THE ROLES OF METHIONINE SULFOXIDE REDUCTASE AND THIOREDOXIN IN *HELICOBACTER PYLORI*

By

LISA GAIL KUHNS

(Under the Direction of Robert J. Maier)

ABSTRACT

Helicobacter pylori is the etiological agent of peptic ulcer disease, chronic gastritis, and gastric cancer. It is well adapted for a successful life in the harsh environment that it faces and combats acidic gastric conditions with the urease enzyme. Urease functions to neutralize the pH. *H. pylori* is able to combat oxidative stress due to the function of many antioxidant enzymes such as methionine sulfoxide reductase (Msr) and the thioredoxin (Trx) proteins. Here I explore both Msr and the Trx proteins in more detail and link both to being important for host colonization. I discovered that Msr plays a significant role in nickel-dependent urease maturation. I determined that urease activity in a Δ*msr* mutant is decreased under elevated oxygen levels. This is not due to urease itself being damaged, as purified urease activity was not decreased upon exposure to oxidants. The nickel levels in the pure urease were decreased due to oxidants, leading to the idea that urease maturation is affected by oxidation. In fact, the urease maturation protein UreG was found to be the oxidizable target and contains an above average methionine content in its primary amino acid sequence. All nine methionines in UreG are susceptible to attack and modification by oxidants, forming the methionine derivative methionine sulfoxide (Met-SO). However, Msr can repair each Met-SO back to the non-oxidized form.

The Trx proteins of *H. pylori* are thought to be involved in the maintenance of the redox balance of the cell. I determined that after deletion of either *trx1* or *trx2*, the macromolecules of the cell are susceptible to damage as a result of oxidant stress. Specific damage to DNA was shown with the formation of 8-oxoguanine in both Δ*trx* strains, but with a greater amount shown in Δ*trx2*. There was also a greater abundance of lipid peroxides formed in the Δ*trx* strains and higher amounts of protein carbonylation in both strains. In addition, a Δ*trx1* mutant was decreased in mouse stomach colonization but more interestingly, the Δ*trx2* mutant was completely unable to colonize the mouse stomach. My study demonstrates the individual importance of the thioredoxins.

INDEX WORDS: *Helicobacter pylori*, oxidative stress, methionine sulfoxide reductase, thioredoxin, urease, 8-oxoguanine, lipid peroxidation, protein damage

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DEDICATION

I dedicate my dissertation to my wonderful parents, Ernie and Nancy Kuhns. They have helped lead me to where I am today and set such a great example to live by. They showed me what it means to be hard working and a person of integrity. I miss my daddy everyday and know he would be very proud of me.

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CHAPTER 1

Introduction and Literature Review

The thought of a bacterium thriving in the acidic environment of the stomach and causing disease was once a hard concept to imagine. That is, until two brilliant Australian researchers by the names of Barry Marshall and Robin Warren convinced people that this idea was plausible. To convince fellow scientists and medical doctors that such a pathogen existed, Dr. Marshall ingested a culture of the organism and subsequently developed peptic ulcer disease. He then had a colleague perform a biopsy on his stomach tissue, isolated and cultured the suspect organism, and fulfilled Koch's postulates to show that a bacterium was in fact the etiologic agent of gastric related diseases (1-4). This bacterium was first known as *Campylobacter pyloridis* and was later named *Helicobacter pylori*. The work carried out by Marshall and Warren sparked the interest of many researchers and the investigation of the bacterium's unique characteristics began soon after. Now we know of over 100 species of *Helicobacters* that infect many animal hosts, and five that colonize humans.

Characteristics such as being a microaerophile, resistance to the host immune response, and unusual nitrogen metabolism attributes all make the members of the genus *Helicobacter* interesting to a broad range of disciplines. Thus, the amount of cited literature has rapidly increased over the years. From 1997 to 2000 there were more articles published on *H. pylori* than on *Salmonella* or *Bacillus*. *H. pylori* has an interesting bacteriology in that it contains unique lipopolysaccharides that play a role in the avoidance of host responses. In addition, it is naturally

competent and easily transformable. Finally, it has a small genome indicating that it is well adapted for the environment it inhabits (5). These, among many other features, make it a truly fascinating bacterium to study just from academic perspectives.

General microbial characteristics of *H. pylori*

Epidemiology of infection. *H. pylori* is the cause of acute or chronic gastritis, peptic ulcer disease and, if left untreated, can lead to gastric carcinoma or B-cell mucosa-associated lymphoid tissue (MALT) lymphoma (4, 6, 7). *H. pylori* can infect anyone, but the highest prevalence of infection is found in people from developing countries (80-90%). This has been attributed to the rate at which the infection is passed to children. Children acquire the infection between the ages of 2 and 8 years, and most are infected by their teens. Our understanding of the transmission route in children is limited, however, and it is unknown when or how the infection is initially acquired (8). In the United States, about 30% of people are positive for *H. pylori*, and among those 1% develop peptic ulcer disease. A smaller percentage will develop gastric cancer and gastric lymphoma. In Western countries, people are more likely to acquire the infection if above the age of 60 years, of low socio-economic status, or are immigrants (9).

Humans are the primary carrier of *H. pylori*, but other reservoirs are still considered to be possible. One possibility is that animals carry the bacterium (10). However, conclusions regarding animals as a likely reservoir are an ongoing argument (11-14). There have also been hypotheses that *H. pylori* is found in the water supply (15, 16). Most of these have been refuted, however, because only the DNA from *H. pylori* has been found in environmental water samples, not viable cells. *H. pylori* has rarely been successfully cultured from any environmental source other than the human stomach (15, 17, 18) although it was isolated from a colony of domestic cats (19). Since there is no concrete evidence that *H. pylori* exists in the environment, the most

likely mode of transmission is from one person to another by direct contact, either by oral-oral or fecal-oral contact (20). Numerous studies have shown that people in close contact with those positive for *H. pylori* also contract the infection (21-24).

Bacteriology and Morphology. *H. pylori* is a member of the family Campylobacteriaceae which includes the genera *Campylobacter*, *Arcobacter*, *Wollinella*, *Thiovulum*, and *Helicobacter*. When *H. pylori* was first cultured, it was given the name *Campylobacter pyloridis* (5). After carefully studying the organism in more detail, it became evident that although it resembled members from the genus *Campylobacter*, it in fact differed in features such as flagellum morphology, fatty acid composition, and 16S rRNA sequence (4, 25). This qualified it to be placed in a new genus, *Helicobacter*, and it was then renamed *Helicobacter pylori*.

The predominant morphological form of *H. pylori* is bacillary, but a coccoid shape has also been observed. The function of each form is not entirely understood. However, it is thought that the bacillary form is the virulent form and the coccoid form occurs during a dormant state (26). There is evidence that *H. pylori* changes from the bacillary to coccoid form in older cultures (27). The bacillary form contains polar-sheathed flagella that, along with its helical morphology, are thought to allow it to move in a screw-like motion through the mucin layer. It also contains the urease and catalase enzymes that allow for its successful transition and survival through the acidic pH of the stomach and protect it from the damaging effects of hydrogen peroxide, respectively (28, 29).

Other features of *H. pylori* include the requirement of a less than atmospheric oxygen tension, it being a gram-negative pathogen, and containing a unique peptidoglycan structure. In the laboratory, *H. pylori* is kept under an atmosphere containing 1-6% O_2 (30). The low oxygen

conditions are thought to mimic the levels of oxygen found in the gastric mucosa. The inner and outer membranes of *H. pylori* are separated by the periplasm, classifying it as a gram-negative bacterium. Interestingly, the peptidoglycan composition is slightly different from that of other gram-negative organisms and less complex structurally (31). The peptidoglycan of *H. pylori* contains a high proportion of muropeptides, with a pentapeptide side chain ending in glycine and containing anhydro-*N*-acetyl muramic acid. It also has a low percentage of cross-linked di-aminopinelic acid (32).

Genetics. Genomes of over 100 strains of *H. pylori* have been sequenced to date, a few representatives of which are 26695, J99, and X47 (19, 33, 34). Strain 26695 was isolated from a patient suffering from gastritis and was sequenced by The Institute for Genomic Research (35). J99 was sequenced by AstraZeneca PLC and Genome Therapeutics Corporation as a collaborative effort and was isolated from a patient suffering from a duodenal ulcer and duodenitis (34). X47 was first isolated from a colony of domestic cats that presented signs of gastritis (19). The average GC content of all three strains is 39% and for all strains about 1,500 open reading frames have been identified. Among these, 60% were attributed to a predicted function whereas 17% were *H. pylori*-specific and have no known function. The entire genome is 1.6 Mb and is one-third smaller than the genome of *Escherichia coli* (36).

Nutritional Requirements and Energy Metabolism. Bacteria require nitrogen to synthesize macromolecules. There are two major sources of nitrogen in the gastric environment: amino acids and urea. *H. pylori* has been shown to be auxotrophic for several amino acids, which suggests that it relies on the gastric environment for nutrients. These amino acids are: arginine, histidine, isoleucine, leucine, methionine, phenylalanine, and valine. Some strains also require alanine or serine (37, 38). Analysis of the genome of *H. pylori* suggests that it can acquire these

amino acids through transporters and use them as carbon or nitrogen sources (33). When grown in a minimal medium lacking glucose, *H. pylori* will utilize arginine, aspartate, asparagine, glutamate, and serine as substrates and convert them to acids. Continuous culture experiments also showed a depletion of these amino acids when *H. pylori* is grown in a defined medium without ammonium or urea (39), further evidence that these are being used for a carbon or nitrogen source. In addition to amino acids, *H. pylori* can use urea as a nitrogen source. *H. pylori* produces large amounts of the enzyme urease, which functions to hydrolyze urea to ammonia and bicarbonate (40). These small molecules have buffering capacity and help keep the microenvironment around the organism at a neutral pH. There is evidence that the ammonia produced from urease can be incorporated into amino acids (41). *H. pylori* can synthesize biotin, folate, heme, molybdopterin, pantothenate, pyridoxal phosphate, riboflavin, and thioredoxin. It does not synthesize vitamin B12 or coenzyme A. *H. pylori* has a limited ability to acquire carbohydrates from the environment, and there are no complex sugar transport or degradation genes present (42). However, ABC-type transporter genes for dipeptide (Dpp) and oligopeptide (Opp) transport have been identified. These systems are encoded by the genes *dppABCDF* and *oppABCD*. In fact, mutational studies determined that the Opp system was involved in the transport of a variety of short peptides. The Dpp system was found to be essential for the transport of some identified di-, hexa-, and nonapeptides (43).

H. pylori does not appear to use complex sugars as a source of energy. It contains only the enzymes present to run glycolysis/gluconeogenesis in reverse, which suggests that it uses these pathways for anabolism rather than catabolism (42, 44). However, it appears to use glucose as an energy source and it contains all the genes required for the Entner-Doudoroff pathway (42, 45-47) although the ability to oxidize glucose is poorly understood. Pyruvate is produced

primarily from lactate, L-alanine, L-serine, and D-amino acids (48). It is converted to acetyl coenzyme A via a pyruvate oxidoreductase and can be dissimilated to produce acetate, formate, succinate, and lactate (48). The tricarboxylic acid (TCA) cycle in *H. pylori* is similar to the branched anaerobic TCA pathway used by *E. coli* (42). *H. pylori* can use oxygen as a terminal electron acceptor but no homologues of fumarate reductase and *N*-oxide reductase are present (42). However, there are terminal respiratory enzymes present that could be involved in anaerobic metabolism (49). This suggests that it is capable of only limited anaerobic metabolism. *H. pylori* was shown to be able to oxidize molecular hydrogen via a cytochrome-containing respiratory chain (50). Indeed, H_2 use is an important factor in conferring colonization ability (see below).

Metal Homeostasis. Metals are required for cell growth and for several cell processes. Therefore, it is important for organisms to have a transport mechanism to direct the flow of ions inside the cell. However, too high of a concentration of ions/metals can be toxic so they must be able to maintain homeostasis. *H. pylori* is well adapted for thriving in the gastric milieu and this includes the adaptation for the uptake of metals from the environment (51).

H. pylori has a high requirement for nickel, largely due to its requirement as a cofactor for two major enzymes, urease and hydrogenase. Urease comprises up to 10% of the total cellular protein and contains 12 nickel atoms per molecule (36). Thus, *H. pylori* must have transport systems to scavenge nickel when its environment is low in nickel (e.g. 2 to 11 nM); the levels measured in human sera (52). In fact, *H. pylori* does contain nickel specific transporters, including both single-component and multicomponent transporters (53). The first one-component transporter identified was NixA (54). This protein is located in the cell membrane and contains eight transmembrane domains (55, 56). NixA has a high affinity for nickel but it can also

transport other metals such as cadmium, cobalt, and zinc (55). A *nixA* mutant showed decreased urease and nickel transport activities; however, these activities were not abolished (42). Thus, it was concluded there are other transporters that allow for the influx of nickel. Later, a gene homologous to an *E. coli* nickel ABC transporter was identified in a sequence homology search (34). This gene was identified as *abcC* and contains an adjacent gene, *abcD*. Mutational studies of *abcD* showed a severe effect on urease activity, while an *abcC* and *nixA* double mutant had almost no urease activity (34). Once nickel is inside the cell, Hpn and Hpn-like are thought to act as metal storage proteins to protect against nickel toxicity (44, 57-59). These proteins are rich in histidine residues that bind nickel and other divalent cations (33). The nickel-responsive regulator NikR, in the presence of nickel, transcriptionally activates both of these proteins (60).

Cells require iron for the proper function of several important enzymes. The iron responsive systems in *H. pylori* are mediated by the ferric uptake regulator (Fur) protein that acts as a transcriptional repressor of several genes under conditions of high iron (61-63). In general, the iron concentration around the cell is low but if it becomes too high, bacteria have the ability to limit its transport (51). This is advantageous to the cell as an abundance of iron can result in the generation of oxidative damage. Several iron transport systems are present in *H. pylori*. Ferrous iron can pass across the outer membrane but requires the Feo system to cross the cytoplasmic membrane (47). Ferric iron requires active transport through both the outer and cytoplasmic membrane. To cross the outer membrane, the ferric enterobactin receptor FrpB and ferric citrate receptor FecA are used (48, 64). Energy for this process is supplied by the TonB/ExbB/ExbD protein complex (65). To cross the cytoplasmic membrane, transfer is mediated by the ABC transport system FecD (permease) and FecE (ATPase) (48, 64). Once

inside the cell, iron is stored via two ferritins, prokaryotic ferritin (Pfr) protein and bacterioferritin (47). Iron stored by these proteins can be released and reused for cellular processes.

Pathogenesis

Following ingestion of the bacteria, *H. pylori* moves into the gastric region and colonizes the gastric mucosa next to the epithelial cells (Figure 1.1a) (66). In this region, the bacterium is able to buffer the surrounding environment due to the function of urease. However, as infection proceeds epithelial cells are damaged, resulting in a lesser amount of mucin production. This then allows for the acidic gastric fluids to contact and further damage epithelial and mucin producing cells, often resulting in host cell death. The host immune response is then induced and inflammatory cells are recruited to the site of infection (Figure 1.1b) (67). Eventually, gastric cancers can result.

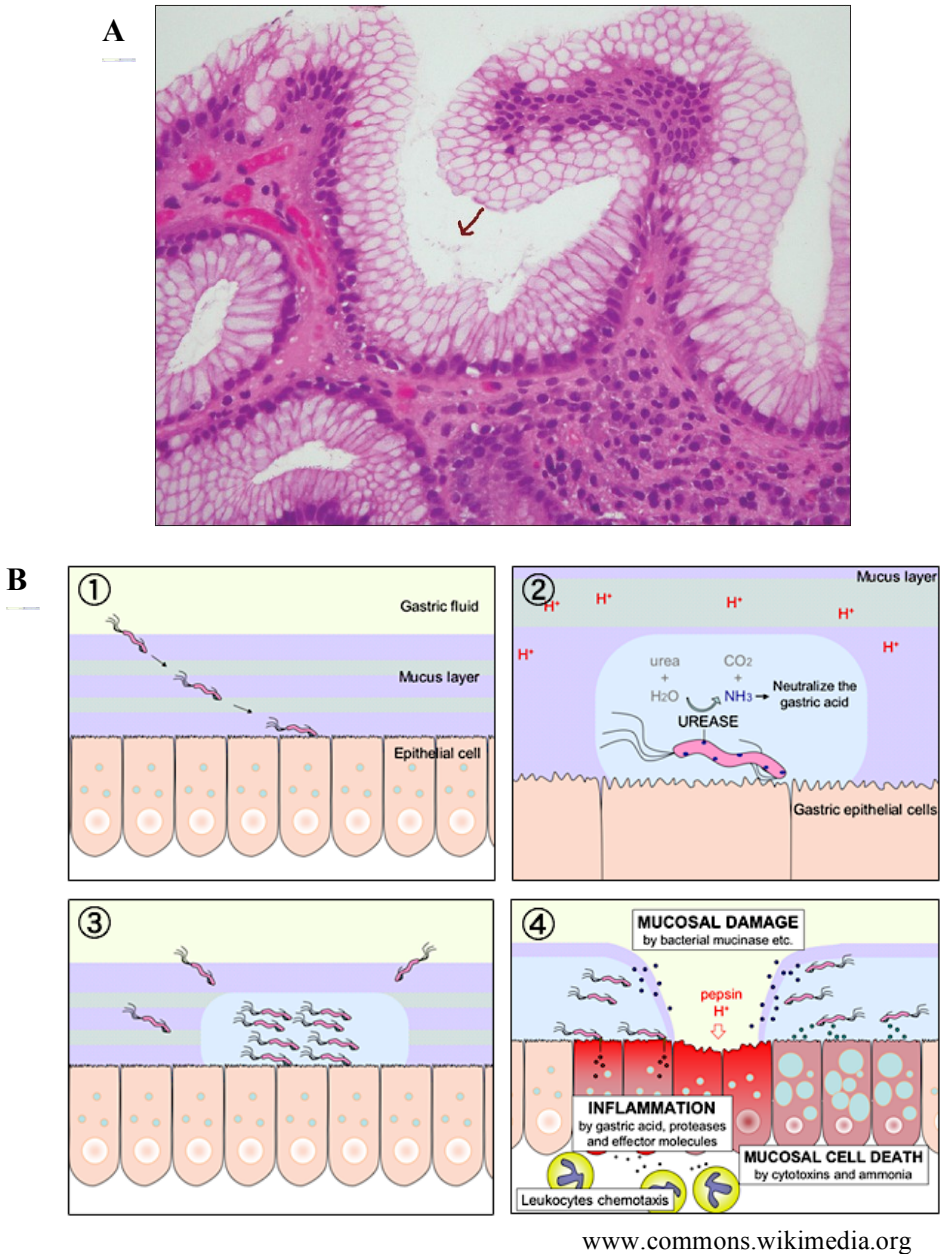

Figure 1.1 *H. pylori* colonization of the gastric mucosa. A) Gastric mucosa biopsy taken from an infected human. The specimen was stained with Hematoxylin and Eosin to detect *H. pylori*. Image shows *H. pylori* infection of the gastric mucosa. *H. pylori* resides next to the epithelial cells in the gastric pit. Arrow points to *H. pylori*. B) Stages 1-4 of *H. pylori* infection. 1. *H. pylori* movement into the mucus layer, 2. Production of urease neutralizes the gastric acid

surrounding the organism, 3. Colonization of the mucus layer, 4. Depletion of the mucus surrounding epithelial cells, recruitment of inflammatory cells, mucosal cell death.

