salmonella typhimurium* was shown to confer resistance to reactive N intermediates (9). *H. pylori* AhpC is closely related to the peroxiredoxin of higher organisms such as those found in *Sacchromyces cerevisiae* and *Caenorhabditis elegans*, but it also has considerable sequence identity to five bacterial AhpC's (2). The reductase system for pure *H. pylori* AhpC was identified, and consists of thioredoxin and thioredoxin reductase; the flavoprotein reductant (AhpF) used by most bacteria to reduce AhpC was shown to be inactive for the *H. pylori* AhpC system (2). The complete system (AhpC with thiol-specific reductant) was capable of reducing a variety of hydroperoxide substrates that include both small aromatic and lipid hydroperoxides. It was concluded that *H. pylori* alkyl hydroperoxide reductase is essential for growth of the bacterium, as gene-disrupted deletion mutants could not be recovered (2, 8, 23).

Based on the genome sequence, *H. pylori* contains a battery of enzymes to combat oxidative stress, but the importance of these for growth of the organism via targeted mutagenesis approaches has been addressed for only a few of these (24, 29); (Seyler and Maier, unpublished, see ASM abstract #D-185, 2001). To address the role of AhpC in *H. pylori*, we obtained mutants in the *ahpC* gene encoding the AhpC, by screening the allelic exchange transformants in 2% O_2 conditions. Surprisingly, two types of mutants were recovered. Both classes lack AhpC, but the major class of mutant recovered was found to

up-regulate another proposed antioxidant protein, an iron-binding (31) neutrophil activating protein (NapA) (14). Consequently, gene disruption mutations were obtained in NapA and a double mutant strain (in NapA and AhpC) was also created. Of the four types of mutants, the double mutant was the most sensitive to oxidative stress, and by far had the highest spontaneous mutation frequency. However, all the mutants could be distinguished from the wild type in some oxidative stress phenotype. The phenotypic results are consistent with the conclusion that *H. pylori* has multiple oxidative stress related proteins and that compensatory mutations occur to circumvent loss of AhpC.

Material and Methods

Bacterial strains and growth conditions. *H. pylori* strain ATCC 43504 was used as the wild type. Cultures of *H. pylori* were grown microaerobically at 37°C in 5% CO_2 (Forma Scientific incubator model 3130) under different continuously controlled levels of oxygen (between 2%-12% partial pressure as indicated). Brucella agar (Difco) supplemented with 10% defibrinated sheep blood (Gibson Laboratories, Inc) plates are called BA , and were used for growth of *H. pylori*. Kanamycin (at 30 μg/ml) or chloramphenicol (at 20 μg/ml) were added as indicated. All strains were confirmed to be *H. pylori* by the presence of urease activity, catalase activity, and helical morphology by phase contrast microscopy (28). Genetic manipulations were performed with *E. coli* strain DH5α. LB agar supplemented with ampicillin (100 μg/ml), chloramphenicol (20 μg/ml), or kanamycin (30 μg/ml) were used for growing various *E. coli* strains, as noted. Strains and their source are given in table 2.1.

Cloning of *ahpC* and construction of *ahpC*:Kan mutant. Oligonucleotide primers for cloning the *ahpC* gene (ahpCF and ahpCR, see table 2.1) were designed from the sequenced strain J99 (1). PCR amplification was performed with *Taq* DNA polymerase (Fisher Scientific, Pittsburgh, PA) using chromosomal DNA from *H. pylori* strain 43504 as a template. The PCR product (851 bp) was then treated with T4 DNA polymerase (Promega) and phosphorylated with T4 polynucleotide kinase (Promega). The fragment was then cloned into a *Sma*I site of pBluescript KS^+ (Stratagene) to give pAhpC. This construct was then sequenced by the University of Georgia Molecular Genetics Instrumentation Facility. *ahpC*:Kan was created via insertion of kanamycin resistance cassette *AphA3* (25) into *Afl*II site of pAhpC. Restriction analysis revealed that the Kan cassette was in the opposite orientation to that of the *ahpC* gene.

Cloning of *nap*A and construction of pNapA:cm. A fragment of *H. pylori* DNA corresponding to the *napA* was amplified from genomic DNA obtained from both *H. pylori* strain 43504 and *ahpC*:Kan (type I) using the primers napAF and napAR (see table 2.1) and *Taq* DNA polymerase (Fisher Biotech). This 809 bp fragment contains the entire *napA* coding region as well as 339 bp upstream of the *napA* start codon. The PCR fragment was treated with T4 DNA polymerase to ensure blunt ends, and phosphorylated with T4 polynucleotide kinase. The fragments were then ligated with pBluescript KS^+ digested with *Sma*I. The resulting clones, pNapa (*napA* cloned from 43504) and pNapAT1 (*napA* cloned from *ahpC*:Kan type I) were then sequenced at the University of Georgia Molecular Genetics Instrumentation Facility. In order to utilize the *Sac*I unique restriction site within the *napA* coding region, it was necessary to excise the fragment with *EcoR*I and *BamH*I and clone into a derivative of pBluescript KS^+ in which the *Sac*I

site had been destroyed (pKSΔS), yielding pNAΔS. *Sac*I digested pNAΔS was then filled with T4 DNA polymerase and ligated to the *cat* chloramphenicol resistance cassette. The resulting plasmid, pNapA:Cm was then electroporated into competent 43504 cells to yield the *napA* mutant *napA*:Cm and into competent *ahpC*:Kan type I cells to yield the double *ahpC napA* mutant. Insertion of the cassette was confirmed by isolation of chromosomal DNA from the mutant strains and PCR amplification of the *napA* locus followed by agarose gel electrophoresis, used to monitor the increase in size of *napA* due to the *cat* insertion.

Transformation of *H. pylori*. Preparation of competent cells involved harvesting and washing cells four times in a solution of (ice-cold) sucrose/glycerol followed by their immediate storage of the cells at -80°C. *H. pylori* was transformed with pAhpCKan by electroporation (27). During transformation, 2 µl of the plasmid was added to 50 µl competent cells and electroporated using transporator plus (BTX, San Diego, CA) with a pulse of 2.5 kV. Immediately after the pulse, 50 µl of Mueller Hinton broth (Difco) was added to the cuvette containing the competent cells. Aliquots of these cells were then immediately plated on non-selective BA plates, and the plates incubated in the CO_2 incubator at 2% O_2 partial pressure. After a 48 hour incubation period, the cells from this non-selective media were transferred to BA plates containing kanamycin. Kan resistant colonies were selected by incubation in the 2% O_2 incubator. Genomic DNA isolated from Kan resistant strains were shown to have an increase in PCR product size for *ahpC* from 851 bp to approximately 2251 bp, as anticipated. Further, SDS-PAGE and 2D gels were used to confirm the lack of synthesis of AhpC protein in the gene-directed mutants (see text).