Adherence and motility. Colonization of a bacterium requires four steps: 1. transmission to a host, 2. adherence inside the host, 3. avoidance of host immune response, and 4. the ability to acquire nutrients to survive. All of these factors are very important for the success of *H. pylori* as a pathogen. Adherence is one of the earliest challenges *H. pylori* must face when infecting a new host. Once it has moved through the mucin layer, it attaches via adhesions. These are bacterial proteins, glycoconjugates, or lipids that make contact with the host receptors. Attachment of these adhesion proteins with host receptors creates a signaling cascade in the host to recruit inflammatory cells such as neutrophils and macrophages (67). Only a small percentage of *H. pylori* cells actually adhere to the epithelial cells (<1%). Most of them reside in the gastric mucus layer or are cleared by the host (68, 69).

***cag* pathogenicity island.** Once *H. pylori* has attached to the host epithelial cell surface, the microvilli are removed and the tight junctions are disrupted (70-86). This results in depletion of the mucus granules. The *cag* (cytotoxin-associated gene) pathogenicity island encodes the CagA protein and translocation of this after depletion of the mucus granules results in the phosphorylation of a tyrosine residue that sparks a signaling cascade in the host and activation of NF-kB, cytokine interleukin (IL)-8, neutrophil infiltration, ROS burst, and apoptosis of the host cell (67).

Urease. During the journey to the gastric mucosa to establish colonization, *H. pylori* is often confronted with the acidic gastric juices. However, *H. pylori* is protected from the gastric acid by the function of the urease enzyme. Urease has been shown to be required for colonization of the stomach (28). It functions to provide a buffer surrounding the cell by hydrolyzing urea to

yield ammonia and bicarbonate (40). Urease is cytoplasmic, and in addition to providing a buffering environment for the cell, it provides ammonium for nitrogen assimilation mediated by glutamine synthetase (87, 88). The urease holoenzyme is made up of six copies of UreA and UreB. In order for the enzyme to become active, nickel must be incorporated into the active site located within the UreB subunit (89). Nickel is shuttled through a battery of accessory proteins: UreEFGH, where two Ni^{2+} ions are loaded into each active site for a total of 12 Ni^{2+} ions per fully loaded urease (40, 90-92). UreI is a membrane protein that is thought to act as an acid-gated urea channel. Nickel is incorporated into apo-urease through the interactions of the accessory proteins (Figure 1.2). These proteins are absolutely required for urease activity. UreH is thought to stabilize the apoprotein. UreF is required for carbamylation of the Ni^{2+} lysine to prevent Ni^{2+} from binding to the active site prematurely. UreG contains a nucleotide-binding motif and contains GTPase activity, possibly to supply energy required during Ni^{2+} transfer. UreE binds nickel and possibly transfers the nickel to the other accessory proteins for loading into the urease active site (93, 94).

Hydrogenase. While colonizing the host, *H. pylori* must have a means to obtain energy from the environment. Since it seems that *H. pylori* is metabolically deficient in carbon source catabolism, the use of H_2 would be a simple way to obtain energy while using amino acids and peptides as carbon sources (49). Hydrogen is known to be a high-energy reductant for use in anabolic reactions and bacterial oxidation of molecular hydrogen commonly occurs in nature. The oxidation of H_2 can provide ATP for the cell as well (49). It has been shown that *H. pylori* possess a membrane-bound uptake-type hydrogenase that is used to power cell-building functions (50). Interestingly, the mucus layer of the stomach contains a surprisingly high concentration of molecular H_2; it results as a byproduct of colonic fermentation from commensal

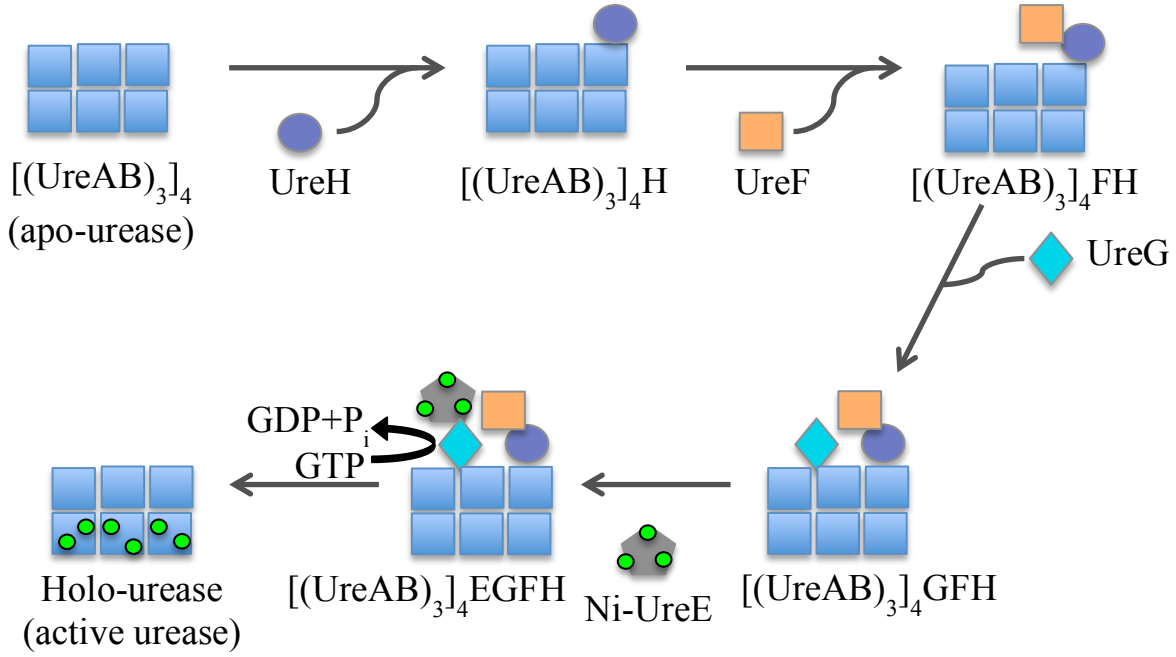

Figure 1.2. Model of urease maturation. Nickel is transferred to the active site of urease by several accessory proteins. UreH binds to UreF and UreG binds to UreE. UreE is the primary carrier of nickel to the complex and UreG supplies the energy for the reaction by carrying out the hydrolysis of GTP to GDP + P_i.

intestinal bacteria that evolve H_2. It is very likely that *H. pylori* obtains a great deal of energy from the oxidation of molecular hydrogen. In fact, a mutant strain unable to oxidize hydrogen is greatly impacted in the ability to colonize mouse stomachs (95).

H. pylori contains a single H_2-oxidizing [NiFe] hydrogenase that is organized as heterotrimeric complexes. It is made up of a small (β) subunit encoded by *hydA*, a large (α) subunit encoded by *hydB* and a membrane anchored cytochrome *b* (γ) subunit encoded by *hydC*. It is hypothesized that the small β subunit contains the [Fe-S] clusters, while the large α subunit contains Ni and Fe. In order for hydrogenase to be active, it must contain nickel in the active site. A battery of accessory proteins, HypABCDEF, carry out the unexpectedly complicated process of hydrogenase maturation. HypA forms dimers in solution and binds two nickel ions per dimer. HypB is a GTPase and is required for hydrogenase activity. Both HypA and HypB also play a role in urease maturation (49).

Oxidative stress

Many organisms often require O_2 for energy production and growth, and O_2 is the most efficient (leads to more ATP production) terminal electron acceptor in biological respiratory pathways. Toxic forms of oxygen can arise however, and these forms can be detrimental to the cell. For example, electron leakage (reducing O_2 to toxic forms) during aerobic respiration is one way that reactive oxygen species (ROS) can be formed in the cell (96). In addition, ROS can be formed by chemical processes or through radiation outside of the cell. More importantly for pathogens is the generation of ROS from the oxidative burst of polymorphonuclear leukocytes (PMN) (97). ROS are usually in the form of superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxide radical (•OH) or hypochlorous acid (HOCl). Following colonization by *H. pylori*, the gastric epithelial cells have been shown to produce an increase in O_2^- and H_2O_2 secretion (98-

102). The production of O_2^- is likely a result of the enzyme NADPH oxidase and H_2O_2 production is due to the spermine oxidase enzyme; both are present in gastric cells (103-105). HOCl can be produced from H_2O_2 through the neutrophil enzyme myeloperoxidase and •OH is produced from H_2O_2 in the presence of transition metals in a process known as the Fenton reactions (Figure 1.2) (106). As a result of these processes occurring in the gastric epithelial cells, *H. pylori* is in constant contact with ROS. Being a microaerophile, one would think the bacteria would be especially sensitive to these forms. However, *H. pylori* produces an impressive array of antioxidant enzymes that counteract the effects of ROS. If ROS are left unmanaged they can cause damage to the macromolecules of the cell.

Oxidative damage of macromolecules. If ROS are not scavenged from the cell or if they accumulate in very high amounts, contact of ROS with the cell material can lead to an array of problems with cellular components. For example, O_2^- can affect many enzymatic processes by causing the release of iron from 4Fe-4S functional groups. Together, the different forms of ROS can lead to DNA, lipid, and protein damage (Figure 1.3) (107). Severe protein oxidation leads to carbonylation, a state that cannot be repaired and that is assigned with cell death.

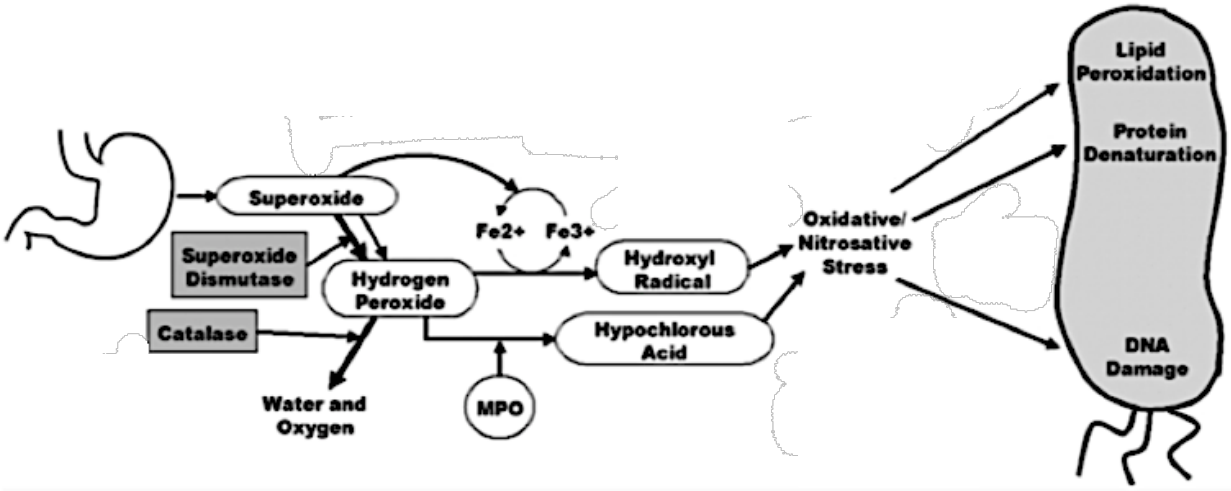

Figure 1.3. Antioxidant pathways of *H. pylori*. Superoxide is produced from the host stomach by the immune responses. This can be converted to hydrogen peroxide by superoxide dismutase. Hydrogen peroxide can be further converted to hypochlorous acid by the host myeloperoxidase or to hydroxyl radical as a result of the Fenton reactions. These forms of oxidants can cause problems for the cell in the form of lipid peroxidation, protein denaturation, and DNA damage.

DNA is often a target of oxidation by ROS such as •OH and superoxide. ROS attacks both the base and sugar moieties and results in a modification of purine and pyrimidine bases, strand breaks, DNA-protein crosslinks, and abasic sites. The most frequently occurring mutagenic lesion is an oxidized form of guanine, 8-oxo-7,8-dihydroguanine (8-oxoG) (108, 109). When 8-oxoG is present in a template strand it induces pairing with adenine during DNA replication resulting in G:C-to-T:A transversions (110, 111). 8-oxoG can pair with either adenine or cytosine (111). It was found that *H. pylori* is, in fact, susceptible to DNA damage from host-derived ROS molecules. Mutants were made in the *nth* gene, making the cell unable to repair oxidized pyrimidines (112). These mutants were significantly more sensitive than the wild-type strain to killing by activated macrophages and were reduced in mouse colonization capacity.

H. pylori has mechanisms to repair damage that occurs to its DNA as a result of oxidation. The DNA methyl-directed mismatch repair (MMR) system consists of three proteins MutS, MutL and MutH. This system maintains genetic stability and functions in post-replication DNA repair. *H. pylori* lacks genes encoding *mutL* and *mutH* but a *mutS* strain is sensitive to oxidative stress (113). When the types of mutations in the *mutS* strain were analyzed it was found that most were in the form of 8-oxoG indicating a role for MutS in the repair of 8-oxoG. A DNA repair system responsible for the removal of 8-oxoG-induced mutations has been described in *Escherichia coli* where it is known as the GO system. This system is initiated by two DNA glycosylases, Fpg and MutY. Fpg recognizes and excises cysteine from an 8-oxoG:C pair. MutY recognizes and excises incorrectly paired adenines opposite from 8-oxoG (114, 115). *H. pylori* has a homologue to MutY that has been shown to behave similarly to the *E. coli* MutY. It has the ability to excise adenine paired with 8-oxo-G and a *mutY* mutant showed a higher spontaneous

mutation frequency as a result of exposure to oxygen. The *mutY* mutants are deficient in mouse colonization compared to the wild-type (116).

Another major target of oxidative attack are lipids (unsaturated phospholipids, glycolipids, and cholesterol) (117, 118). Oxidation of lipids results in loss of membrane fluidity and can interfere with the function of membrane-bound proteins. Lipid peroxidation is a free radical chain reaction that is initiated when free radicals attack polyunsaturated fatty acids in membranes. This results in the formation of a fatty acid radical that is unstable. This molecule then reacts with molecular oxygen to produce a peroxyl-fatty acid radical that further reacts with a lipid molecule, producing a lipid peroxide (119). Lipid peroxide is not a radical molecule *per se* but it promotes the radical chain reaction to proceed (120). The reaction will continue until the concentration of lipid radicals is so high that they react with each other to produce a non-radical species (119). The radicals that are produced during this process are highly toxic, much like superoxide, and cause severe damage to the cell (120).

Proteins are especially susceptible to attack by ROS due to their abundance and reactivity. Many amino acids can be modified by oxidation; some of these modifications are irreversible and others are reversible (121). Upon modification of the protein side chains, a loss of function can occur so these oxidative modifications often result in serious consequences for the cell. The sulfur containing amino acids methionine and cysteine are especially susceptible to attack, but these modifications are reversible. When oxidized, methionine and cysteine form methionine sulfoxide and cysteine disulfides, respectively. These forms of oxidative modification can be repaired back to their native forms. Prolonged oxidation of methionine leads to the formation of methionine sulfone but this form is rarely found in biological systems (122). More severe oxidation leads to the formation of protein carbonyls, an irreversible modification.

Carbonyl groups are aldehydes and ketones that are produced on protein side chains, especially on proline, arginine, lysine and threonine (123). Carbonyl groups can also be formed on lysine, cysteine and histidine by secondary reactions as a result of lipid peroxidation. The cell removes carbonylated proteins by degrading them (124).

Oxidative stress resistance in *H. pylori*

Despite being a microaerophile and being in almost constant contact with ROS during infection of the host, *H. pylori* is able to survive and thrive in this environment. This is due to the production of a battery of antioxidant enzymes: superoxide dismutase (SOD), catalase, peroxidases and a variety of reductases. Catalase and SOD function to neutralize ROS (29, 125-128). Catalase protects the cell by catalyzing the dismutation of H_2O_2 into water and oxygen (97). Catalase itself can become inactivated by oxidants, but it is protected by methionine sulfoxide reductase (Msr) (129). SOD also functions to degrade oxidants. It functions by catalyzing the dismutation of superoxide to H_2O_2 that is then deactivated by catalase or peroxidase (97). *H. pylori* only produces one SOD, as opposed to *E. coli* that produces three (127, 128, 130). Alkylhydroperoxide reductase (AhpC) catalyzes the reduction of alkylhydroperoxide to the corresponding alcohol (131). AhpC is one peroxiredoxin among three found in *H. pylori*. The other two are thiol-peroxidase (Tpx) and bacterioferritin co-migratory protein (BCP). These enzymes catalyze the reduction of H_2O_2, peroxynitrite, and organic hydroperoxides into their corresponding alcohols (132). *H. pylori* also has two thioredoxin (Trx) proteins, Trx1 and Trx2, which function in the maintenance of a reduced cellular environment by acting on oxidized cysteine residues (33, 133).

Methionine sulfoxide reductase. Msr plays a large role in *H. pylori* resistance to oxidative stress. As mentioned above, Msr reduces oxidized methionine back to the native form

resulting in repair of the target protein (134) (Figure 1.4). A *H. pylori* Δ*msr* mutant strain has been shown to be very sensitive to oxidative stress and further studies on this strain showed Msr is an important colonization factor, especially in long-term colonization (135). *H. pylori* Msr protects several proteins from oxidative inactivation. One important protein, catalase, has been shown to be repaired by Msr thus protecting the enzyme from oxidative inactivation (129). Msr in *H. pylori* is composed of MsrA and MsrB proteins fused together as a single enzyme (136). This is the Msr type present in most pathogens. Msr transfers electrons from the oxidized protein to the cysteines present in Msr forming a disulfide bond. Thioredoxin reductase then catalyzes the transfer of these electrons from the disulfide bond to thioredoxin at the expense of NADPH (137, 138). The Msr protein is thus repaired and can continue to repair other oxidized proteins.

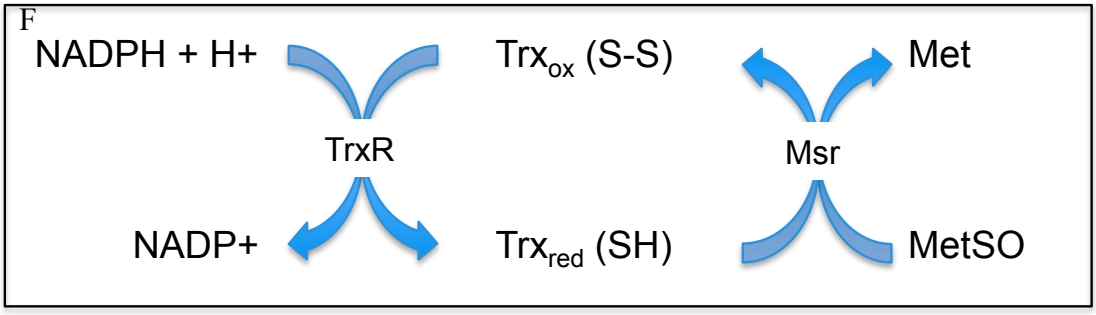

Figure 1.4. Msr reduction of an oxidized protein. The protein's methionine residue is converted to methionine sulfoxide (MetSO) via oxidant attack. During this processes, Msr itself becomes oxidized. Thioredoxin (Trx) then accepts electrons from Msr, resulting in the formation of a disulfide bond (Trx_{ox} (S-S)). Trx is then reduced in a reaction catalyzed by thioredoxin reductase (TrxR) at the expense of a NADPH molecule.

Thioredoxin system. The oxidation-reduction status of *H. pylori* is important due to its exposure to a constantly changing environment. In most cells, the oxidation of thiols leads to a buildup of the disulfide forms of proteins and accumulation of other small compounds such as glutathionine (GSH) or cysteine (97). GSH plays a large role in the maintenance of redox balance in cells but there is no evidence of this compound being present in *H. pylori* (33, 34). There is evidence that free cysteine plays a role in the maintenance of redox balance in *H. pylori*, but this is from unpublished data (97). In fact, cysteine is not an appropriate free thiol compound in aerobic organisms as it is oxidized very rapidly in the presence of a metal catalyst. Thus, it does not seem likely that this would result in maintaining redox balance since *H. pylori* is under constant ROS stress. However, *H. pylori* does have a thioredoxin (Trx) system that is involved in protecting the cell from oxidative stress. A *trx1* and *trx2* deletion mutant showed sensitivity to a variety of oxidants tested (139). It seems likely that the Trx system is the major player in maintaining redox homeostasis in *H. pylori*.

The thioredoxin system is highly conserved across all organisms from humans to bacteria. Many organisms contain several Trx proteins and sometimes these proteins play separate and distinct roles in protecting the cell from oxidative stress. The Trx system functions to reduce cysteine residues at the expense of NADPH. Trx reductase catalyzes the transfer of electrons and functions to keep the Trx proteins in a reduced state (140). *H. pylori* contains two Trx proteins, Trx1 and Trx2. Trx1 has been shown to be the electron donor to Msr and AhpC but the function of Trx2 remains unknown (131, 136).

Scope of study

H. pylori has a variety of unique characteristics that allow it to be a successful pathogen. One of the most interesting features is the ability to withstand a harsh environment and to evade or combat the host immune system, especially combating toxic oxygen species. This attribute is especially surprising since the bacterium is a microaerophile, and a high oxygen concentration leads to the organism being unable to survive for more than a few hours. *H. pylori* is highly adapted to the gastric niche, and it is able to use several strategies to detoxify oxygen during exposures to an apparently unique environment. It neutralizes oxygen by the use of enzymes such as catalase and superoxide dismutase, modulating redox potential, or through repair of damaged DNA or proteins.

While we understand the general functions of Msr and the thioredoxin system, we don't know in how many cellular processes they are involved to maintain viable *H. pylori*. In fact, we have only identified a few proteins with which either Msr or Trx interact. It is likely that Msr plays a role in reducing many proteins in the cell, as a deletion mutant strain shows greatly enhanced sensitivity to oxidant stress, and the methionine repair process is required for colonization of the mouse stomach (135). My studies on Msr revealed a new role for the enzyme. In addition, the Trx system has not been studied in *H. pylori* in great detail and we do not know the function of Trx2 in the cell. A previous study showed a Δ*trx1* mutant strain was oxidant stress sensitive, but that a Δ*trx2* strain was not very stress sensitive (139). However, no further studies were performed and it is likely that both of the Trx proteins play a role in *H. pylori* survival. My work on the thioredoxin proteins identifies for the first time that Trx2 plays a major role in protecting the macromolecules from oxidative damage. I also identify a role for Trx2 in colonization of the mouse stomach.

References

1. **Marshall BJ.** 1986. *Campylobacter pyloridis* and gastritis. J Infect Dis **153:**650-657.

2. **Marshall BJ.** 1988. The *Campylobacter pylori* story. Scand J Gastroenterol **146:**58-66.

3. **Marshall BJ, Armstrong JA, McGechie DB, Glancy RJ.** 1985. Attempt to fulfil Koch's postulates for pyloric *Campylobacter*. Med J Aust **142:**436-439.

4. **Marshall BJ, McGechie DB, Rogers PA, Glancy RJ.** 1985. Pyloric *Campylobacter* infection and gastroduodenal disease. Med J Aust **142:**439-444.

5. **Mobley HLT, Mendz GL, Hazell SL.** 2001. Overview. *In* Mobley HLT, Mendz GL, Hazell SL (ed.), *Helicobacter pylori*: Physiology and Genetics, Washington (DC).

6. **Graham DY, Lew GM, Klein PD, Evans DG, Evans DJ, Jr., Saeed ZA, Malaty HM.** 1992. Effect of treatment of *Helicobacter pylori* infection on the long-term recurrence of gastric or duodenal ulcer. A randomized, controlled study. Ann Intern Med **116:**705-708.

7. **Parsonnet J, Shmuely H, Haggerty T.** 1999. Fecal and oral shedding of *Helicobacter pylori* from healthy infected adults. JAMA **282:**2240-2245.

8. **Ernst PB, Gold BD.** 2000. The disease spectrum of *Helicobacter pylori*: the immunopathogenesis of gastroduodenal ulcer and gastric cancer. Annu Rev Microbiol **54:**615-640.

9. **Foundation TH** 2014, posting date. Epidemiology. [Online.]

10. **Handt LK, Fox JG, Dewhirst FE, Fraser GJ, Paster BJ, Yan LL, Rozmiarek H, Rufo R, Stalis IH.** 1994. *Helicobacter pylori* isolated from the domestic cat: public health implications. Infect Immun **62:**2367-2374.

11. **Ansorg R, Von Heinegg EH, Von Recklinghausen G.** 1995. Cat owners' risk of acquiring a *Helicobacter pylori* infection. Int J Med Microbiol **283:**122-126.

12. **Bode G, Rothenbacher D, Brenner H, Adler G.** 1998. Pets are not a risk factor for *Helicobacter pylori* infection in young children: results of a population-based study in Southern Germany. Pediatr Infect Dis J **17:**909-912.

13. **Osato MS, Ayub K, Le HH, Reddy R, Graham DY.** 1998. Houseflies are an unlikely reservoir or vector for *Helicobacter pylori*. J Clin Microbiol **36:**2786-2788.

14. **Sathar MA, Gouws E, Simjee AE, Mayat AM.** 1997. Seroepidemiological study of *Helicobacter pylori* infection in South African children. Trans R Soc Trop Med Hyg **91:**393-395.

15. **Hulten K, Han SW, Enroth H, Klein PD, Opekun AR, Gilman RH, Evans DG, Engstrand L, Graham DY, El-Zaatari FA.** 1996. *Helicobacter pylori* in the drinking water in Peru. Gastroenterology **110:**1031-1035.

16. **Klein PD, Graham DY, Gaillour A, Opekun AR, Smith EO.** 1991. Water source as risk factor for *Helicobacter pylori* infection in Peruvian children. Gastrointestinal Physiology Working Group. Lancet **337:**1503-1506.

17. **Hulten K, Enroth H, Nystrom T, Engstrand L.** 1998. Presence of *Helicobacter* species DNA in Swedish water. J Appl Microbiol **85:**282-286.

18. **Sasaki K, Tajiri Y, Sata M, Fujii Y, Matsubara F, Zhao M, Shimizu S, Toyonaga A, Tanikawa K.** 1999. *Helicobacter pylori* in the natural environment. Scand J Infect Dis **31:**275-279.

19. **Veyrier FJ, Ecobichon C, Boneca IG.** 2013. Draft Genome Sequence of Strain X47-2AL, a Feline *Helicobacter pylori* Isolate. Genome Announc **1**.

20. **Megraud F.** 1995. Transmission of *Helicobacter pylori*: faecal-oral versus oral-oral route. Aliment Pharmacol Ther **9 Suppl 2:**85-91.

21. **Berkowicz J, Lee A.** 1987. Person-to-person transmission of *Campylobacter pylori*. Lancet **2:**680-681.

22. **Kimura A, Matsubasa T, Kinoshita H, Kuriya N, Yamashita Y, Fujisawa T, Terakura H, Shinohara M.** 1999. *Helicobacter pylori* seropositivity in patients with severe neurologic impairment. Brain Dev **21:**113-117.

23. **Lambert JR, Lin SK, Sievert W, Nicholson L, Schembri M, Guest C.** 1995. High prevalence of *Helicobacter pylori* antibodies in an institutionalized population: evidence for person-to-person transmission. Am J Gastroent **90:**2167-2171.

24. **Vincent P, Gottrand F, Pernes P, Husson MO, Lecomte-Houcke M, Turck D, Leclerc H.** 1994. High prevalence of *Helicobacter pylori* infection in cohabiting children. Epidemiology of a cluster, with special emphasis on molecular typing. Gut **35:**313-316.

25. **Goodwin CS, McConnell W, McCulloch RK, McCullough C, Hill R, Bronsdon MA, Kasper G.** 1989. Cellular fatty acid composition of *Campylobacter pylori* from primates and ferrets compared with those of other campylobacters. J Clin Microbiol **27:**938-943.

26. **Chan WY, Hui PK, Leung KM, Chow J, Kwok F, Ng CS.** 1994. Coccoid forms of *Helicobacter pylori* in the human stomach. Am J Clin Pathol **102:**503-507.

27. **Catrenich CE, Makin KM.** 1991. Characterization of the morphologic conversion of *Helicobacter pylori* from bacillary to coccoid forms. Scand J Gastroenterol **181:**58-64.

28. **Tsuda M, Karita M, Morshed MG, Okita K, Nakazawa T.** 1994. A urease-negative mutant of *Helicobacter pylori* constructed by allelic exchange mutagenesis lacks the ability to colonize the nude mouse stomach. Infect Immun **62:**3586-3589.

29. **Hazell SL, Evans DJ, Jr., Graham DY.** 1991. *Helicobacter pylori* catalase. J Gen Microbiol **137:**57-61.

30. **Owen RJ.** 1995. Bacteriology of *Helicobacter pylori*. Baillieres Clin Gastroenterol **9:**415-446.

31. **Costa K, Bacher G, Allmaier G, Dominguez-Bello MG, Engstrand L, Falk P, de Pedro MA, Garcia-del Portillo F.** 1999. The morphological transition of *Helicobacter pylori* cells from spiral to coccoid is preceded by a substantial modification of the cell wall. J Bacteriol **181:**3710-3715.

32. **Krishnamurthy P, Phadnis SH, DeLoney CR, Rosenthal RS, Dunn BE.** 2001. Biosynthetic Pathways Related to Cell Structure and Function. *In* Mobley HLT, Mendz GL, Hazell SL (ed.), *Helicobacter pylori*: Physiology and Genetics, Washington (DC).

33. **Tomb JF, White O, Kerlavage AR, Clayton RA, Sutton GG, Fleischmann RD, Ketchum KA, Klenk HP, Gill S, Dougherty BA, Nelson K, Quackenbush J, Zhou L, Kirkness EF, Peterson S, Loftus B, Richardson D, Dodson R, Khalak HG, Glodek A, McKenney K, Fitzegerald LM, Lee N, Adams MD, Hickey EK, Berg DE, Gocayne JD, Utterback TR, Peterson JD, Kelley JM, Cotton MD, Weidman JM, Fujii C, Bowman C, Watthey L, Wallin E, Hayes WS, Borodovsky M, Karp PD, Smith HO, Fraser CM, Venter JC.** 1997. The complete genome sequence of the gastric pathogen *Helicobacter pylori*. Nature **388:**539-547.

34. **Alm RA, Ling LS, Moir DT, King BL, Brown ED, Doig PC, Smith DR, Noonan B, Guild BC, deJonge BL, Carmel G, Tummino PJ, Caruso A, Uria-Nickelsen M, Mills DM, Ives C, Gibson R, Merberg D, Mills SD, Jiang Q, Taylor DE, Vovis GF, Trust TJ.** 1999. Genomic-sequence comparison of two unrelated isolates of the human gastric pathogen *Helicobacter pylori*. Nature **397:**176-180.

35. **Eaton KA, Morgan DR, Krakowka S.** 1989. *Campylobacter pylori* virulence factors in gnotobiotic piglets. Infect Immun **57:**1119-1125.

36. **Alm RA, Noonan B.** 2001. The Genome. *In* Mobley HLT, Mendz GL, Hazell SL (ed.), *Helicobacter pylori*: Physiology and Genetics, Washington (DC).

37. **Nedenskov P.** 1994. Nutritional requirements for growth of *Helicobacter pylori*. Appl Environ Microbiol **60:**3450-3453.

38. **Reynolds DJ, Penn CW.** 1994. Characteristics of *Helicobacter pylori* growth in a defined medium and determination of its amino acid requirements. Microbiology **140 (Pt 10):**2649-2656.

39. **Stark RM, Suleiman MS, Hassan IJ, Greenman J, Millar MR.** 1997. Amino acid utilisation and deamination of glutamine and asparagine by *Helicobacter pylori*. J Med Microbiol **46:**793-800.

40. **Mobley HLT.** 2001. Urease. *In* Mobley HLT, Mendz GL, Hazell SL (ed.), *Helicobacter pylori*: Physiology and Genetics, Washington (DC).

41. **Williams CL, Preston T, Hossack M, Slater C, McColl KE.** 1996. *Helicobacter pylori* utilises urea for amino acid synthesis. FEMS Immunol Med Microbiol **13:**87-94.

42. **Doig P, de Jonge BL, Alm RA, Brown ED, Uria-Nickelsen M, Noonan B, Mills SD, Tummino P, Carmel G, Guild BC, Moir DT, Vovis GF, Trust TJ.** 1999. *Helicobacter pylori* physiology predicted from genomic comparison of two strains. Microbiol Mol Biol Rev **63:**675-707.

43. **Weinberg MV, Maier RJ.** 2007. Peptide transport in *Helicobacter pylori*: roles of *dpp* and *opp* systems and evidence for additional peptide transporters. J Bacteriol **189:**3392-3402.

44. **Hoffman PS, Goodwin A, Johnsen J, Magee K, Veldhuyzen van Zanten SJ.** 1996. Metabolic activities of metronidazole-sensitive and -resistant strains of *Helicobacter pylori*: repression of pyruvate oxidoreductase and expression of isocitrate lyase activity correlate with resistance. J Bacteriol **178:**4822-4829.