Gel electrophoresis. Plate-grown cells (2% O_2 condition) were harvested by a swab from the plate and resuspended in 50 mM Tris (pH 7.4) buffer containing 50 mM NaCl (TN). The cells were collected by centrifugation (10,000 x g for 10 min), resuspended in TN, and broken by two passages through a French pressure cell at 138,000 kPa (SLM Instruments, Inc). Crude extracts were then cleared of unbroken cells by centrifugation at 10, 000 x g for 10 min. The protein concentrations of the cell free extracts were determined by the BCA protein assay kit (Pierce, Rockford, IL). For SDS-PAGE, 5 μg of cell free extract was placed into SDS buffer and boiled for 5 min, and applied to a denaturing 12.5% acrylamide gel as described (21). For two-dimensional gel electrophoresis, 50 μg total protein (crude extract) was added to 1.125X IPG buffer (1X concentration: 8 M urea, 2 % Chaps (3-[(3-Cholamidopropyl)-dimethylammonio]-1-propanesulfonate), and 40 mM Tris (hydroxymethyl)-aminomethane). The immobilized pH gradient (IPG) strips (pH 3-10, BioRad, Hercules, CA) were then rehydrated overnight at room temperature with the IPG buffer treated protein. Proteins were focused for 5 hours at 3000 Volts using a Multiphore II isoelectric focusing apparatus (Pharmacia, Piscataway, NJ). The strips were then overlaid onto a 12.5% acrylamide gel and separated in the second dimension using SDS-PAGE (sodium dodecylsulfate Polyacrylamide Gel Electrophoresis), which was performed according to the recommendation of the manufacturer (BioRad). Protein spots were visualized by silver stain as described (26).

Protein fractionation and sequencing. Crude extracts of *H. pylori* wild-type cells were subjected to partial purification by FPLC Q–Sepharose ion exchange chromatography (Pharmacia Biotech, Piscataway, NJ). Proteins were prepared for

sequencing by subjecting the starting material (partially purified *ahpC*:Kan, type I crude extract for NapA, or the *ahpC napA* crude extract for HP 0630) to 12.5% acrylamide SDS-PAGE, followed by electroblotting onto polyvinylidene difluoride (PVDF) membrane (32). The appropriate bands were then excised and subjected to 8 cycles of N-terminal protein sequencing. Protein sequencing was performed by the Molecular Genetics Instrumentation Facility at the University of Georgia.

RNA isolation and primer extension analysis. Plates were streaked and incubated at 2%O_2 for 3 days. The harvested cells of wild type, *napA* and *ahpC napA* double mutant were resuspend in 1 ml PBS. RNA was isolated as described previously (2). For the primer extension analysis, 10 pmol of primers NapART and HP0630RT (see table 2.1) were 5' labeled in reaction with 5 µl [γ-^{32}P] ATP (6,000 Ci/mmol; Amersham) and 10 U of T4 polynucleotide kinase (Promega). An RNA sample (11 µl at 200 µg/ml) was incubated with 3 µl of ^{32}P-labeled primers and allowed to anneal at 70°C for 5 min followed by cooling slowly to 50 °C for 3 hours. 1 µl of AMV RT(Avian Myeloblastosis Virus Reverse Transcriptase) (Promega), 4 µl of 5x RT buffer (supplied with enzyme), 1 µl of 10 mM dNTP's were added to the annealed primers for 1 hour at 42 °C. DNA sequencing reactions of *napA* and HP0630 were performed simultaneously using the same primers and the fmol DNA sequencing system (Promega). Aliquots of primer extension and DNA sequencing reactions were heated prior to loading onto a 6% PAGE, 7 M urea gel. Gels were run for 2 hours at 65 Watts, dried, and bands were visualized by exposure to film.

Growth sensitivity to oxygen. *H.pylori* mutant and wild-type strains were streaked for individual colony observation by use of 3-way streaks onto BA plate. These

plates were placed into a CO_2 incubator under controlled O_2 concentrations. The incubators were adjusted to different (but continuously controlled) oxygen levels (2, 3, 4, 5, 7.5, 10, or 12% partial pressure) and the plates were observed for the presence of isolated colonies in the area of the final streak, after 3 days in the incubator. Individual colony sizes described in the table 2 are from the third streak, where individual colonies are the farthest apart.

Tolerance to oxygen. BA plates were heavily streaked and incubated at 2% O_2 for 3 days to obtain confluent growth. *ahpC*:Kan and wild type cells from these plates were harvested and cell suspensions prepared in PBS (phosphate buffered saline). The optical density of the cells was measured (600 nm), and adjusted to 0.1, and 5 ml aliquots were exposed to either anaerobic or aerobic conditions at 37˚C, as previously described (29). Undiluted samples from each set of conditions were plated hourly for a period of 6 hr onto BA plates. CFU (survivors) were counted after 3-5 days incubation at the 2% condition.

Frequency of spontaneous mutations. The frequency of spontaneous mutations was monitored by quantitating mutation to rifampin resistance (29). The strains were plated on BA plates supplemented with or without rifampin (5 μg/ml). Cells were first harvested from the plates and resuspended in PBS buffer. Optical density (at 600 nm) was measured and dilutions were plated to determine the number of viable cells in the original (undiluted) suspension. The mutant was plated on BA plates containing kanamycin and the wild type plated on the same medium but without the antibiotic. The original suspension of wild-type and mutant cells were also plated on BA plates, but supplemented with rifampin. The number of wild type and mutant cells was then

compared with the number of colonies identified on rifampin plates, and the mutation frequency was calculated (29).

Peroxides sensitivity. Sensitivity to three different cytotoxic agents was evaluated by disk assays (29). Sterile filter paper disks of 7.5 mm diameter containing one of the agents in water (5% v/v cumene hydroperoxide, 5% v/v t-butyl hydroperoxide, or 2 mM paraquat) were placed on BA plates (100 x 15 mm, 25 ml volume) that had been previously streaked for confluent growth with either the mutant or the wild-type cells. The plates were then placed in the 2% O_2 incubator. Following a 2 day incubation period, the clear zones surrounding the disks were measured. The data shown is the distance from the edge of the disk to the end of the clear zone, where growth begins. No growth inhibition was seen with disks containing H_2O.

Results

Sequencing and cloning of the *ahpC* gene. *H. pylori* gene # HP1563 *ahpC* encodes an alkyl hydroperoxide reductase, known as AhpC (30). Our goal was to clone this gene to enable the generation of gene-directed mutants for physiological studies. The cloned gene was then used to make a Kan^r *ahpC*-disrupted mutant by allelic exchange (see experimental procedures). Importantly, the mutant strains were obtained by screening the transformants in a low O_2 condition, and Kan^r transformants were not recovered when the transformants were screened in 12% O_2. SDS-PAGE gels were done to compare AhpC levels in wild-type and mutants. Alkyl hydroperoxide reductase was previously shown to be a predominant protein (third most abundant) in *H. pylori* and it could be readily identified in 2-D gels (18). In single dimension gels of crude extracts of

the wild type we observed a predominant peptide that migrated at 26 kDa (see figure 2.1, band of interest identified by arrow); this band represented the putative AhpC absent in extracts from *ahpC*:Kan mutants.

FPLC-Q Sepharose ion exchange chromatography was used to fractionate AhpC from extracts of the wild type (see figure 2.1). Fraction 23 contained abundant 26 kDa protein that was well-separated from other bands, so the polypeptides in this fraction were separated by SDS-PAGE and transblotted onto a PVDF (polyvinylidene difluoride) type membrane. The excised 26 kDa band was subjected to 8 cycles of N-terminal sequencing. The sequence result was in excellent agreement with that predicted for AhpC, with complete identity in the first 8 amino acids (MLVTKLAP). Based on SDS-PAGE analysis, all mutants chosen (as Kan^r colonies) lacked the 26 kDa protein (i.e. AhpC). However, from the gel analysis, we also observed that the AhpC mutant (lane 2, Figure 2.2) contained higher than wild-type levels of another protein (of an approximate molecular weight 17 kDa). This type of mutant was common (see text below) and is referred to as type I. Sequencing of the first 8 amino acids of the up-regulated protein (by transblotting and excising) revealed 100% identity to *H. pylori* NapA (neutrophil activating protein). Densitometric scanning of all the proteins in each lane (of figure 2.2) showed that the NapA band (normalized to all protein bands) was 5-fold greater in comparison to its intensity determined for the wild type. Apparently, the loss of AhpC has caused the concomitant overproduction of another oxygen stress related protein, NapA. NapA in *H. pylori* is a ferritin-like iron binding protein (31) and it could play a role in reducing toxic oxygen radical production via free iron removal.

Two types of mutant strains. In further analysis of a series of transformant colonies, it was observed that another type of AhpC mutant was obtained in which NapA was not up-regulated. Therefore, two types of mutants are discernible. To determine the prevalence of the two types of mutants, 36 individual colonies were chosen from 3 complete and independent transformation procedures (selecting for kanamycin insertions). The Kan^r transformants from all 3 procedures were selected in the 2% partial pressure O_2 condition. Out of the 36 *ahpC*:Kan mutants, only 2 lacked the up-regulation of NapA phenotype. These two isolates still made NapA, but at the wild-type level. These will be referred to as type II mutants and SDS gel of a representative of each of the two types of mutants is shown in figure 2.2. As a control, *modA*:Kan mutants were obtained by screening for transformants in the same manner (in 2% O_2). In this case, no up-regulation of NapA was visible for any of the 6 mutants chosen; all *modA* mutants had normal (like wild-type) NapA levels. Therefore, the up-regulation phenotype is associated with loss of AhpC, even though the second class of mutant was obtained in the same screening procedure. As further confirmation of the correlation between lack of AhpC and a concomitant up-regulation of NapA, we prepared samples of the wild-type and both types of mutant cells and subjected them to 2 dimensional gel electrophoresis. The result (figure 2.3) confirmed the loss of AhpC in the *ahpC* gene-directed mutants, and documented the up-regulation of NapA in the type I mutant, and the normal levels of NapA in type II strains (data not shown for type II).

The difference in NapA levels between the parent strain and the type I mutants is not due to a change in the *napA* promoter region. The DNA sequence of the intergenic region (209 bp) between HP0244 and the start of *napA* was sequenced from clones

derived from both the wild-type 43504 and the type I *ahpC:*Kan mutant, and they were determined to be identical.

Due to the observed up-regulation of NapA and our desire to identify roles of oxidative stress resistance proteins, we created more mutant strains. These were a gene-disruption mutant in *napA*, designated *napA*:Cm, and a double mutant in both *napA* and *ahpC*, designated *ahpC napA*. Conferring additional stress on *H. pylori* via mutation of oxidative stress resistance genes created another compensatory change; in the double mutant strains (*ahpC napA*) the 2D gels revealed induction of another protein in size of about 22 kDa. N-terminal sequencing of this protein showed 100% homology with HP0630 of *H. pylori* 26695 (30), a 21.8 kDa protein. Interestingly, a conserved domain search of the Pfam database (US, version 6.6) (3) revealed this protein is part of a family of NAD(P)H dehydrogenases (pfam02525) which in eukaryotic cells catalyzes the 2-electron reduction of quinone, and is involved in cellular protection against damage by free radicals and reactive oxygen species (22). The only bacterial member of the family to be characterized is the Mda66 protein of *E. coli*, which has been shown to be a NADPH quinone reductase, but to which no physiological role has been assigned (16). Further analysis of this protein will bring a deeper understanding of its role in *H. pylori*, perhaps as an oxidative stress resistance factor.

Primer extension analysis of *napA* and HP0630. The transcription start sites of *napA* and HP0630 genes were identified by primer extension analysis. Two possible transcription start sites of *napA* were identifed 53 and 54 bp upstream of the translation initiation codon. The most prominent band indicating base C located at 53 bp upstream, is designated +1 (figure 2.4). All the strains analyzed (wt, *ahpC*:Kan type I, and

ahpC:Kan type II) utilized the same transcription start site for *napA*. Similarly, the wt, *ahpC*:Kan type I and *ahpC napA* strains had the same start site for the HP0630 gene at 16 bp upstream of the translation start site (data not shown). The extended product for the *ahpC*:Kan type I mutant was more intense than that of the type II mutant (figure 2.4), in agreement with the increased amount of protein observed for the type I mutant. Analysis of the promoter region for the two new genes of interest with that of the previously determined *ahpC* promoter (2) revealed strong homology in the –35 regions among the three genes, and some (but less) similarity in the location and sequence of the –10 regions among the three genes (figure 2.4). The inverted repeat identified in the *ahpC* promoter (2) does not appear to be conserved in either *napA* or HP0630.