45. **Chalk PA, Roberts AD, Blows WM.** 1994. Metabolism of pyruvate and glucose by intact cells of *Helicobacter pylori* studied by ^{13}C NMR spectroscopy. Microbiology **140 (Pt 8):**2085-2092.

46. **Mendz GL, Hazell SL, Burns BP.** 1994. The Entner-Doudoroff pathway in *Helicobacter pylori*. Arch Biochem Biophys **312:**349-356.

47. **Mendz GL, Hazell SL, Burns BP.** 1993. Glucose utilization and lactate production by *Helicobacter pylori*. J Gen Microbiol **139:**3023-3028.

48. **Mendz GL, Hazell SL, van Gorkom L.** 1994. Pyruvate metabolism in *Helicobacter pylori*. Arch Microbiol **162:**187-192.

49. **Benoit SL, Maier RJ.** 2008. Hydrogen and nickel metabolism in *Helicobacter* species. Ann N Y Acad Sci **1125:**242-251.

50. **Maier RJ, Fu C, Gilbert J, Moshiri F, Olson J, Plaut AG.** 1996. Hydrogen uptake hydrogenase in *Helicobacter pylori*. FEMS Microbiol Lett **141:**71-76.

51. **van Vliet AHM, Bereswill S, Kusters JG.** 2001. Ion Metabolism and Transport. *In* Mobley HLT, Mendz GL, Hazell SL (ed.), *Helicobacter pylori*: Physiology and Genetics, Washington (DC).

52. **Christensen JM, Kristiansen J, Nielsen NH, Menne T, Byrialsen K.** 1999. Nickel concentrations in serum and urine of patients with nickel eczema. Toxicol Lett **108:**185-189.

53. **Wood DW, Setubal JC, Kaul R, Monks DE, Kitajima JP, Okura VK, Zhou Y, Chen L, Wood GE, Almeida NF, Jr., Woo L, Chen Y, Paulsen IT, Eisen JA, Karp PD, Bovee D, Sr., Chapman P, Clendenning J, Deatherage G, Gillet W, Grant C, Kutyavin T, Levy R, Li MJ, McClelland E, Palmieri A, Raymond C, Rouse G, Saenphimmachak C, Wu Z, Romero P, Gordon D, Zhang S, Yoo H, Tao Y, Biddle P, Jung M, Krespan W, Perry M, Gordon-Kamm B, Liao L, Kim S, Hendrick C, Zhao ZY, Dolan M, Chumley F, Tingey SV, Tomb JF, Gordon MP, Olson MV, Nester EW.** 2001. The genome of the natural genetic engineer *Agrobacterium tumefaciens* C58. Science **294:**2317-2323.

54. **Rubio MP, Guerra S, Almendral JM.** 2001. Genome replication and postencapsidation functions mapping to the nonstructural gene restrict the host range of a murine parvovirus in human cells. J Virol **75:**11573-11582.

55. **Fulkerson JF, Jr., Garner RM, Mobley HL.** 1998. Conserved residues and motifs in the NixA protein of *Helicobacter pylori* are critical for the high affinity transport of nickel ions. J Biol Chem **273:**235-241.

56. **Penzes Z, Gonzalez JM, Calvo E, Izeta A, Smerdou C, Mendez A, Sanchez CM, Sola I, Almazan F, Enjuanes L.** 2001. Complete genome sequence of transmissible gastroenteritis coronavirus PUR46-MAD clone and evolution of the purdue virus cluster. Virus genes **23:**105-118.

57. **Huesca M, Borgia S, Hoffman P, Lingwood CA.** 1996. Acidic pH changes receptor binding specificity of *Helicobacter pylori*: a binary adhesion model in which surface heat shock (stress) proteins mediate sulfatide recognition in gastric colonization. Infect Immun **64:**2643-2648.

58. **Gilbert JV, Ramakrishna J, Sunderman FW, Jr., Wright A, Plaut AG.** 1995. Protein Hpn: cloning and characterization of a histidine-rich metal-binding polypeptide in *Helicobacter pylori* and *Helicobacter mustelae*. Infect Immun **63:**2682-2688.

59. **Seshadri S, Benoit SL, Maier RJ.** 2007. Roles of His-rich *hpn* and *hpn*-like proteins in *Helicobacter pylori* nickel physiology. J Bacteriol **189:**4120-4126.

60. **Peura DA, Pambianco DJ, Dye KR, Lind C, Frierson HF, Hoffman SR, Combs MJ, Guilfoyle E, Marshall BJ.** 1996. Microdose ^{14}C-urea breath test offers diagnosis of *Helicobacter pylori* in 10 minutes. Am J Gastroent **91:**233-238.

61. **Cave DR, Hoffman JS.** 1996. Management of *Helicobacter pylori* infection in ulcer disease. Hosp Pract (1995) **31:**63-64, 67-69, 73-65.

62. **Mendz GL, Hazell SL.** 1993. Fumarate catabolism in *Helicobacter pylori*. Biochem Mol Biol Int **31:**325-332.

63. **Mendz GL, Hazell SL.** 1993. Glucose phosphorylation in *Helicobacter pylori*. Arch Biochem Biophys **300:**522-525.

64. **Pressler U, Staudenmaier H, Zimmermann L, Braun V.** 1988. Genetics of the iron dicitrate transport system of *Escherichia coli*. J Bacteriol **170:**2716-2724.

65. **Schauer K, Gouget B, Carriere M, Labigne A, de Reuse H.** 2007. Novel nickel transport mechanism across the bacterial outer membrane energized by the TonB/ExbB/ExbD machinery. Mol Microbiol **63:**1054-1068.

66. **Yakoob J, Abbas Z, Jafri W, Usman MW, Jafri F, Awan S.** 2013. Comparison of the virulence markers of *Helicobacter pylori* and their associated diseases in patients from Pakistan and Afghanistan. Saudi J Gastroenterol **19:**211-218.

67. **Testerman TL, McGee DJ, Mobley HLT.** 2001. Adherence and Colonization. *In* Mobley HLT, Mendz GL, Hazell SL (ed.), *Helicobacter pylori*: Physiology and Genetics, Washington (DC).

68. **Hazell SL, Lee A, Brady L, Hennessy W.** 1986. *Campylobacter pyloridis* and gastritis: association with intercellular spaces and adaptation to an environment of mucus as important factors in colonization of the gastric epithelium. J Infect Dis **153:**658-663.

69. **Tricottet V, Bruneval P, Vire O, Camilleri JP, Bloch F, Bonte N, Roge J.** 1986. *Campylobacter*-like organisms and surface epithelium abnormalities in active, chronic gastritis in humans: an ultrastructural study. Ultrastruct Pathol **10:**113-122.

70. **Allen LA.** 1999. Intracellular niches for extracellular bacteria: lessons from *Helicobacter pylori*. J Leukoc Biol **66:**753-756.

71. **Bode G, Malfertheiner P, Ditschuneit H.** 1988. Pathogenetic implications of ultrastructural findings in *Campylobacter pylori* related gastroduodenal disease. Scand J Gastroenterol **142:**25-39.

72. **Boren T, Falk P, Roth KA, Larson G, Normark S.** 1993. Attachment of *Helicobacter pylori* to human gastric epithelium mediated by blood group antigens. Science **262:**1892-1895.

73. **Caselli M, Figura N, Trevisani L, Pazzi P, Guglielmetti P, Bovolenta MR, Stabellini G.** 1989. Patterns of physical modes of contact between *Campylobacter pylori* and gastric epithelium: implications about the bacterial pathogenicity. Am J Gastroent **84:**511-513.

74. **Clyne M, Drumm B.** 1996. Cell envelope characteristics of *Helicobacter pylori*: their role in adherence to mucosal surfaces and virulence. FEMS Immunol Med Microbiol **16:**141-155.

75. **Corthesy-Theulaz I, Porta N, Pringault E, Racine L, Bogdanova A, Kraehenbuhl JP, Blum AL, Michetti P.** 1996. Adhesion of *Helicobacter pylori* to polarized T84 human intestinal cell monolayers is pH dependent. Infect Immun **64:**3827-3832.

76. **Dytoc M, Gold B, Louie M, Huesca M, Fedorko L, Crowe S, Lingwood C, Brunton J, Sherman P.** 1993. Comparison of *Helicobacter pylori* and attaching-effacing *Escherichia coli* adhesion to eukaryotic cells. Infect Immun **61:**448-456.

77. **Goodwin CS, Armstrong JA, Marshall BJ.** 1986. *Campylobacter pyloridis*, gastritis, and peptic ulceration. J Clin Pathol **39:**353-365.

78. **Hemalatha SG, Drumm B, Sherman P.** 1991. Adherence of *Helicobacter pylori* to human gastric epithelial cells in vitro. J Med Microbiol **35:**197-202.

79. **Hessey SJ, Spencer J, Wyatt JI, Sobala G, Rathbone BJ, Axon AT, Dixon MF.** 1990. Bacterial adhesion and disease activity in *Helicobacter* associated chronic gastritis. Gut **31:**134-138.

80. **Kobayashi Y, Okazaki K, Murakami K.** 1993. Adhesion of *Helicobacter pylori* to gastric epithelial cells in primary cultures obtained from stomachs of various animals. Infect Immun **61:**4058-4063.

81. **Neman-Simha V, Megraud F.** 1988. In vitro model for *Campylobacter pylori* adherence properties. Infect Immun **56:**3329-3333.

82. **Noach LA, Rolf TM, Tytgat GN.** 1994. Electron microscopic study of association between *Helicobacter pylori* and gastric and duodenal mucosa. J Clin Pathol **47:**699-704.

83. **Rosberg K, Berglindh T, Gustavsson S, Hubinette R, Rolfsen W.** 1991. Adhesion of *Helicobacter pylori* to human gastric mucosal biopsy specimens cultivated in vitro. Scand J Gastroenterol **26:**1179-1187.

84. **Rudmann DG, Eaton KA, Krakowka S.** 1992. Ultrastructural study of *Helicobacter pylori* adherence properties in gnotobiotic piglets. Infect Immun **60:**2121-2124.

85. **Segal ED, Falkow S, Tompkins LS.** 1996. *Helicobacter pylori* attachment to gastric cells induces cytoskeletal rearrangements and tyrosine phosphorylation of host cell proteins. Proc Natl Acad Sci U S A **93:**1259-1264.

86. **Smoot DT, Resau JH, Naab T, Desbordes BC, Gilliam T, Bull-Henry K, Curry SB, Nidiry J, Sewchand J, Mills-Robertson K, et al.** 1993. Adherence of *Helicobacter pylori* to cultured human gastric epithelial cells. Infect Immun **61:**350-355.

87. **Garner RM, Fulkerson J, Jr., Mobley HL.** 1998. *Helicobacter pylori* glutamine synthetase lacks features associated with transcriptional and posttranslational regulation. Infect Immun **66:**1839-1847.

88. **Marcus EA, Scott DR.** 2001. Cell lysis is responsible for the appearance of extracellular urease in *Helicobacter pylori*. Helicobacter **6:**93-99.

89. **Jabri E, Carr MB, Hausinger RP, Karplus PA.** 1995. The crystal structure of urease from *Klebsiella aerogenes*. Science **268:**998-1004.

90. **Cussac V, Ferrero RL, Labigne A.** 1992. Expression of *Helicobacter pylori* urease genes in *Escherichia coli* grown under nitrogen-limiting conditions. J Bacteriol **174:**2466-2473.

91. **Hu LT, Foxall PA, Russell R, Mobley HL.** 1992. Purification of recombinant *Helicobacter pylori* urease apoenzyme encoded by *ureA* and *ureB*. Infect Immun **60:**2657-2666.

92. **Hu LT, Mobley HL.** 1993. Expression of catalytically active recombinant *Helicobacter pylori* urease at wild-type levels in *Escherichia coli*. Infect Immun **61:**2563-2569.

93. **Stingl K, De Reuse H.** 2005. Staying alive overdosed: how does *Helicobacter pylori* control urease activity? Int J Med Microbiol **295:**307-315.

94. **Stingl K, Schauer K, Ecobichon C, Labigne A, Lenormand P, Rousselle JC, Namane A, de Reuse H.** 2008. In vivo interactome of *Helicobacter pylori* urease revealed by tandem affinity purification. Mol Cell Proteomics **7:**2429-2441.

95. **Olson JW, Maier RJ.** 2002. Molecular hydrogen as an energy source for *Helicobacter pylori*. Science **298:**1788-1790.

96. **Ksenzenko M, Vygodina TV, Berka V, Ruuge EK, Konstantinov AA.** 1992. Cytochrome oxidase-catalyzed superoxide generation from hydrogen peroxide. FEBS letters **297:**63-66.

97. **Hazell SL, Harris AG, Trend MA.** 2001. Evasion of the Toxic Effects of Oxygen. *In* Mobley HLT, Mendz GL, Hazell SL (ed.), *Helicobacter pylori*: Physiology and Genetics, Washington (DC).

98. **Bagchi D, Bhattacharya G, Stohs SJ.** 1996. Production of reactive oxygen species by gastric cells in association with *Helicobacter pylori*. Free Radic Res **24:**439-450.

99. **Davies GR, Banatvala N, Collins CE, Sheaff MT, Abdi Y, Clements L, Rampton DS.** 1994. Relationship between infective load of *Helicobacter pylori* and reactive oxygen metabolite production in antral mucosa. Scand J Gastroenterol **29:**419-424.

100. **Crabtree JE.** 1996. Immune and inflammatory responses to *Helicobacter pylori* infection. Scand J Gastroenterol **215:**3-10.

101. **Davies GR, Simmonds NJ, Stevens TR, Grandison A, Blake DR, Rampton DS.** 1992. Mucosal reactive oxygen metabolite production in duodenal ulcer disease. Gut **33:**1467-1472.

102. **Nalini S, Ramakrishna BS, Mohanty A, Balasubramanian KA.** 1992. Hydroxyl radical formation in human gastric juice. J Gastroenterol Hepatol **7:**497-501.

103. **Ben-Hamida A, Man WK, McNeil N, Spencer J.** 1998. Histamine, xanthine oxidase generated oxygen-derived free radicals and *Helicobacter pylori* in gastroduodenal inflammation and ulceration. Inflamm Res **47:**193-199.

104. **Teshima S, Tsunawaki S, Rokutan K.** 1999. *Helicobacter pylori* lipopolysaccharide enhances the expression of NADPH oxidase components in cultured guinea pig gastric mucosal cells. FEBS Lett **452:**243-246.

105. **Chaturvedi R, Asim M, Romero-Gallo J, Barry DP, Hoge S, de Sablet T, Delgado AG, Wroblewski LE, Piazuelo MB, Yan F, Israel DA, Casero RA, Jr., Correa P, Gobert AP, Polk DB, Peek RM, Jr., Wilson KT.** 2011. Spermine oxidase mediates the gastric cancer risk associated with *Helicobacter pylori* CagA. Gastroenterology **141:**1696-1708 e1691-1692.

106. **Kettle AJ, Winterbourn CC.** 1988. Superoxide modulates the activity of myeloperoxidase and optimizes the production of hypochlorous acid. Biochem J **252:**529-536.

107. **Stent A, Every AL, Sutton P.** 2012. *Helicobacter pylori* defense against oxidative attack. Am J Physiol Gastrointest Liver Physiol **302:**G579-587.

108. **Cheng KC, Cahill DS, Kasai H, Nishimura S, Loeb LA.** 1992. 8-Hydroxyguanine, an abundant form of oxidative DNA damage, causes G----T and A----C substitutions. J Biol Chem **267:**166-172.

109. **Halliwell B, Dizdaroglu M.** 1992. The measurement of oxidative damage to DNA by HPLC and GC/MS techniques. Free Radic Res Commun **16:**75-87.

110. **Grollman AP, Moriya M.** 1993. Mutagenesis by 8-oxoguanine: an enemy within. Trends Genet **9:**246-249.

111. **Shibutani S, Takeshita M, Grollman AP.** 1991. Insertion of specific bases during DNA synthesis past the oxidation-damaged base 8-oxodG. Nature **349:**431-434.

112. **O'Rourke EJ, Chevalier C, Pinto AV, Thiberge JM, Ielpi L, Labigne A, Radicella JP.** 2003. Pathogen DNA as target for host-generated oxidative stress: role for repair of bacterial DNA damage in *Helicobacter pylori* colonization. Proc Natl Acad Sci U S A **100:**2789-2794.

113. **Wang G, Alamuri P, Humayun MZ, Taylor DE, Maier RJ.** 2005. The *Helicobacter pylori* MutS protein confers protection from oxidative DNA damage. Mol Microbiol **58:**166-176.

114. **Michaels ML, Miller JH.** 1992. The GO system protects organisms from the mutagenic effect of the spontaneous lesion 8-hydroxyguanine (7,8-dihydro-8-oxoguanine). J Bacteriol **174:**6321-6325.

115. **Michaels ML, Tchou J, Grollman AP, Miller JH.** 1992. A repair system for 8-oxo-7,8-dihydrodeoxyguanine. Biochemistry **31:**10964-10968.

116. **Eutsey R, Wang G, Maier RJ.** 2007. Role of a MutY DNA glycosylase in combating oxidative DNA damage in *Helicobacter pylori*. DNA repair **6:**19-26.

117. **Girotti AW.** 1985. Mechanisms of lipid peroxidation. J Free Radic Biol Med **1:**87-95.

118. **Halliwell B, Gutteridge JM.** 1990. Role of free radicals and catalytic metal ions in human disease: an overview. Methods Enzymol **186:**1-85.

119. **Girotti AW.** 1998. Lipid hydroperoxide generation, turnover, and effector action in biological systems. J Lipid Res **39:**1529-1542.

120. **Wang G, Hong Y, Johnson MK, Maier RJ.** 2006. Lipid peroxidation as a source of oxidative damage in *Helicobacter pylori*: protective roles of peroxiredoxins. Biochim Biophys Acta **1760:**1596-1603.

121. **Cabiscol E, Tamarit J, Ros J.** 2000. Oxidative stress in bacteria and protein damage by reactive oxygen species. Int Microbiol **3:**3-8.

122. **Vogt W.** 1995. Oxidation of methionyl residues in proteins: tools, targets, and reversal. Free Radic Biol Med **18:**93-105.

123. **Dalle-Donne I, Giustarini D, Colombo R, Rossi R, Milzani A.** 2003. Protein carbonylation in human diseases. Trends Mol Med **9:**169-176.

124. **Nystrom T.** 2005. Role of oxidative carbonylation in protein quality control and senescence. EMBO J **24:**1311-1317.

125. **Manos J, Kolesnikow T, Hazell SL.** 1998. An investigation of the molecular basis of the spontaneous occurrence of a catalase-negative phenotype in *Helicobacter pylori*. Helicobacter **3:**28-38.

126. **Odenbreit S, Wieland B, Haas R.** 1996. Cloning and genetic characterization of *Helicobacter pylori* catalase and construction of a catalase-deficient mutant strain. J Bacteriol **178:**6960-6967.

127. **Pesci EC, Pickett CL.** 1994. Genetic organization and enzymatic activity of a superoxide dismutase from the microaerophilic human pathogen, *Helicobacter pylori*. Gene **143:**111-116.

128. **Spiegelhalder C, Gerstenecker B, Kersten A, Schiltz E, Kist M.** 1993. Purification of *Helicobacter pylori* superoxide dismutase and cloning and sequencing of the gene. Infect Immun **61:**5315-5325.

129. **Mahawar M, Tran V, Sharp JS, Maier RJ.** 2011. Synergistic roles of *Helicobacter pylori* methionine sulfoxide reductase and GroEL in repairing oxidant-damaged catalase. J Biol Chem **286:**19159-19169.

130. **Benov LT, Fridovich I.** 1994. Escherichia coli expresses a copper- and zinc-containing superoxide dismutase. J Biol Chem **269:**25310-25314.

131. **Baker LM, Raudonikiene A, Hoffman PS, Poole LB.** 2001. Essential thioredoxin-dependent peroxiredoxin system from *Helicobacter pylori*: genetic and kinetic characterization. J Bacteriol **183:**1961-1973.

132. **Wang G, Alamuri P, Maier RJ.** 2006. The diverse antioxidant systems of *Helicobacter pylori*. Mol Microbiol **61:**847-860.

133. **Windle HJ, Fox A, Ni Eidhin D, Kelleher D.** 2000. The thioredoxin system of *Helicobacter pylori*. J Biol Chem **275:**5081-5089.

134. **Brot N, Weissbach L, Werth J, Weissbach H.** 1981. Enzymatic reduction of protein-bound methionine sulfoxide. Proc Natl Acad Sci U S A **78:**2155-2158.

135. **Alamuri P, Maier RJ.** 2004. Methionine sulphoxide reductase is an important antioxidant enzyme in the gastric pathogen *Helicobacter pylori*. Mol Microbiol **53:**1397-1406.

136. **Alamuri P, Maier RJ.** 2006. Methionine sulfoxide reductase in *Helicobacter pylori*: interaction with methionine-rich proteins and stress-induced expression. J Bacteriol **188:**5839-5850.

137. **Boschi-Muller S, Azza S, Sanglier-Cianferani S, Talfournier F, Van Dorsselear A, Branlant G.** 2000. A sulfenic acid enzyme intermediate is involved in the catalytic mechanism of peptide methionine sulfoxide reductase from *Escherichia coli*. J Biol Chem **275:**35908-35913.

138. **Olry A, Boschi-Muller S, Marraud M, Sanglier-Cianferani S, Van Dorsselear A, Branlant G.** 2002. Characterization of the methionine sulfoxide reductase activities of PILB, a probable virulence factor from Neisseria meningitidis. J Biol Chem **277:**12016-12022.

139. **Comtois SL, Gidley MD, Kelly DJ.** 2003. Role of the thioredoxin system and the thiol-peroxidases Tpx and Bcp in mediating resistance to oxidative and nitrosative stress in *Helicobacter pylori*. Microbiology **149:**121-129.

140. **Holmgren A.** 1985. Thioredoxin. Annu Rev Biochem **54:**237-271.

CHAPTER 2

THE ROLE OF *HELICOBACTER PYLORI* METHIONINE SULFOXIDE REDUCTASE IN UREASE MATURATION [1]

[1] Kuhns, L.G, Mahawar, M., Sharp, J.S., Benoit, S and R. J. Maier. 2013. *Biochem. J.* 450:141-148

Abstract

The persistence of the gastric pathogen *Helicobacter pylori* is due in part to urease and methionine sulfoxide reductase (Msr). Upon exposure to relatively mild (21% partial pressure O_2) oxidative stress, a Δ*msr* mutant showed both decreased urease specific activity in cell free extracts and decreased nickel associated with the partially purified urease fraction as compared to the parent strain; yet urease apo-protein levels were the same for the Δ*msr* and wild type extracts. Urease activity of the Δ*msr* mutant was not significantly different than the wild-type upon non-stress microaerobic incubation of strains. Urease maturation occurs through Ni mobilization via a suite of known accessory proteins, one being the GTPase UreG. Treatment of UreG with hydrogen peroxide (H_2O_2) resulted in oxidation of MS-identified methionine residues and loss of up to 70% of its GTPase activity. Incubation of pure H_2O_2-treated UreG with Msr led to reductive repair of nine Met residues and recovery of up to full enzyme activity. Binding of Msr to both oxidized and non-oxidized UreG was observed by crosslinking. Therefore, we conclude Msr aids the survival of *H. pylori* in part by ensuring continual UreG-mediated urease maturation under stress conditions.

Introduction

Helicobacter pylori is a Gram-negative, microaerophilic bacterium that colonizes the gastric mucosa (1) of approximately one-half of the world's population (2). The World Health Organization classifies *H. pylori* as a carcinogen and it is the causative agent of most peptic ulcer disease, chronic gastritis, and gastric cancer in humans (3). The pathogenesis and persistence attributes of *H. pylori* rely on its ability to combat harsh conditions; these include both the acidic environment of the gastric lumen and chronic exposure to reactive oxygen species (ROS) (3). During colonization, *H. pylori* induces an inflammatory response from the host in which ROS are produced by gastric cells (4), phagocytes (5), and other immune cells. ROS such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hypochlorous acid (HOCl) (6) oxidize free amino acid residues or the residues within proteins, which oftentimes renders the proteins nonfunctional (7). However, oxidation of a methionine (Met) residue can be enzymatically reversed, resulting in restoration of protein function (8). This is accomplished by methionine sulfoxide reductase, Msr (9-11). Other important enzymes involved in combating oxidative stress in *H. pylori* include superoxide dismutase (SodB), catalase (KatA), alkyl hydroperoxide reductase (AhpC), neutrophil activating protein (NapA), and NADPH quinone reductase (MdaB) (9, 11).

In bacteria and other organisms, two types of Msr proteins have been described, MsrA and MsrB. These two forms of Msr reduce the two isomers Met(S)O and Met(R)O of methionine sulfoxide, respectively (12, 13). In *H. pylori*, MsrA and MsrB are fused to constitute a 42 kDa protein (14). An *H. pylori msr* deficient strain has been shown to be highly sensitive to oxidative stress, and it has greatly diminished ability to colonize the stomach (15). *H. pylori* Msr has also

been shown to play a role in protecting catalase from oxidative damage (16). However, only a few Met-rich proteins have been identified as Msr interacting (16, 17) and the full extent of the physiological roles of Msr remain unknown.

H. pylori resists the acidic environment of the gastric region by producing urease, which hydrolyzes urea to bicarbonate and ammonia (18, 19). Urease is the most abundant protein made by *H. pylori*, as it accounts for more than 10% of the total protein synthesized by the bacterium (20, 21). Urease is composed of only two subunits, UreA and UreB (19), unlike other bacterial ureases that are composed of UreA, UreB, and UreC subunits (25). The maturation of urease requires incorporation of nickel into the active site, which is accomplished by several accessory proteins (22, 23). In *H. pylori*, these include UreE, UreF, UreG, and UreH (24). Based on studies in *K. aerogenes*, it is generally accepted that UreD (UreH in *H. pylori*), UreF, and UreG drive protein conformational change, lysine carbamylation, and GTP hydrolysis, respectively, while UreE functions as a metallochaperone of the maturation system (25-28).

Of the accessory proteins, UreG is the most highly conserved and shares sequence homology with ATP and GTP-binding proteins (29). UreG belongs to the group of homologous P-loop GTPases (26). Loss of all urease activity occurs upon introduction of site-directed mutations at the nucleotide-binding domain for both *H. pylori* (30) as well as *K. aerogenes* UreG (26). In addition to the nucleotide binding domain, UreG is a Met-rich protein with Met comprising ~4.5% of the primary amino acid sequence. From a tandem affinity purification (TAP) approach with UreG as the bait protein, UreG was proposed to interact with up to 33 different proteins, one of which was Msr (27). However, the role of Msr in this possible interaction had not been studied. The TAP results combined with the high proportion of Met residues in UreG caused us

to examine a role for Msr in urease maturation. The role of Msr was addressed by studying a Δ*msr* mutant and by assessing the ability of Msr to repair the oxidized Met residues of UreG. Finally, we demonstrated the intimate interaction between purified UreG and Msr.

Materials and Methods

Bacterial strains and growth conditions. *Helicobacter pylori* strain SS1 was used as the parental strain for all studies. *H. pylori* were routinely grown on Brucella agar (Oxoid Ltd., Hampshire, England) plates containing 10% defribrinated sheep blood (BA plates) (QuadFive, Ryegate, MT) and maintained at 37°C under 5% CO_2, 4% O_2, balanced with N_2, in a microaerobic humidified chamber. The Δ*msr* mutant was described previously (11, 15). *Escherichia coli* cultures were grown aerobically in Luria-Bertani (LB) broth or agar and ampicillin, kanamycin, and chloramphenicol were added when needed at final concentration of 100, 30, and 30 μg/ml, respectively.

Protein purification. *H. pylori* UreG was expressed as a hexahistidine-tagged protein in *E. coli* BL21 RIL (Novagen, Gibbstown, NJ, USA). Briefly, the *ureG* gene was amplified by PCR using genomic DNA from strain 43504 as a template and primers NdeUreG (5'-ACGGCCTCATATGGTAAAAATTGGAG-3') and XhoUreG (5'-GCGTAAGCTCGAGATCTTCCAATAAAGCGTTG-3', designed to amplify *ureG* without its stop codon). The resulting 0.6 kb-DNA fragment was digested with *Nde*I and *Xho*I and ligated into the similarly digested expression vector pET21b (Novagen, Madison, WI). The recombinant plasmid was sequenced at the Georgia Genomics Facility, University of Georgia, to ensure that no error was introduced by PCR. *E. coli* cultures harboring the recombinant plasmid were grown to an optical density at 600 nm of 0.6 at 37°C in 500 ml of LB medium with chloramphenicol and ampicillin. Cultures were then induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside

(IPTG) at 37°C for 3 h. Cells were harvested by centrifugation and the pellet resuspended in buffer A (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 40 mM imidazole), then broken by four passages through a cold French Pressure cell. The cell debris was removed by centrifugation at 15,000 x *g* for 20 min at 4°C. The supernatant was loaded onto a Ni-nitrilotriacetic acid-agarose (Ni-NTA) (Qiagen, Valencia, CA) column pre-equilibrated with buffer A. Unbound proteins were removed by washing with the same buffer. Following washing, UreG was unbound from the column with buffer B (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 250 mM imidazole). UreG-containing fractions were dialyzed against 50 mM Tris-HCl, pH 7.5. The dialyzed samples were then subjected to 12.5% SDS-PAGE to assess purity. Proteins were visualized with Coomassie brilliant blue R-250. Pure UreG was then concentrated using an Amicon Ultra Centrifugal filter (Millipore, Billerica, MA) and protein concentration determined with the BCA protein kit (Thermo Scientific Pierce, Rockford, IL) with bovine serum albumin as the standard.

H. pylori Msr was expressed as a hexahistidine-tagged protein and purified in *E. coli* BL21 (DE3) RIL as described previously (17). Briefly, *E. coli* cultures harboring the recombinant plasmid were grown to an optical density at 600 nm of 0.6 at 37°C in 1 liter of LB medium with ampicillin and kanamycin. Msr was then purified using a Ni-NTA column as described above for UreG. Msr-containing fractions were pooled and loaded onto a Q-sepharose Fast Flow column (Sigma, Saint Louis, MO). Msr was washed from the column with 50 mM Tris-HCl, pH 7.5. *H. pylori* thioredoxin (Trx) and thioredoxin reductase (TrxR) were expressed and isolated from *E. coli* as described previously (17).

GTPase assay. GTPase assays were performed using a malachite green-based kit (Innova Biosciences, United Kingdom) following the manufacturer's instructions. Briefly, 6 μM untreated, oxidized, or repaired (see section on Msr repair) UreG was incubated with 50 mM

Tris, pH 7.5, 2.5 mM $MgCl_2$, 0.5 mM GTP for 30 min. Next, the gold mix (P_iColorLock™ Gold plus accelerator) was added to the mixture and incubated for an additional 2 min. The stabilizer buffer was then incubated with the mixture 30 min. Finally, optical density was determined at 595 nm using a Molecular Devices plate reader. The readings obtained at OD_{595} were compared to a standard curve with known amounts of phosphate. All steps were carried out at room temperature.

Exposure of SS1 and Δ*msr* strains to oxygen stress and measurement of urease activity. *H. pylori* strain SS1 wild type or the Δ*msr* mutant was grown for 48 h, resuspended in brain heart infusion (BHI) broth with 0.4% β-cyclodextrin, pH 7.0, and exposed to 21% O_2 (air) for 2 h while shaking (200 rpm) at 37°C. Cells were harvested by centrifugation and broken by sonication. Cell debris was removed via centrifugation at 14,000 x *g* for 10 min, and the supernatant was assayed for urease activity according to the method of Weatherburn (31).

Partial purification of urease and nickel determination. Urease was partially purified from the oxygen stressed SS1 wild type and Δ*msr* strains as described previously (32) with the following modification. Briefly, 20 mM HEPES, pH 7.2 was used as the starting buffer and the protein was washed from the Q-sepharose HiTrap column (GE healthcare, Piscataway, NJ) with 0-600 mM NaCl gradient. The peak urease-containing fractions were pooled, dialyzed overnight against 20 mM NaCl (atomic absorption grade) assayed for protein concentration (BCA protein assay kit; Pierce) and nickel levels were measured by graphite furnace atomic absorption spectrometry (Shimadzu AA-6701F) as described previously (32).

Immunoblot and quantification of urease in *H. pylori* crude extracts. *H. pylori* SS1 wild type or the Δ*msr* mutant was exposed to 21% O_2 as described above. Cells were then resuspended in BHI with 0.4% β-cyclodextrin and broken by sonication. Approximately 10 μg of

crude extract from both strains was then loaded onto 12.5% SDS polyacrylamide gels. One gel was stained with Coomassie blue silver and proteins from the other were electrophoretically transferred onto a nitrocellulose membrane. Immunoblotting was carried out as described previously (33). Briefly, the membranes were blocked in 3% gelatin prepared in Tris buffered saline (TBS). Following blocking, the membranes were probed with rabbit anti-UreA antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:2,000 in tween 20-TBS (TTBS), 1% gelatin for 1 h and then washed with TTBS. The membranes were then incubated with goat anti-rabbit IgG conjugated to alkaline phosphatase (Bio-Rad, Hercules, CA) diluted 1:2,000 in TTBS, gelatin 1%. After 2 h incubation, the blot was developed with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate. Relative quantity of the urease structural subunit bands (UreA and UreB) was calculated by densitometry.

Oxidation of purified UreG. Purified UreG at 27 μM was treated with 25-50 mM H_2O_2 for 3 h in the dark and at room temperature under aseptic conditions. Excess H_2O_2 was removed by overnight dialysis against 50 mM Tris-HCl, pH 7.5.

Repair of oxidant damaged UreG. UreG or H_2O_2-treated (50 and 100 mM) UreG was repaired with Msr (equimolar concentration relative to UreG) or buffer (50 mM Tris-HCl, pH 7.5) in the presence of 400 μM NADPH (Sigma), 5 μM Trx, and 100 nM TrxR for 1 h at 37°C. GTPase activities were then measured.

Repair of oxidant damaged urease. The Δ*msr* mutant was exposed to 2 h oxygen stress and then urease was partially purified from that mutant as described above. Partially purified urease (14 μg of total protein) was incubated with 10 mM DTT alone or with 10 mM DTT and 14 μg Msr at 37°C for 1 h. Urease activity was then measured according to the method of Weatherburn (31).

Asp-N endoproteinase (Asp-N) digestion and pseudo-multiple reaction monitoring (MRM) LC-MS/MS analysis. UreG samples were digested with Asp-N and analyzed by C18 reverse phase LC coupled with pseudo-MRM MS/MS as described previously (16). Briefly, samples were heated to 60°C in 50 mM Tris HCl, 0.5 mM zinc acetate at pH 8.0 in the presence of 10 mM DTT for 45 min. They were then allowed to cool to room temperature and AspN protease (Thermo Scientific Pierce. Rockford, IL) was added 1:100 (protease:UreG) and digested overnight at 37°C. Samples were stored at -20°C until analysis.