Sensitivity to oxygen. Oxidative stress responses are usually related to the organism's ability to tolerate oxygen and to combat the toxic oxygen radicals and other related oxidative species that may arise. One way in which we sought to characterize mutants was to determine their sensitivity to oxygen. Initially, we measured growth of the wild-type and mutant cells in different oxygen level environments. Brucella agar plates supplemented with 10% sheep blood were incubated at 2%, 3%, 4%, 5%, 7.5%, 10% or 12% O_2. The presence of isolated colonies as well as growth at the inoculant (initial streak site) at each O_2 level was evaluated after 3 days (see table 2.2). In the wild type strain of *H. pylori*, isolated colonies were visible at all concentrations of O_2. On plates streaked with either of the AhpC type mutants, small isolated colonies were visible from the 2% O_2 condition incubator. No isolated colonies were visible when the plates were incubated above 2% O_2, and slight growth (but not individual colonies) was visible in O_2 conditions of 3,4, 5 and 7.5% partial pressure O_2. Still, this growth was only at the

site of heaviest inoculum. No growth was visible (for any of the AhpC mutants) at 10% O_2 or higher, O_2 levels fully permissible for growth of wild type. NapA deficient cells showed growth with appearance of individual colonies in microaerobic conditions up to 3% O_2, like the wild-type, and this strain began to suffer growth inhibitions at 4% O_2. The double mutant (*ahpC napA*) cells were highly oxygen sensitive; we observed no individual colonies of this strain at any O_2 concentration after 3 days of incubation, however, there was growth at the heaviest inoculum. We previously reported that a *sodB* mutant was considerably more sensitive to oxygen killing than the wild type strain, in experiments measuring viability loss of non-growing cells exposed to air. Interestingly, unlike the *sodB* mutant (29), both types of *ahpC:*Kan mutants showed only slightly greater viability loss than the wild-type when subjected to these non-growing, air exposure conditions (data not shown).

Sensitivity to cytotoxic agents. A previously studied *sodB* mutant of *H. pylori* was shown to have increased susceptibility to hydrogen peroxide (29). To test whether our *ahpC*:Kan mutant showed increased sensitivity to an oxidative stress reagent (such as peroxide exposure), a series of disk inhibition assays were performed. Inhibition zones were measured around agent–saturated disks (see table 2.3). Both types of AhpC mutants were more sensitive to cumene hydroperoxide than the wild type. However, with *t*-butyl hydroperoxide the type I mutant was less sensitive than type II, (as shown by a slight decrease in the size of the inhibition zone). This tolerance of type I mutant to *t*-butyl hydroperoxide is probably related to the increased NapA level, as the type II mutant was considerably more sensitive to this peroxide (than the wt). Both types of AhpC mutants were more sensitive to paraquat in comparison to the wild-type strain. The *napA*:Cm

mutant was similar to the wild-type in its sensitivity to peroxides and paraquat, presumably as this strain contains abundant AhpC. The double mutant (*ahpC napA*) showed the greatest sensitivity of all the mutants to both *t*-butyl hydroperoxide and to paraquat.

Spontaneous rifampin resistance. Mutant strains of *H. pylori* lacking superoxide dismutase had a higher spontaneous mutation frequency than the wild-type (29). *H. pylori* deprived of functional AhpC may be more prone to DNA damage caused by ROS as well. In three independent experiments on the AhpC mutants we found they had a significantly elevated spontaneous mutation frequency compared with the wild-type strain. The average frequency of Rif^r mutation was 133 and 20 times greater for the type II (40 Rif^r cfu per 10^8 cells) and type I (6 Rif^r cfu per 10^8 cells) mutant respectively than for the wild-type strain. The *ahpC napA* double mutation strains' frequency was 350 fold greater than the wild-type whereas the *napA*:Cm single mutant had no greater mutation frequency than the wild-type. Lipid hydroperoxides are known to undergo metal ion-mediated decomposition which results in modification of DNA and formation of so called adducts. This type of alteration can cause frequent mutations (7) and could be one explanation of why the double mutant had a much higher mutation frequency than the other strains. The double mutant strain clearly has a deficiency in dissipating organic hydroperoxides (table 2.3).

Discussion

In order to combat reactive oxygen species, and therefore persist in the gastric mucosa, pathogenic *H. pylori* is equipped with a number of putative detoxifying proteins (11, 24, 25). These include not only enzymes to detoxify oxygen-related radicals, but N-containing reactive species as well. One of the enzymes observed in high abundance by *H. pylori* grown outside of the host is alkyl hydroperoxide reductase (18) and several groups concluded the organic peroxide detoxifying activity is important to *H. pylori* survival, as mutants in AhpC could not be recovered (2, 8, 23). From our results, such mutants can be recovered under low O_2 conditions. AhpC clearly plays a role in oxidative stress resistance but whether this is primarily due to minimizing the level of organic peroxides (17) is unclear. For example, it was recently suggested that H_2O_2 may be the primary substrate for AhpC (10).

Two classes of mutants, both lacking AhpC, were obtained. The type I mutants contained increased levels of NapA protein, whereas type II mutants did not. This increased NapA expression was associated with an increased resistance to *t*-butyl hydroperoxide and a lower spontaneous mutation frequency compared to the type II mutants. Previously, it was found that overexpressed levels of alkyl hydroperoxide reductase were able to overcome the high frequency of spontaneous mutations implicating the reductase in playing an important role against oxidative DNA damage (17). The same may be true for our (*ahpC*:Kan type I) mutants where NapA appears to be playing a role in minimizing spontaneous mutation frequency.

In other systems a loss of oxidative stress resistance function has been reported to cause a concomitant increase in levels of another oxidative stress resistance function.

Mutation of AhpCF of *Bacillus subtilis* led to increased expression of some factors that may help confer H_2O_2 resistance (6). Similarly, the antioxidant protein Dpr of *Streptococcus mutans* was identified due to its production upon mutation of other antioxidant encoding genes (33). Dpr, a homologue of NapA, was proposed to help confer aerotolerance to *S. mutans* by keeping free iron levels low, thus minimizing hydroxyl radical production (34).

Increased expression of an oxidative resistance factor appears to be a compensatory response used by oxidative stress deficient *H. pylori* to ensure that oxidative damage resistance is maintained. The peroxide sensitivity phenotype associated with AhpC mutants supports this compensatory up-regulation conclusion. Our results on the up-regulation phenomena prompted us to obtain additional mutants, namely a *napA*:Cm mutant and a double gene disruption (*ahpC napA*) strain. NapA is an iron-binding protein that may protect the cells by sequestering ferric iron released from iron sulfur clusters (20). The need for sequestering this type of iron is related to Fenton reactions that produce DNA-damaging hydroxyl radicals (15). Most of the results on the *H. pylori napA*:Cm mutants do not clearly define an oxidative stress role for this protein, but the *napA*:Cm mutant strain was O_2 sensitive compared to wild type. However, the high spontaneous mutation frequency and marked sensitivity to organic peroxides of the double mutant are in agreement with the conclusion that both proteins (AhpC and NapA) play oxidative agent detoxification roles. Further experiments on the role of the protein (HP0630) that is up-regulated in the *ahpC napA* mutant may prove to be useful in furthering our knowledge of oxidative stress resistance in the gastric pathogen.

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Table 2.1.Strains and plasmids.

Name	Description	Source
H. pylori		
43504	Parent strain for all *H. pylori* strains	ATCC
ahpC:Kan type I	*AphA3* insertion within *ahpC*	This study
ahpC:Kan type II	*AphA3* insertion within *ahpC*	This study
napA:Cm	*cat* insertion within *napA*	This study
ahpC napA	*ahpCnapA* double mutant	This study
E. coli		
DH5α	Cloning strain	Lab stock
Plasmids		
pBluescript KS^+	Cloning vector	Stratagene
pKSΔS	pBluescript KS^+ with destroyed *Sac*I site	This study
pAhpC	*ahpC* inserted into *Sma*I site of pBluescript KS^+	This study
pAhpCKan	*AphA3* inserted into *Afl*II site of pAhpC	This study
pNapA	*nap*A cloned from 43504 into pBluescript KS^+	This study
pNapAT1	*nap*A cloned from *ahpC*Kan typeI mutant	This study
pNAΔS	*nap*A cloned into pKSΔS	This study
pNapA:cm	*cat* inserted in *Sac*I site of pNAΔS	This study
Primers		
ahpCF	5’ AGCCACGCCCAATAACGATG 3’	IDT, Coralville, IA
ahpCR	5’ TCGCCTTTTCTCCAACCTGCTG 3’	IDT, Coralville, IA
NapAF	5' AACCACTAAATTAAAGGGTAACGGC 3'	IDT, Coralville, IA
NapAR	5' CGCTGTAATCTCTCATGCTGGC 3'	IDT, Coralville, IA
NapART	5’ ACACGATCGCATCCGCTTGC 3’	IDT, Coralville, IA
HP0630RT	5’ AGAATGCCCGAACGCTTTGG 3’	IDT, Coralville, IA

Table 2.2 Growth sensitivity[a] to oxygen

Strain	Oxygen concentration						
	2%	3%	4%	5%	7.5%	10%	12%
WT	++	+++	+++	+++	+++	+++	+++
ahpC:Kan type I	++	+	+/-	+/-	+/-	-	-
ahpC:Kan type II	++	+	+/-	+/-	+/-	-	-
*nap*A:Cm	++	+++	++	+	+	+/-	+/-
ahpC napA	+	+	+/-	+/-	-	-	-

[a] scored at 3 days by the following criteria:
-, no growth
+/-, slight growth at the site of heaviest inoculum, no individual colonies
+, significant growth at the site of heaviest inoculum, no individual colonies
++, significant growth and development of isolated colonies (pinpoint like colonies, less than 0.5 mm)
+++, healthy growth with individual colonies (more than 0.5 mm in size)

Table 2.3 Disk sensitivity assay[a]

Strain	Cumene hydro peroxide[b]	*t*-butyl hydro peroxide[c]	paraquat[d]
Wild-type	7.3 ± 1.5	13.5 ± 1.2	1.1 ± 0.1
ahpC:Kan type I	11.7 ± 2.4	9.0 ± 0.2	9.5 ± 0.5
ahpC:Kan type II	12.9 ± 2.3	19.8 ± 1.0	10.4 ± 1.1
napA:Cm	7.3 ± 1.3	14 ± 1.7	1.1 ± 0.1
ahpC napA	9.9 ± 1.3	20.5 ± 1.7	13.7 ± 0.9

[a] Zones of inhibition were measured (in mm) around filter paper disks saturated with 10 µL of the indicated compounds. Water as a control did not show any zones of growth inhibition. Results represent the average (± std. deviation) from five independent experiments.
[b] 5% cumene hydroperixide.
[c] 5% *t*-butyl hydroperoxide.
[d] 2 mM paraquat.

Figure 2.1 Fractionation of AhpC via Q-Sepharose chromatography. The arrow delineates the protein of interest, AhpC. Lane labeled as 'M', low range standards composed of Phosphorylase b (97.4 kD), BSA (66.2 kDa), Ovalbumin (45 kDa), Carbonic anhydrase (31 kDa), Soybean trypsin inhibitor (21.5 kDa), and Lysozyme (14.4 kDa); Lane 1, *H. pylori* crude extract; lane 2, flow-through eluent; lanes 3 through 9, fractions 20 through 26.

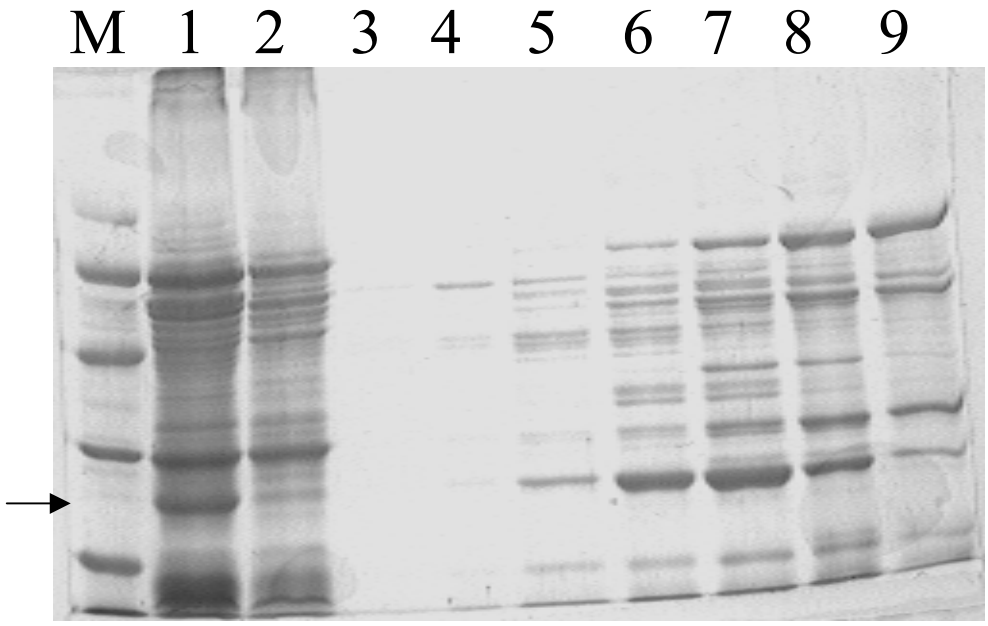

M 1 2 3 4 5 6 7 8 9

Figure 2.2 SDS-PAGE of *H. pylori* strains. 5 μg of crude extract were loaded in each lane of the following: lane indicated as 'M' represents low range markers (size indicated in figure 1); lane 1, strain 43504; lane 2, *ahpC*:Kan type I; lane 3, *ahpC*:Kan type II. The arrows indicate proteins AhpC (26 kDa) and NapA (17 kDa).