After digestion, samples were analyzed using the LTQ front-end of an LTQ-FT mass spectrometer (Thermo Scientific) coupled to an Agilent 1100 HPLC system with CaptiveSpray ionization using an Advance Ion Source for Thermo MS (Michrom Bioresources Inc.) Samples were autoinjected onto a C18 trapping cartridge for desalting, followed by separation on a C18 capillary column (Michrom Bioresources, 0.2 x 50 mm, 3 µm, 200 Å) using a linear gradient from 95% Buffer A (water, 0.1% formic acid) and 5% Buffer B (acetonitrile, 0.1% formic acid) to 40% Buffer A, 60% Buffer B, at a flow rate of 3 µl/min for 70 minutes. Elution times and MS/MS transitions specific for unoxidized and oxidized methionine-containing peptides at 35 V collision energy were manually determined based on MS/MS analysis. For peptides containing two methionines, transitions were chosen that differentiated between oxidation on each methionine based on fragment ion mass. For quantitative analysis, oxidized and unoxidized methionine-containing peptide *m/z* values were placed on an include list, and oxidation was carried out based on the abundance of the selected MS/MS product ions determined as specific transitions for that peptide at the appropriate elution time.

Cross-linking and biotin label transfer. Purified UreG or 50 mM H_2O_2-treated UreG (oxUreG) was conjugated to Sulfo-SBED using the Sulfo-SBED biotin label transfer kit from (Thermo scientific Pierce). Sulfo-SBED was dissolved in dimethylformamide at 40 μg/ml and then added to UreG in 1x phosphate buffered saline (PBS) at 5-fold molar excess. The mixture was incubated at room temperature for 30 min in the dark to preserve the aryl azide group. Excess Sulfo-SBED was removed via overnight dialysis against 50 mM Tris, pH 7.5. UreG or oxUreG conjugated to Sulfo-SBED (4 μM) was then incubated with equal molar concentrations of Msr, lysozyme, or alone in a final volume of 40 μl for 2 h at room temperature in the dark. The binding of Msr to UreG was captured upon ultraviolet photoactivation of the aryl azide moiety. UV photoactivation was carried out using a 365 nm UV lamp held 5 cm from the mixture for 15 min on ice. The cross-linked mixture (20 μl) was then reduced with 0.5 M DTT in the dark for 10 min. The proteins were then resolved via SDS-PAGE followed by transfer to a nitrocellulose membrane. The biotin label was detected by incubating the membrane bound proteins with streptavidin-horseradish peroxidase (HRP) for 1 h. The membrane was developed by enhanced chemiluminescence (GE healthcare).

Statistical significance. Data are presented as the means ± S.D. All data comparisons were performed using the Student's *t* test. These values were calculated using the GraphPad QuickCalcs website: http://graphpad.com/quickcalcs/ttest.cfm.

Results

Purification of UreG. To study *H. pylori* UreG biochemically, the protein was overexpressed and purified as a recombinant his-tagged protein from *E. coli*. The purity of the protein was assessed via SDS-PAGE analysis. The purified UreG migrated at a mass of about 24 kDa (Figure 1).

Interaction between UreG and Msr. To analyze the direct binding of Msr to UreG we used a Sulfo-SBED trifunctional crosslinking reagent. Sulfo-SBED contains biotin, a sulfonated *N*-hydroxysuccinimide (Sulfo-NHS) active ester, and a photoactivatable aryl azide. Upon incubation of untreated "as-purified" UreG and separately H_2O_2-treated UreG (oxUreG) with Sulfo-SBED, the Sulfo-NHS ester reacted with the amine groups of UreG at neutral pH, resulting in conjugation of UreG and the crosslinking agent. Unlabeled Msr or lysozyme (as a control) was added to the Sulfo-SBED conjugated UreG or oxUreG and the aryl azide moiety within Sulfo-SBED was photoactivated with UV light to promote crosslinking. The binding event of Msr with UreG is thus captured so that disulfide bond cleavage by the addition of DTT then results in the transfer of the biotin tag to Msr. Samples were taken before and after UV crosslinking. The biotin label transfer is detected by Western blot using Streptavidin-HRP and enhanced chemiluminescence. Msr bound to both oxidized and non-oxidized UreG as shown by the presence of an approximate 42 kDa band in lanes 5 and 6 (Figure 2). These bands were not present when non-oxidized UreG or oxUreG was incubated alone (lanes 1 and 2) or with lysozyme (lanes 3 and 4). The absence of these bands in the samples incubated with lysozyme suggests this 42 kDa band is not caused by protein aggregation and further confirms the specificity of the interaction between UreG and Msr. Higher molecular weight bands observed in all lanes are believed to be UreG oligomers. UV-treated UreG contained more biotin label than the non-UV treated, presumably due to UV light mediated promotion of intramolecular tagging (34). No intermolecular tag transfer to Msr was detected in the absence of UV light (lanes 9 and 10).

UreG activities in oxidized and repaired samples. To determine the susceptibility of *H. pylori* UreG to oxidant damage, we incubated purified UreG with H_2O_2 and measured the

resulting GTPase activity. Incubation of purified UreG with H_2O_2 resulted in a dose-dependent decrease in GTPase activity (Figure 3). After incubation of 6 μM UreG with 25, 50, and 100 mM H_2O_2, the GTPase activity was significantly decreased to ~44, 35, and 28% of the untreated sample, respectively. These oxidant levels seem high, but are the amounts sometimes used to oxidize repair targets. It is also important to note that pathogens are subject to a variety of oxidants simultaneously that likely exert a cumulative protein oxidation affect. GTPase activity of *H. pylori* UreG has previously been shown to be negligible (30, 35). However, the calculated k_{cat} of 0.032 min^{-1} for the untreated *H. pylori* UreG used in this study is comparable to the measured GTPase activities of UreG in other organisms (*Bp*UreG k_{cat}=0.04 min^{-1}, *Mt*UreG k_{cat}=0.01 min^{-1}, *Gm*UreG k_{cat}=0.01 min^{-1}) (36-38). To determine whether the decrease in GTPase activity after UreG incubation with H_2O_2 was due to Met oxidation and also whether Msr is capable of restoring activity, oxidized UreG samples were incubated with Msr (plus repair system) and the resulting GTPase activities were measured. As a control, UreG was incubated with the repair components (Trx, TrxR, NADPH) without Msr. The latter sample is considered the untreated sample and is given 100% activity (in earlier experiments it had the same activity as untreated UreG with none of the repair mixture components added). Upon incubation with Msr-containing repair components (Msr, Trx, TrxR, and NADPH) the activity of the oxidant-damaged UreG was restored (Figure 4); it achieved full (non-oxidant treated) levels for the 50 mM H_2O_2-oxidized sample and ~80% of the non-oxidant treated levels for the 100 mM H_2O_2-treated sample. GTPase restoration (i.e. repair) was never seen when the oxidized UreG samples were incubated with the repair system without Msr. These results indicate that Msr can repair

oxidatively damaged UreG and restore enzyme activity. Interestingly, a 20% increase of activity with the addition of Msr to the untreated sample was sometimes observed indicating some spontaneous UreG oxidation during preparation and storage.

MS/MS identification of Mets oxidized and repaired in UreG. UreG samples were digested with Asp-N and the resulting peptide mix was then subjected to LC-MS/MS. All 9 Mets of UreG could be identified and they all showed significant oxidation after treatment with 50 mM H_2O_2 (Figure 5). For all untreated samples, the oxidation for all Met residues remained less than 10%. Met^{1}, Met^{25}, Met^{47}, Met^{77}, Met^{85}, and Met^{167} were all greater than 80% oxidized after treatment with H_2O_2. Met^{31}, Met^{53}, and Met^{160} were ~70-80% oxidized. After incubation of the oxidized samples with Msr plus 10 mM DTT as the reducing agent, all Mets remained less than 20% oxidized (Figure 5). DTT alone did not cause Met-SO repair of any residues. The results indicate that Msr is able to repair all Mets of UreG.

Urease activities in wild type and Δ*msr* strains after exposure to mild oxidative stress. Despite expressing a broad repertoire of stress-combating enzymes and being an obligate aerobe, *H. pylori* is sensitive to oxidative molecules, including high oxygen. All wild type strains of the bacterium are routinely grown in controlled atmospheres maintained below 12% partial pressure O_2 (10). We thus addressed the physiological importance of UreG repair under a relatively mild stress condition, ambient O_2 levels. Since the UreG GTPase activity is susceptible to oxidation (Figure 3), and the activity is restored by Msr (Figure 4), we reasoned the importance of UreG repair could be assessed by comparing the Δ*msr* mutant with the parent in urease activity upon cell exposure to 2 h of ambient O_2. This mild oxidant treatment has been used previously to study roles of oxidative stress enzymes in whole *H. pylori* cells (39, 40); the 2 h treatment causes viability loss of some oxidative stress sensitive mutants, but the parent strain

maintains full viability (but not growth) by this incubation (15, 40). A significant decrease in cell free extract urease activity to 64% of the parent strain level was observed in the Δ*msr* mutant (Figure 6A) upon 2 h air exposure. As a control, the wild-type and Δ*msr* strains were left at 4% partial pressure oxygen and the Δ*msr* mutant showed no statistically significant difference from the wild-type in activity over the 2-h period (Figure 6A). If urease maturation is the deficiency associated with the Δ*msr* mutant, we would expect urease apo-protein levels to be equal in that mutant and the parent. Urease apo-protein levels indeed appear to be similar in the wild-type and Δ*msr* strains (see Figure 6B and C), and densitometric scanning (32) of these gels (from Figure 6B) confirmed that UreA and UreB subunits did not differ by more than 5% among the two strains.

Evidence to suggest this decrease in activity is due to a deficiency of urease maturation was obtained by measuring the nickel content associated with partially purified urease of both parent and mutant strains. Urease was partially purified and the urease fractions analyzed for nickel levels. The Δ*msr* mutant contained 33% less nickel content than the wild-type (30 ± 1.6 ng Ni per mg of protein for the wild-type and 20 ± 0.8 ng Ni per mg of protein for Δ*msr*). These data were based on 13 replicates for each strain and the difference between the two strains was statistically significant ($p < 0.01$). Still, as urease was only partially purified some of the nickel measurement could be associated with other proteins in both strains. The decreased urease specific activity shown in the oxygen stressed Δ*msr* mutant raises the possibility that urease itself could be damaged by Met oxidation during this treatment. To address this concern, partially purified urease from the oxygen stressed Δ*msr* mutant was incubated with DTT, or with DTT and Msr. No gain in urease activity was seen in either case (data not shown). These results suggest that the decrease in urease activity observed in the oxidant stressed strain is not due to

methionine oxidation of urease. Rather, UreG and possibly other proteins involved in urease maturation are prone to methionine oxidation and can be repaired by Msr.

Discussion

The long-term survivability of *H. pylori* in the host requires that the bacterium survive harsh conditions, including the host inflammatory responses. The pathogens persistence is key to the most severe disease symptoms, and is in part due to the battery of DNA and protein repair enzymes that confer oxidative stress resistance to the pathogen *in vivo*. Proteins are often the targets of ROS due to their abundance in cells and their ease of reactivity with oxidants and Met is one of the most sensitive amino acids to oxidation. It is of obvious benefit for the bacterium to repair damaged Met-SO-containing proteins rather than synthesize new proteins due to energy input costs for synthesis and in order to rapidly maintain key enzyme function.

Many oxidative stress-combating enzymes have been described in *H. pylori,* but our knowledge of the significance and physiological role of Msr continues to expand. An *H. pylori* Δ*msr* mutant shows attenuated growth in the presence of chemical oxidants and the strain is severely deficient in ability to colonize the mouse stomach (15). In addition, carbonylated proteins have been shown to accumulate in a Δ*msr* mutant after exposure of cells to oxidant damage (16). These proteins were not oxidized in the parent strain (15). However, only a few specific targets of Msr-mediated repair in *H. pylori* have been identified. These include GroEL, SSR, AhpC, and catalase (16, 17). Msr-repair targets in other bacteria include GroEL and Ffh (41, 42).

In the host gastric region, *H. pylori* is in frequent contact with acid and oxidative molecules. Urease is essential for *H. pylori* to survive the acid stress and it is a highly abundant protein that requires nickel to function. Inside the host, *H. pylori* likely encounters varying levels of Ni so it

employs many Ni sequestering proteins (HspA, Hpn, Hpn-like) thought to gather the ion for storage and possible transfer to the metalloenzymes (43-45). To achieve optimal urease activity, the active sites must be fully loaded with Ni and the Ni demand is thus high; Ni-saturated urease contains 12 Ni atoms per molecule (46). As Hu and Mobley (47) demonstrated, higher counts of Ni in recombinant urease correlated with higher urease activities. We observed a 31% decrease in urease activity and a concomitant 33% decrease of urease-associated Ni levels in the Δ*msr* mutant compared to the parent after exposure to mild oxidative conditions (e.g. air). Although modest, we propose this is significant to the *in vivo* situation, where Met damage could be much greater, and more potent oxidants exist. Indeed, Stingl and de Reuse (48) calculated that under *in vitro* conditions without added nickel only a small proportion of the urease active sites are filled with nickel but that is sufficient for full acid resistance. Under similiar conditions (no added nickel) we still see a significant difference in the nickel load between the wild-type and Δ*msr* mutant, which further suggests a role of Msr in protecting urease maturation. In addition, HOCl is a highly potent oxidant (much more potent than H_2O_2) and it can achieve levels of 5 mM at sites of inflammation within the host (49). The Msr-repairable Met residues in *E. coli* GroEL were shown to be much more sensitive to oxidation by phagocyte-produced oxidants like HOCl and peroxynitrite than to oxidants produced by bacterial metabolism such as H_2O_2 (42). It seems possible that urease *per se* could be a target for oxidative inactivation, but a previous study using co-immunoprecipitation found no evidence for urease-Msr interactions (50). Furthermore, in this study we saw no enhancement of activity when Msr was added to urease purified from oxygen stressed cells. Taken together, our results indicate that non-lethal elevated oxygen (~21% partial pressure) caused a urease maturation defect.

Interestingly, the *H. pylori* urease maturation proteins have been reported to assemble at the cytoplasmic membrane in a pH-dependent manner when urease is also undergoing increased Ni-dependent maturation (51, 52). *H. pylori* Msr has also been shown to be membrane associated (15), so studies of Msr-UreG recognition (and perhaps roles of membrane proteins) as a function of pH may be informative. Although UreG complexes with UreE to function in Ni delivery (53, 54), UreE does not seem to be a target for Msr; crosslinking approaches to identify such an interaction between the two proteins were negative (17). Also, UreE is not a Met-rich protein (~1.8% Met), and other Msr targets are rich in Met. In this study, we found that UreG is sensitive to inactivation by H_2O_2 and given that UreG is rich in Met residues (4.5%), we speculated that it was inactivated due to Met oxidation. We found that H_2O_2 greatly inhibited UreG GTPase activity compared to the untreated UreG sample. It is likely that the bulk of inactivation of enzyme activity is due to Met-SO formation since the other residues susceptible to oxidation (Tyr and Cys) occur in small amounts within UreG (1.5% each) and Msr (known to repair Met residues only) restored the oxidized protein's GTPase activity.

In *H. pylori*, Msr was able to restore full UreG enzyme activity. This is similar to Msr-mediated catalase repair in which Msr restored up to 82% of full enzyme activity (16). All nine Met residues in UreG showed susceptibility to oxidation, and all were repaired with the addition of Msr. Interestingly, almost all (eight out of nine) Met residues in UreG are predicted to be surface or solvent-exposed. This is consistent with evidence that exposed Met residues are more readily oxidized by oxidants and would be expected to be accessible to Msr (55-58). A few prior accounts of Met-SO protein repair upon oxidative inactivation have been demonstrated in other systems such as *E. coli* L12 protein (59) and *H. pylori* catalase (16). Oxidation and/or repair of every Met residue within an enzyme has not been shown previously in any other system. For *E.*

coli GroEL twelve out of twenty-three Mets were converted to Met-SO (42). Some activity (70% compared to untreated) of GroEL was restored by Msr when the lowest oxidant concentration was used) but harsher oxidation led to the production of some Met-sulfone residues. In this case no activity could be restored (40). Analysis of oxidized or Msr-repaired *E. coli* Ffh showed four out of five Met-containing peptides were oxidized and repaired (41). Not all Mets of *H. pylori* catalase were susceptible to HOCl-mediated oxidation, but those that were indeed became targets of repair (16). Our experiments expand our understanding of the unusual recognition flexibility of Msr, in that all nine Met residues are repaired, and another target enzyme with a defined physiological role in pathogenesis is identified.

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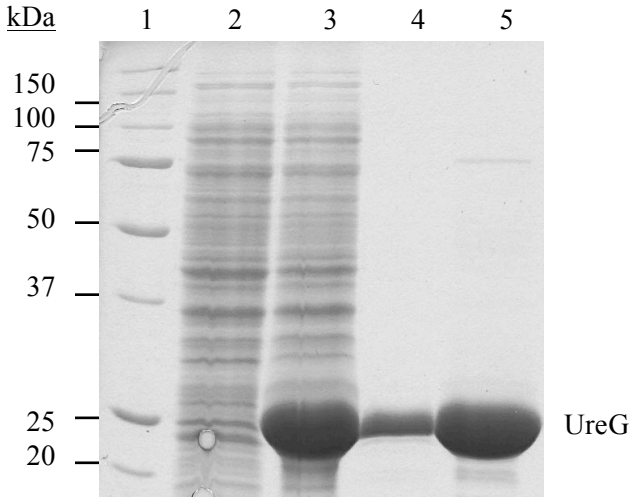

Figure 2.1. SDS-PAGE of purified UreG. Lane 1, molecular weight marker; lane 2, cell extract from non-induced *E. coli* BL21 RIL harboring pET21b-*ureG*; lane 3, cell extract from IPTG induced *E. coli* BL21 RIL harboring pET21b-*ureG*; lane 4, purified UreG after Ni-NTA; lane 5, concentrated pure UreG. The arrow to the right indicates UreG.