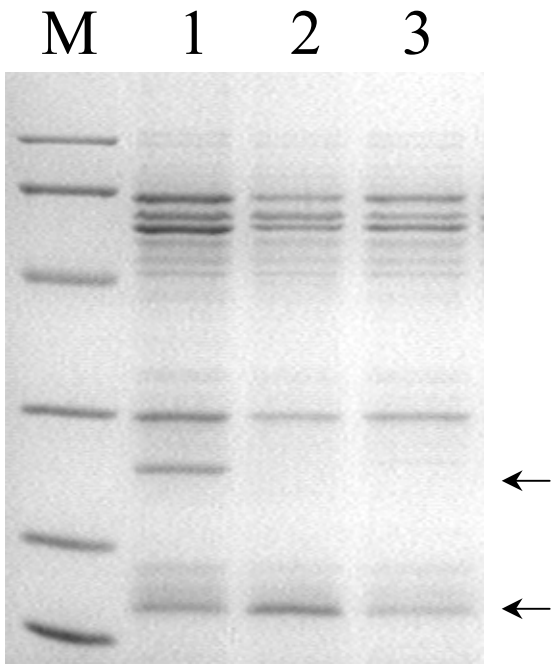

M
1
2
3

Figure 2.3 Two dimensional separation of crude extracts of wild type (ATCC 43504) (A), *ahpC*:Kan type I (B), *napA*:Cm (C), *ahpC napA* (D). The first dimension (isoelectric focusing) was carried out between the pH range of 3 (left) and 10 (right). The identified proteins are AhpC (contained within ovals), NapA (within the rectangles), and HP 0630 (circled).

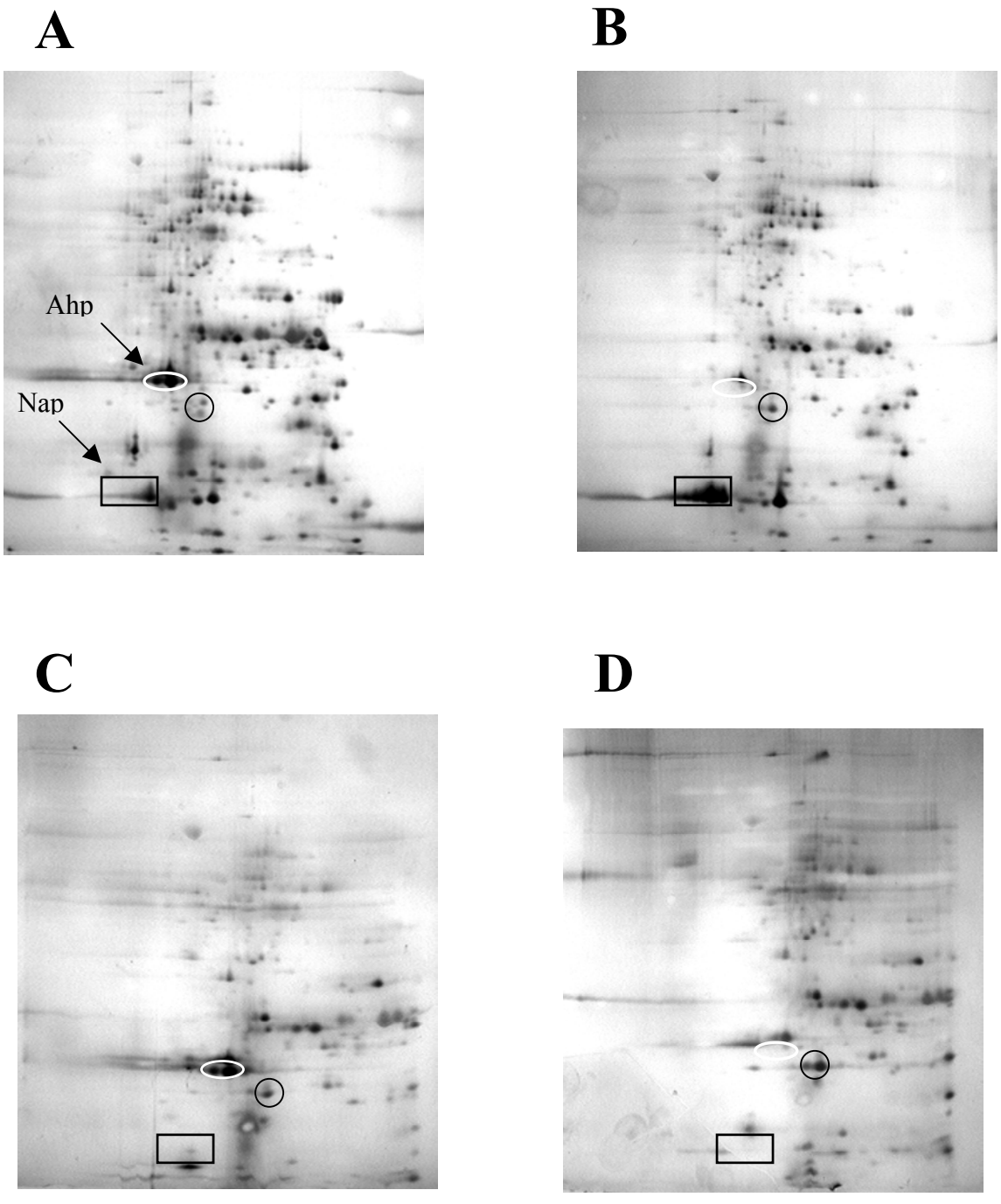
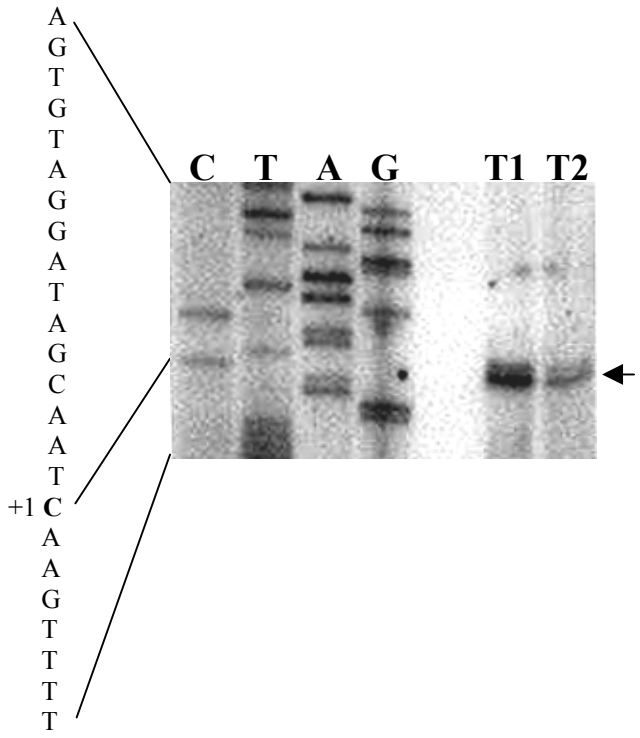