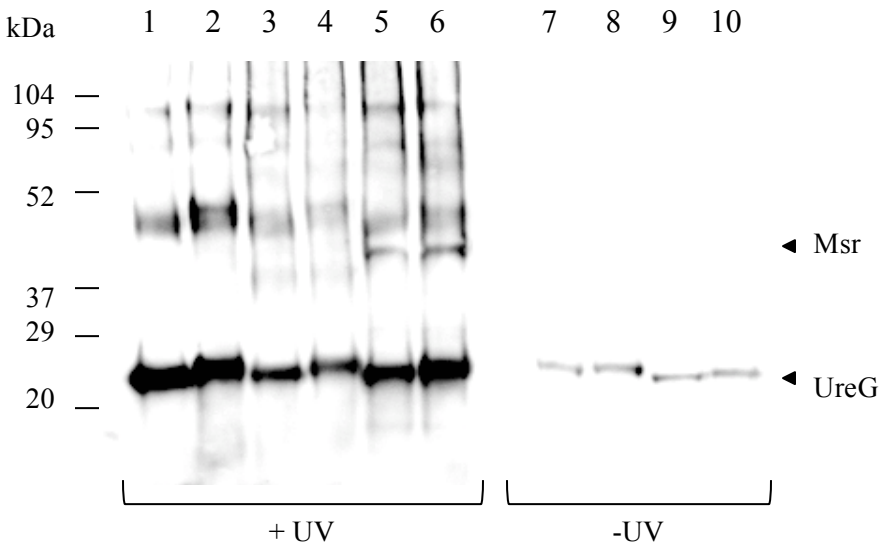

Figure 2.2. Interaction between UreG and Msr identified by biotin transfer. Sulfo-SBED conjugated UreG or 0.6% H_2O_2-oxidized UreG was incubated with Msr, lysozyme, or alone and then the mixture was subjected to UV-crosslinking. Samples were taken before and after exposure to UV light. The conjugated samples were reduced with 0.5 M DTT for label transfer and the proteins were resolved via SDS-PAGE, transferred to a nitrocellulose membrane, and probed with streptavidin-horseradish peroxidase. Lane 1, UreG; lane 2, oxidized UreG; lane 3, UreG and lysozyme; lane 4, oxidized UreG and lysozyme; lane 5, UreG and Msr; lane 6, oxidized UreG and Msr; lane 7, UreG; lane 8, oxidized UreG; lane 9, UreG and Msr; lane 10, oxidized UreG and Msr. Lanes 7-10 were not exposed to UV light. The molecular weight marker is displayed to the left. The arrows to the right indicate Msr or UreG. UreG is ~ 22 kDa and Msr is ~ 42 kDa.

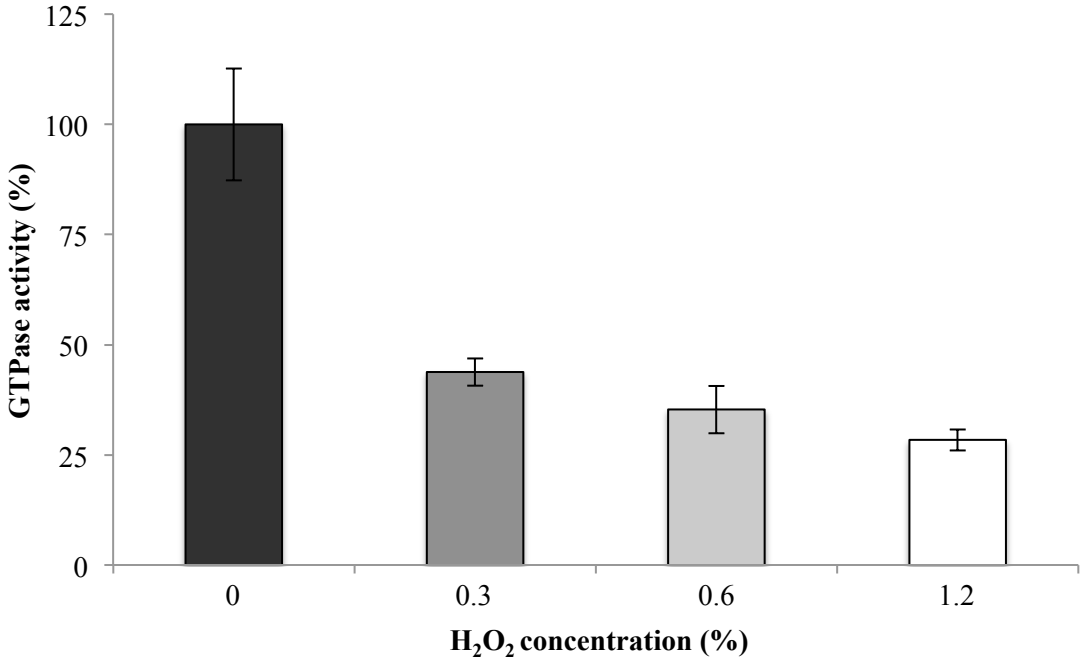

Figure 2.3. H_2O_2 inactivation of UreG GTPase activity. Purified UreG (6 μM) was incubated with varying concentrations of H_2O_2 (0, 0.3, 0.6, 1.2%) for 3 h. Excess oxidant was removed via overnight dialysis. GTPase activities were measured using a colorimetric assay to detect the release of P_i and presented as % activity of the untreated sample. Each concentration is statistically significantly less than every higher concentration shown in the figure at $p < 0.05$. Results are the mean ± S.D. for n=8, two independent experiments each sampled four times.

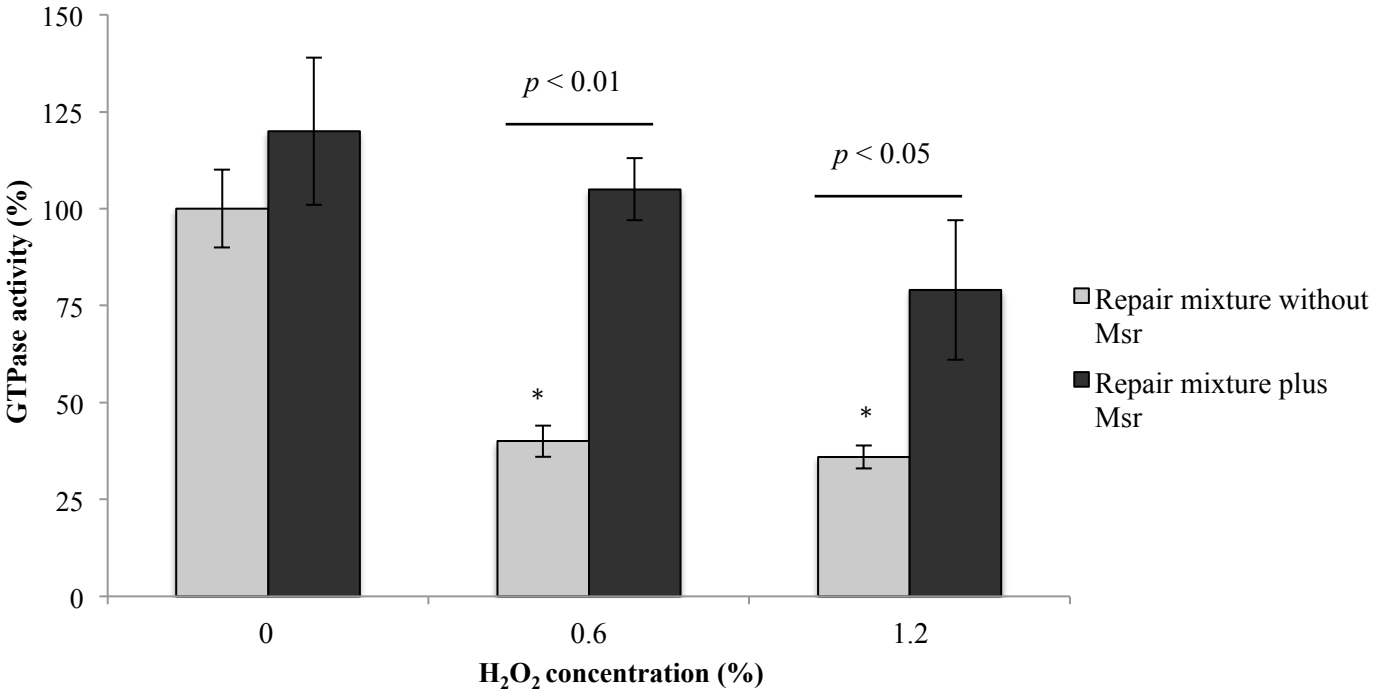

Figure 2.4. Msr repair of H_2O_2 damaged UreG. H_2O_2-treated UreG (6 μM) was incubated with equimolar amounts of Msr or buffer, along with the Msr repair components (400 μM NADPH, 5 μM Trx, and 100 nM TrxR) at 37°C for 1 h. The samples were then assayed for GTPase activity spectrophotometrically. Data are presented as mean ± S.D. with n=8, three independent experiments sampled in duplicate. * indicates $p < 0.01$.

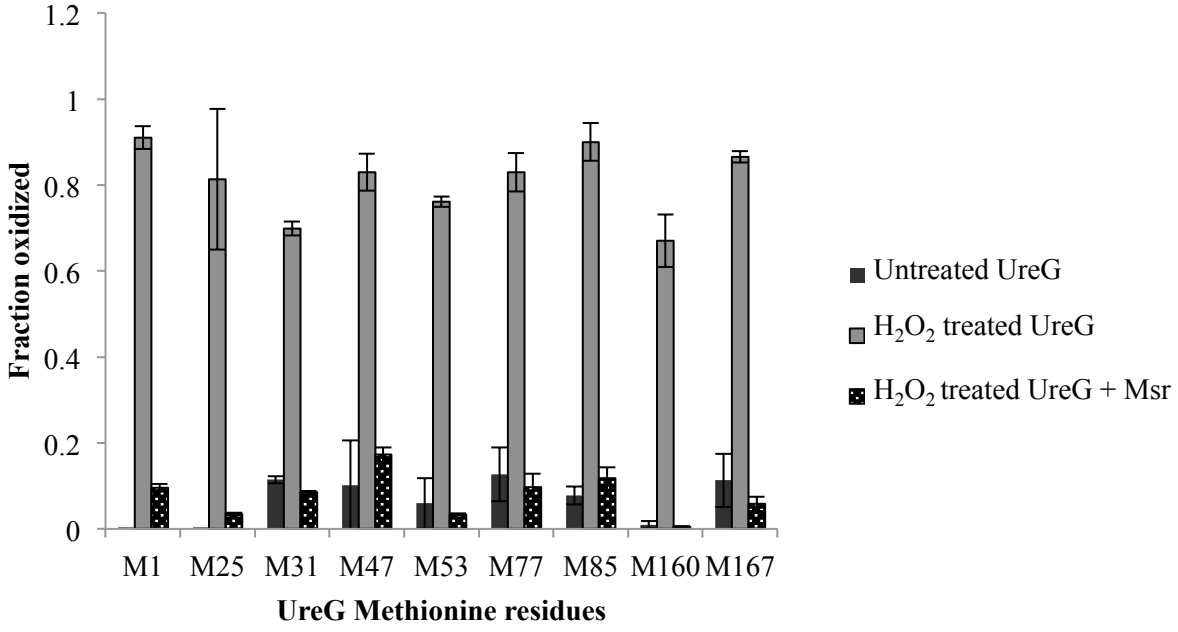

Figure 2.5. MS/MS Identification of Met residues after oxidation and repair of UreG. UreG was incubated with buffer or 0.6% H_2O_2 in that buffer for 3 h. Excess H_2O_2 was removed via overnight dialysis. Following dialysis, oxidized UreG samples were incubated with or without Msr in the presence of DTT at 37°C for 1 h. DTT alone did not result in repair of any Met residues. No oxidized Met^1 or Met^{25} could be detected in the untreated sample. Samples were digested with Asp-N and Met residues were identified and quantified by LC-MS/MS.

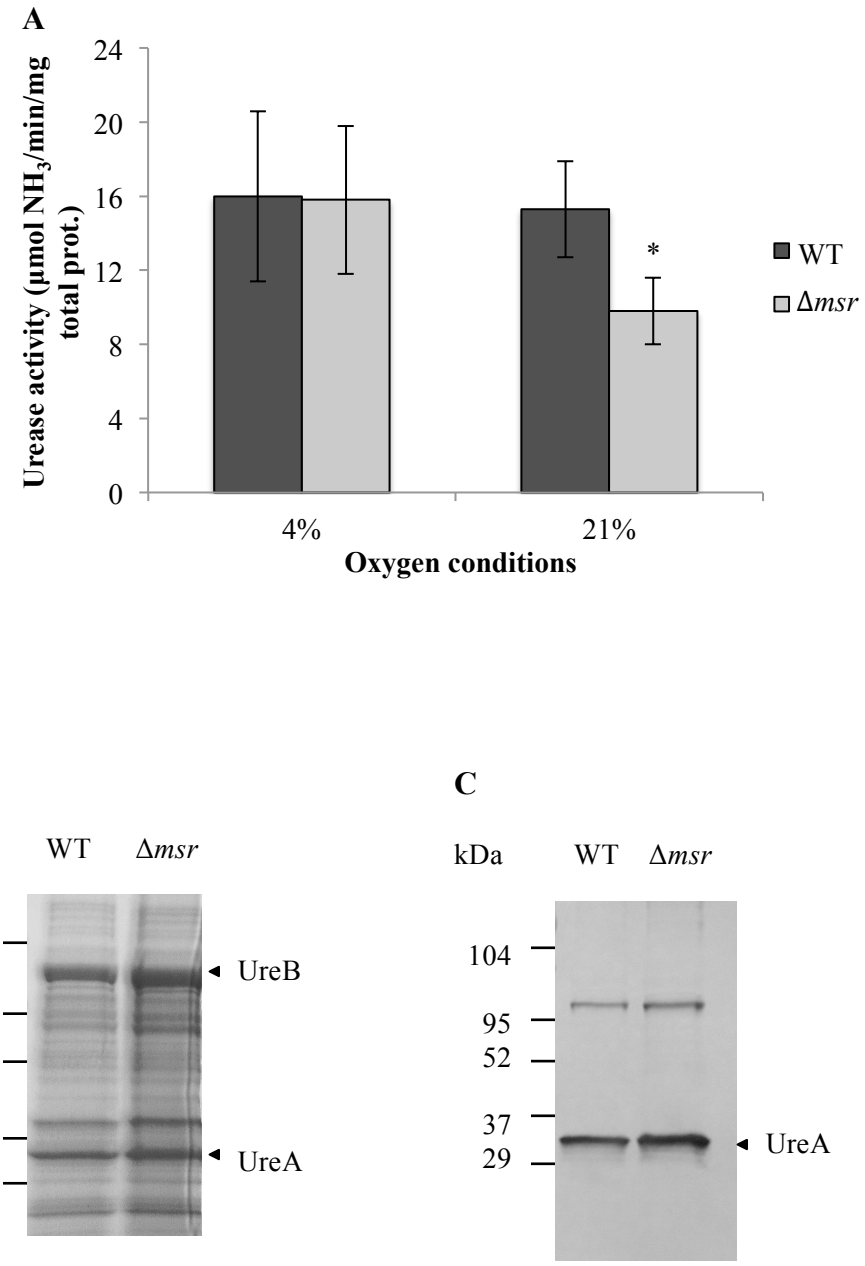

Figure 2.6. Urease activity and expression after oxidant stress. (**A**) Urease activity after exposure to oxidative stress. *H. pylori* SS1 wild type and Δ*msr* strains were exposed to 21% O_2 (air) for 2 h or left at 4% O_2 as a control. Cells were then lysed by sonication and urease activity was measured in cell free extracts. Results are the mean ± S.D of n=12, based on four independent

experiments each sampled in triplicate. *indicates $p < 0.01$. (**B**) SDS-PAGE analysis of cell free extract from SS1 wild type (9 μg) and Δ*msr* (10 μg) after exposure to 21% O_2. (**C**) Immunoblot analysis of SS1 wild type and Δ*msr* cell free extract after exposure to 21% O_2. Whole cell extract were resolved via SDS-PAGE, transferred to a nitrocellulose membrane, and blotted with anti-UreA antibodies.

References

1. **Marshall BJ, Warren JR.** 1984. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. Lancet **1:**1311-1315.

2. **Blaser MJ, Atherton JC.** 2004. *Helicobacter pylori* persistence: biology and disease. J Clin Invest **113:**321-333.

3. **Dunn BE, Cohen H, Blaser MJ.** 1997. *Helicobacter pylori*. Clin Microbiol Rev **10:**720-741.

4. **Bagchi D, Bhattacharya G, Stohs SJ.** 1996. Production of reactive oxygen species by gastric cells in association with *Helicobacter pylori*. Free Radic Res **24:**439-450.

5. **Ramarao N, Gray-Owen SD, Meyer TF.** 2000. *Helicobacter pylori* induces but survives the extracellular release of oxygen radicals from professional phagocytes using its catalase activity. Mol Microbiol **38:**103-113.

6. **Handa O, Naito Y, Yoshikawa T.** 2010. *Helicobacter pylori*: a ROS-inducing bacterial species in the stomach. Inflamm Res **59:**997-1003.