A
Ahp
Nap
B
C
D

Figure 2.4 (A) Primer extension analysis of *napA* in *ahpC*:Kan type I and type II mutants. The extended product (designated by the arrow) is located 53 bp upstream from the translation initiation codon AUG, and is indicated as +1. (B) Alignment of -35 and -10 promoter regions of *napA* and HP0630 with the previously identified *ahpC* regions (2). The -35 heptamers begin 33, 30, and 33 bp upstream from the transcriptional start site of *napA*, HP0630, and *ahpC* respectively.

A



B

	-35		-10
napA	TTTCATT	N_{11}	TGTA
HP0630	TTTCATT	N_{17}	TATT
ahpC	TTTCATT	N_{13}	TATA

CHAPTER 3

COLONIZATION BY *HELICOBACTER PYLORI* STRAINS LACKING FUNCTIONAL ALKYL HYDROPEROXIDE REDUCTASE.

Helicobacter pylori is a human pathogen that colonizes the gastric mucosa. Once *H. pylori* establishes a successful infection, the host is usually colonized for life if they do not seek treatment. Although this is an extremely persistent infection, there are many obstacles for a bacterium to overcome in order to maintain residence in the gastric environment. Before *H. pylori* can attach to the gastric epithelial cells, it has to be able to overcome the acidic environment of the stomach. This is why *H. pylori* produces urease, the enzyme which breaks down urea present in the gastric juice. The presence of generated ammonia maintains intracellular pH level above 5 (7). This makes it possible for the pathogen to survive in gastric acid during the time necessary for colonization of gastric mucosa (6). With the help of external adhesins, the pathogen attaches to the receptors on the gastric epithelial cells lining the stomach. Phagocytes like neutrophils guard the skin and mucous membranes against invasion by microorganisms. When microbes enter tissues, neutrophils circulating in blood migrate to the site of the attack. The phagocytic cell must first find the invader, adhere to it, destroy it and finally digest. During the destruction process, macrophages may generate reactive oxygen species like hydrogen peroxide (H_2O_2), nitric oxide (NO), superoxide ions (O_2^-), and hypochloride

ions (OCl^-). These molecules are actively involved in damaging plasma membranes of the ingested pathogen (2).

In order to be a successful pathogen, *H. pylori* must possess the ability to tolerate the multitude of reactive oxygen species it encounters during the colonization process. Toxic oxygen defense systems seem to play a significant role in virulence of the pathogen. It was previously established that *sodB* encoding superoxide dismutase (surface-associated, iron containing enzyme) of *H. pylori* gave the bacterium not only the ability to deal with toxic oxygen species when grown in the lab, but also to survive in the host and thus colonize the stomach successfully (8). Mutational analysis of *ahpC* in another microaerophilic pathogen like *Campylobacter jejuni* has been performed (1). Although colonization assays with the *C. jejuni ahpC* mutants were not done, a phenotype of the mutant strain in AhpC was obtained in oxidative stress; cells containing nonfunctional AhpC were shown to exhibit decreased resistance to killing by cumene hydroperoxide. The *ahpC* mutant was also less able to survive periods of exposure to atmospheric oxygen levels than the wild-type. This implies that the AhpC protein may be involved in protecting *C. jejuni* from oxidative damage during the exposure to extracellular oxygen metabolites of stimulated polymorphonuclear phagocytes in the inflamed mucosa of the infected gut. In order to determine the role of alkyl hydroperoxide reductase in host colonization, we constructed the *ahpC* mutation in *H. pylori* strain SS1, which is a strain that is capable of colonizing the mouse stomach (5).

H. pylori strain SS1 carries the *vacA* and *cagA* genes (5). VacA is a vacuolating cytotoxin which is endocytosed by epithelial cells where it causes endosome-lysosome fusion (vacuoles) (4). CagA is a cytotoxin associated gene which forms an island of 30

genes which when injected into the epithelial cell will induce production of interleukin-8. This will then trigger neutrophil infiltration resulting in duodenal ulcer symptoms (3).

The chromosomal copy of the *ahpC* gene was replaced with a disrupted copy of the targeted gene by homologous recombination. The *ahpC* gene was cloned into pBluescriptKS and interrupted with kanamycin cassette at the *AflII* restriction site. *H. pylori* was then transformed with this plasmid and colonies were selected for kanamycin resistance. The interruption was then confirmed by PCR and SDS-PAGE electrophoresis. Similarly to the previous results, two different classes of oxygen sensitive *ahpC*:Kan mutants were recovered. Although both types of strains were lacking functional AhpC, once again one of the mutant types (type I) synthesized increased levels of protein NapA while the other (type II) displayed a wild-type level of NapA (Figure 3.1).

Both mutated strains were inoculated directly into mouse stomachs and the colonization frequency was measured. The assay was carried out with 6-week-old female mice. The strains of the wild-type SS1 and the mutant to be inoculated were harvested from the blood agar plates and blood agar plates with kanamycin respectively. The cells were then resuspended in PBS (phosphate buffered saline) to a concentration of approximately 10^9 CFU /ml. The head space of the suspension was sparged with Argon gas in order to minimize oxygen exposure from the time of harvest until mice inoculation could proceed. The bacterial suspension was then delivered orally to the (1hr) starved mice. After three weeks, the mice were starved again for 3 hours and sacrificed. Their stomachs were removed, weighed, and homogenized in 5 ml of Ar-sparged PBS. Samples of the stomach homogenate (100 μl) were then plated on blood agar plates

supplemented with bacitracin (200 μg/ml) and naladixic acid (10 μg/ml). Plates were then incubated for 5-7 days at 37°C in a 2% O_2 partial pressure atmosphere and the colonies were counted.

Neither of the two types of *ahpC* mutants were able to colonize the mice while 78% of the mice inoculated with the parental strain became *H. pylori* positive (table 3.1). These results suggest that oxidative stress resistance plays an important role in virulence of *H. pylori*.

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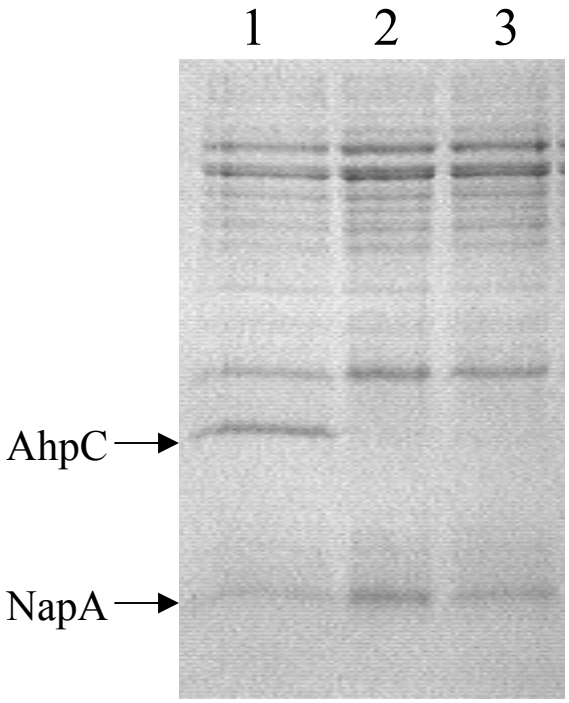
Table 3.1 Mouse colonization of wild type and *ahpC*:Kan mutant

Experiment #	*ahpC*:Kan	SS1 parent strain	SS1 mutant strain
1	type I	18/22	0/20
2	type I	14/17	0/17
1	type II	8/12	0/15

Figure 3.1 SDS – polyacrylamide(12%) gel electrophoresis of *H. pylori* SS1 wt, *ahpC*:Kan type I and II mutant cell extracts. 5 μg crude extract was loaded in each lane. Lane 1, strain SS1; lane 2, SS1 *ahpC*:Kan type I; lane 3, SS1 *ahpC*:Kan type II. Arrows indicate AhpC (26kDa) and NapA (17kDa) proteins.


1
2
3
AhpC
NapA

CHAPTER 4

CONCLUSION

Oxidative stress is caused when the presence of reactive oxygen species (ROS) overwhelms the defenses of the cell. The normal physiological processes of microorganisms may often become a source of ROS. The emerging reactive species include superoxides, hydrogen peroxides or hydroxyl radicals. The protective as well as reparative response to the oxidative stress comes from the battery of enzymes responsible for preventing the cell from being damaged by reactive oxygen species (3). For *H. pylori* these include superoxide dismutase (*sod*), catalase (*kat*), thiolperoxidase (*tpx*), and alkyl hydroperoxide reductase (*ahp*). Superoxide dismutase has been previously shown to have a significant effect on the ability of the microorganism to survive in oxygen atmosphere as well as in the host (5). Surface located catalase has also been previously characterized in *H. pylori* and it seems to play an important role in the defense against phagocytes (4). Tpx or TagD of *H. pylori*, namely, thiolperoxidase uses thioredoxin to reduce potentially harmful peroxides (6). Interruption of *tpx* has caused a moderate increase in sensitivity to oxygen and prevention of mouse stomach colonization (Sayler and Maier, unpublished data). Finally, my data clearly implicated the involvement of alkyl hydroperoxide reductase of *H. pylori* in oxidative stress resistance and colonization of mouse.

Alkyl hydroperoxide reductase uses thioredoxin and thioredoxin reductase for its activity (1). The gene-disruption mutation in *ahpC* encoding alkyl hydroperoxide reductase was obtained by screening the transformants in 2% O_2 partial pressure. The major (typeI) class of recovered mutants showed increased levels of neutrophil activating protein NapA by 5-fold. This mutant showed lower mutation frequency and less sensitivity to *t* -butyl hydroperoxides than type II mutants containing wild-type levels of NapA. Both types of mutants were more sensitive to paraquat and cumene hydroperoxide. The primer extension analysis of the promoter region of the *napA* in both mutants appeared to be identical to the wild-type. We have also measured the colonization frequency of both types of *ahpC*Kan mutants. From the colonization results, one may conclude that alkyl hydroperoxide reductase plays a significant role in achieving successful colonization by the pathogen. None of the survived mice infected with the mutant cells of *H. pylori* were colonized. The reactive oxygen species produced upon the inflammation are overwhelming to the infectious organisms like *H. pylori* thus the colonization becomes impossible in the absence of functional peroxidase like AhpC. Also, two other mutants deficient in *napA* only as well as double mutant lacking both *ahpC* and *napA* were also constructed. The sensitivity of *napA*:Cm to oxygen has been higher than of the wild-type. The double mutant *ahpC napA* had not only the highest frequency of spontaneous mutations but also it showed the highest sensitivity to oxygen and organic peroxides. From the analysis of one and two dimensional protein separation one can easily observe the up-regulation of another protein in the absence of alkyl hydroperoxide reductase in the type I mutant. The up-regulated protein is neutrophil

activating protein NapA. Similar was seen in the double mutant where up-regulation of yet unknown HP0630 protein of *H. pylori* became prominent.

A similar phenomenon has been observed in other microorganisms. In *Bacillus subtilis*, the loss of functional alkyl hydroperoxide reductase has resulted in the increase of another oxidative stress resistance protein in order to overcome the presence of hydrogen peroxide (2). Also, in *Streptococcus mutans* mutation of other antioxidant encoding genes showed the up-regulated level of dps like peroxide resistant protein Dpr (7). It has been suggested that Dpr (homologue to NapA of *H. pylori*) diminishes production of hydroxyl radicals by sequestering free iron (8).

In both *ahpC*Kan type I mutant as well as double mutant *ahpC napA*, we can see up-regulation of another oxidative protection protein. It seems that *H. pylori* can utilize other proteins in order to compensate for the lack of systems involved in resistance to oxidative stress. Still this up-regulation (type I mutant) does not fully restore the oxidative stress resistance to a wild-type phenotype.

In conclusion, the strains deficient in AhpC have lost their ability to overcome the oxidative stress implemented by phagocytic cells thus they lost ability to colonize mouse stomach. Although mutants in *ahpC* have been obtained in another microaerophilic human pathogen-*Campylobacter jejeuni*, no effects on virulence of alkyl hydroperoxide reductase have been determined. Our results clearly indicated, however, that *H.pylori*'s ability to resist toxin oxygen species is essential for successful colonization. In our studies, the deficiency in AhpC most predominantly resulted in upregulation of neutrophil activating protein (NapA). This may be caused by mutations within the coding region of NapA repressor since no difference was observed within the promoter

region of *napA* for both types of AhpC mutants. Because NapA is capable of binding free iron, it is most probably involved in sequestering iron during the oxidative stress thus preventing further generation of hydroxyl radicals via Fenton reaction.

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