7. **Stadtman ER, Levine RL.** 2000. Protein oxidation. Ann N Y Acad Sci **899:**191-208.

8. **Brot N, Fliss H, Coleman T, Weissbach H.** 1984. Enzymatic reduction of methionine sulfoxide residues in proteins and peptides. Methods Enzymol **107:**352-360.

9. **Olczak AA, Olson JW, Maier RJ.** 2002. Oxidative-stress resistance mutants of *Helicobacter pylori*. J Bacteriol **184:**3186-3193.

10. **Wang G, Alamuri P, Maier RJ.** 2006. The diverse antioxidant systems of *Helicobacter pylori*. Mol Microbiol **61:**847-860.

11. **Wang G, Conover RC, Benoit S, Olczak AA, Olson JW, Johnson MK, Maier RJ.** 2004. Role of a bacterial organic hydroperoxide detoxification system in preventing catalase inactivation. J Biol Chem **279:**51908-51914.

12. **Grimaud R, Ezraty B, Mitchell JK, Lafitte D, Briand C, Derrick PJ, Barras F.** 2001. Repair of oxidized proteins. Identification of a new methionine sulfoxide reductase. J Biol Chem **276:**48915-48920.

13. **Kauffmann B, Aubry A, Favier F.** 2005. The three-dimensional structures of peptide methionine sulfoxide reductases: current knowledge and open questions. Biochim Biophys Acta **1703:**249-260.

14. **Tomb JF, White O, Kerlavage AR, Clayton RA, Sutton GG, Fleischmann RD, Ketchum KA, Klenk HP, Gill S, Dougherty BA, Nelson K, Quackenbush J, Zhou L, Kirkness EF, Peterson S, Loftus B, Richardson D, Dodson R, Khalak HG, Glodek A, McKenney K, Fitzegerald LM, Lee N, Adams MD, Hickey EK, Berg DE, Gocayne JD, Utterback TR, Peterson JD, Kelley JM, Cotton MD, Weidman JM, Fujii C, Bowman C, Watthey L, Wallin E, Hayes WS, Borodovsky M, Karp PD, Smith HO, Fraser CM, Venter JC.** 1997. The complete genome sequence of the gastric pathogen *Helicobacter pylori*. Nature **388:**539-547.

15. **Alamuri P, Maier RJ.** 2004. Methionine sulphoxide reductase is an important antioxidant enzyme in the gastric pathogen *Helicobacter pylori*. Mol Microbiol **53:**1397-1406.

16. **Mahawar M, Tran V, Sharp JS, Maier RJ.** 2011. Synergistic roles of *Helicobacter pylori* methionine sulfoxide reductase and GroEL in repairing oxidant-damaged catalase. J Biol Chem **286:**19159-19169.

17. **Alamuri P, Maier RJ.** 2006. Methionine sulfoxide reductase in *Helicobacter pylori*: interaction with methionine-rich proteins and stress-induced expression. J Bacteriol **188:**5839-5850.

18. **Dunn BE, Campbell GP, Perez-Perez GI, Blaser MJ.** 1990. Purification and characterization of urease from *Helicobacter pylori*. J Biol Chem **265:**9464-9469.

19. **Hu LT, Mobley HL.** 1990. Purification and N-terminal analysis of urease from *Helicobacter pylori*. Infect Immun **58:**992-998.

20. **Mobley HL, Garner RM, Bauerfeind P.** 1995. *Helicobacter pylori* nickel-transport gene *nixA*: synthesis of catalytically active urease in *Escherichia coli* independent of growth conditions. Mol Microbiol **16:**97-109.

21. **Bauerfeind P, Garner R, Dunn BE, Mobley HL.** 1997. Synthesis and activity of *Helicobacter pylori* urease and catalase at low pH. Gut **40:**25-30.

22. **Lee MH, Mulrooney SB, Renner MJ, Markowicz Y, Hausinger RP.** 1992. *Klebsiella aerogenes* urease gene cluster: sequence of *ureD* and demonstration that four accessory genes (*ureD*, *ureE*, *ureF*, and *ureG*) are involved in nickel metallocenter biosynthesis. J Bacteriol **174:**4324-4330.

23. **Cussac V, Ferrero RL, Labigne A.** 1992. Expression of *Helicobacter pylori* urease genes in *Escherichia coli* grown under nitrogen-limiting conditions. J Bacteriol **174:**2466-2473.

24. **Clayton CL, Pallen MJ, Kleanthous H, Wren BW, Tabaqchali S.** 1990. Nucleotide sequence of two genes from *Helicobacter pylori* encoding for urease subunits. Nucleic Acids Res **18:**362.

25. **Park IS, Hausinger RP.** 1995. Evidence for the presence of urease apoprotein complexes containing UreD, UreF, and UreG in cells that are competent for in vivo enzyme activation. J Bacteriol **177:**1947-1951.

26. **Moncrief MB, Hausinger RP.** 1997. Characterization of UreG, identification of a UreD-UreF-UreG complex, and evidence suggesting that a nucleotide-binding site in UreG is required for in vivo metallocenter assembly of *Klebsiella aerogenes* urease. J Bacteriol **179:**4081-4086.

27. **Stingl K, Schauer K, Ecobichon C, Labigne A, Lenormand P, Rousselle JC, Namane A, de Reuse H.** 2008. In vivo interactome of *Helicobacter pylori* urease revealed by tandem affinity purification. Mol Cell Proteomics **7:**2429-2441.

28. **Soriano A, Colpas GJ, Hausinger RP.** 2000. UreE stimulation of GTP-dependent urease activation in the UreD-UreF-UreG-urease apoprotein complex. Biochemistry **39:**12435-12440.

29. **Moncrief MB, Hausinger RP.** 1996. Purification and activation properties of UreD-UreF-urease apoprotein complexes. J Bacteriol **178:**5417-5421.

30. **Mehta N, Benoit S, Maier RJ.** 2003. Roles of conserved nucleotide-binding domains in accessory proteins, HypB and UreG, in the maturation of nickel-enzymes required for efficient *Helicobacter pylori* colonization. Microb Pathog **35:**229-234.

31. **Weatherburn MW.** 1967. Phenol-hypochlorite reaction for determination of ammonia. Anal Chem **39:**971-974.

32. **Olson JW, Mehta NS, Maier RJ.** 2001. Requirement of nickel metabolism proteins HypA and HypB for full activity of both hydrogenase and urease in *Helicobacter pylori*. Mol Microbiol **39:**176-182.

33. **Minamisawa S, Hoshijima M, Chu G, Ward CA, Frank K, Gu Y, Martone ME, Wang Y, Ross J, Jr., Kranias EG, Giles WR, Chien KR.** 1999. Chronic phospholamban-sarcoplasmic reticulum calcium ATPase interaction is the critical calcium cycling defect in dilated cardiomyopathy. Cell **99:**313-322.

34. **Minami Y, Kawasaki H, Minami M, Tanahashi N, Tanaka K, Yahara I.** 2000. A critical role for the proteasome activator PA28 in the Hsp90-dependent protein refolding. J Biol Chem **275:**9055-9061.

35. **Minami Y, Minami M.** 1999. Hsc70/Hsp40 chaperone system mediates the Hsp90-dependent refolding of firefly luciferase. Genes Cells **4:**721-729.

36. **Zambelli B, Stola M, Musiani F, De Vriendt K, Samyn B, Devreese B, Van Beeumen J, Turano P, Dikiy A, Bryant DA, Ciurli S.** 2005. UreG, a chaperone in the urease assembly process, is an intrinsically unstructured GTPase that specifically binds Zn^{2+}. J Biol Chem **280:**4684-4695.

37. **Zambelli B, Musiani F, Savini M, Tucker P, Ciurli S.** 2007. Biochemical studies on Mycobacterium tuberculosis UreG and comparative modeling reveal structural and functional conservation among the bacterial UreG family. Biochemistry **46:**3171-3182.

38. **Real-Guerra R, Staniscuaski F, Zambelli B, Musiani F, Ciurli S, Carlini CR.** 2012. Biochemical and structural studies on native and recombinant *Glycine max* UreG: a detailed characterization of a plant urease accessory protein. Plant Mol Biol **78:**461-475.

39. **Wang G, Olczak AA, Walton JP, Maier RJ.** 2005. Contribution of the *Helicobacter pylori* thiol peroxidase bacterioferritin comigratory protein to oxidative stress resistance and host colonization. Infect Immun **73:**378-384.

40. **Wang G, Maier RJ.** 2004. An NADPH quinone reductase of *Helicobacter pylori* plays an important role in oxidative stress resistance and host colonization. Infect Immun **72:**1391-1396.

41. **Ezraty B, Grimaud R, El Hassouni M, Moinier D, Barras F.** 2004. Methionine sulfoxide reductases protect Ffh from oxidative damages in *Escherichia coli*. EMBO J **23:**1868-1877.

42. **Khor HK, Fisher MT, Schoneich C.** 2004. Potential role of methionine sulfoxide in the inactivation of the chaperone GroEL by hypochlorous acid (HOCl) and peroxynitrite (ONOO-). J Biol Chem **279:**19486-19493.

43. **Seshadri S, Benoit SL, Maier RJ.** 2007. Roles of His-rich hpn and hpn-like proteins in *Helicobacter pylori* nickel physiology. J Bacteriol **189:**4120-4126.

44. **Kansau I, Labigne A.** 1996. Heat shock proteins of *Helicobacter pylori*. Aliment Pharmacol Ther **10 Suppl 1:**51-56.

45. **Gilbert JV, Ramakrishna J, Sunderman FW, Jr., Wright A, Plaut AG.** 1995. Protein Hpn: cloning and characterization of a histidine-rich metal-binding polypeptide in *Helicobacter pylori* and *Helicobacter mustelae*. Infect Immun **63:**2682-2688.

46. **Mobley HLT.** 2001. Urease. *In* Mobley HLT, Mendz GL, Hazell SL (ed.), *Helicobacter pylori*: Physiology and Genetics, Washington (DC).

47. **Mobley HL, Hu LT, Foxal PA.** 1991. *Helicobacter pylori* urease: properties and role in pathogenesis. Scand J Gastroenterol **187:**39-46.

48. **Stingl K, De Reuse H.** 2005. Staying alive overdosed: how does *Helicobacter pylori* control urease activity? Int J Med Microbiol **295:**307-315.

49. **Weiss SJ.** 1989. Tissue destruction by neutrophils. N Engl J Med **320:**365-376.

50. **Alamuri P.** 2006. The Role of Methionine Sulfoxide Reductase in *Helicobacter pylori*. University of Georgia.

51. **Scott DR, Marcus EA, Wen Y, Singh S, Feng J, Sachs G.** 2010. Cytoplasmic histidine kinase (HP0244)-regulated assembly of urease with UreI, a channel for urea and its metabolites, CO_2, NH_3, and $NH_4^{(+)}$, is necessary for acid survival of *Helicobacter pylori*. J Bacteriol **192:**94-103.

52. **Scott DR, Marcus EA, Weeks DL, Sachs G.** 2002. Mechanisms of acid resistance due to the urease system of *Helicobacter pylori*. Gastroenterology **123:**187-195.

53. **Rain JC, Selig L, De Reuse H, Battaglia V, Reverdy C, Simon S, Lenzen G, Petel F, Wojcik J, Schachter V, Chemama Y, Labigne A, Legrain P.** 2001. The protein-protein interaction map of *Helicobacter pylori*. Nature **409:**211-215.

54. **Voland P, Weeks DL, Marcus EA, Prinz C, Sachs G, Scott D.** 2003. Interactions among the seven *Helicobacter pylori* proteins encoded by the urease gene cluster. Am J Physiol Gastrointest **Liver** Physiol **284:**G96-G106.

55. **Levine RL, Mosoni L, Berlett BS, Stadtman ER.** 1996. Methionine residues as endogenous antioxidants in proteins. Proc Natl Acad Sci U S A **93:**15036-15040.

56. **Luo S, Levine RL.** 2009. Methionine in proteins defends against oxidative stress. FASEB J **23:**464-472.

57. **Vogt W.** 1995. Oxidation of methionyl residues in proteins: tools, targets, and reversal. Free Radic Biol Med **18:**93-105.

58. **Xiong Y, Chen B, Smallwood HS, Urbauer RJ, Markille LM, Galeva N, Williams TD, Squier TC.** 2006. High-affinity and cooperative binding of oxidized calmodulin by methionine sulfoxide reductase. Biochemistry **45:**14642-14654.

59. **Caldwell P, Luk DC, Weissbach H, Brot N.** 1978. Oxidation of the methionine residues of *Escherichia coli* ribosomal protein L12 decreases the protein's biological activity. Proc Natl Acad Sci U S A **75:**5349-5352.

CHAPTER 3

COMPARATIVE ROLES OF THE TWO *HELICOBACTER PYLORI* THIOREDOXINS IN PREVENTING MACROMOLECULE DAMAGE [2]

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Abstract

Thioredoxins are highly conserved throughout a wide range of organisms and are essential for survival of oxygen-sensitive cells. The gastric pathogen *Helicobacter pylori* uses the thioredoxin system to maintain its thiol/disulfide balance. There are two thioredoxins present in *H. pylori*, Trx1 and Trx2. Trx1 has been shown to be important as an electron donor for some antioxidant enzymes, but the function of Trx2 remains unknown (Baker LM, Raudonikiene A, Hoffman PS, Poole LB. J Bacteriol 2001. 183:1961-1973, Alamuri P, Maier RJ. 2006. J Bacteriol 188:5839-5850). We demonstrate that both Trx1 and Trx2 are important in protecting *H. pylori* from oxidative stress. Individual deletion mutant strains Δ*trx1* or Δ*trx2* each showed a greater abundance of lipid peroxides and suffered more DNA damage and protein carbonylation than the parent. Both deletion mutants were much more sensitive to O_2-mediated viability-loss than the parent. Unexpectedly, the oxidative DNA damage and protein carbonylation was more severe in Δ*trx2* than in Δ*trx1* with 20-fold and 4-fold more carbonylated protein content than the wild-type and Δ*trx1*, respectively, after 4 h of atmospheric O_2 stress. *trx* transcript abundance was altered by the deletion of the heterologous *trx*. Δ*trx2* lacked mouse colonization ability while Δ*trx1* was significantly reduced in ability to colonize mice.

Introduction

Oxidative stress is a problem encountered by most organisms, and anaerobic and microaerophilic microorganisms frequently have a limited tolerance for oxygen. Reactive oxygen species (ROS) are abundantly generated in the environment, which leads to every living organism, at least transiently, having contact with different reactive oxygen molecules. ROS are generated in the form of superoxide (O_2^-), hydroxyl radical (•OH), hydrogen peroxide (H_2O_2), and hypochlorous acid (HOCl). All of these can be generated by irradiation (x-rays, γ-rays, and ultraviolent radiation) or by normal metabolic processes including autoxidation of reduced electron carriers, lipid peroxidation and metal-catalyzed reactions (1). These forms of ROS can be detrimental to organisms, as they react with and damage macromolecules (DNA, lipids, and proteins) of the cell if not scavenged by antioxidants. Pathogens must combat an additional source of ROS, the oxidative burst generated by host immune cells (2-6).

Helicobacter pylori is a gram-negative microaerophilic organism that colonizes the gastric mucosa and is the causative agent of peptic ulcers and chronic gastritis. It is chronically in contact with an array of ROS during colonization due to the host inflammatory response (2, 7-10). The oxidative stress response of *H. pylori* is made up of many enzymatic systems including superoxide dismutase (SodB), catalase (KatA), methionine sulfoxide reductase (Msr), alkyl hydroperoxide reductase (AhpC), thiol-peroxidase (Tpx) and bacterioferritin co-migratory protein (Bcp) (11-20). These enzymes function in concert as the primary means to combat oxidative stress in the cell. These systems, combined with the Trx system as the reductant, provide *H. pylori* with the tools needed to maintain a reduced environment in times of oxygen stress. *H. pylori* lacks genes for oxidative stress regulators and does not contain the glutathione-glutaredoxin reduction system (GSH) (21). The GSH reduction system is often used in addition

to the Trx system in bacteria to maintain a reduced state inside the cell (22). Organisms that lack GSH such as *Lactobacillus casei, Bacillus subtilis, Bacteroides fragilis, Staphylococcus aureus* and *H. pylori* presumably must rely on the Trx system to maintain thiol/disulfide balance in the cell (21, 23-26). The mechanisms for the maintenance of this balance in *H. pylori* have not been studied in great detail.

The Trx system is highly conserved throughout many organisms and is comprised of thioredoxin reductase (TrxR), NADPH, and Trx. *H. pylori* contains two genes that encode for Trx; Trx1 (HP0824) and Trx2 (HP1458), but only a single gene that encodes the reductase TrxR (HP0825) (13). The first known function of Trx1 was identified in *E. coli*, where it was found to provide electrons to ribonucleotide reductase (RNR). RNR is an essential enzyme in the replication of DNA, and functions to catalyze the synthesis of deoxyribonucleotides from ribonucleotides. Evidence for this function in *H. pylori* has not been presented. However, *H. pylori* Trx1 is expressed in greater amounts as a response to stress agents (27), and it is the electron donor for two important antioxidant enzymes, AhpC and Msr (13, 19). Trx1 but not Trx2 also acts as a chaperone in *H. pylori*, whereby it renatures denatured arginase into its active form (28).

Notably, a *H. pylori* Δ*trx1* mutant was shown to be more sensitive to oxidative stress agents (such as paraquat, S-nitrosoglutathione and O_2) compared to the wild-type (strain 26695), while Δ*trx2* was much less sensitive to these agents than the Δ*trx1* strain (15). No specific roles (e.g. electron donor) for *H. pylori* Trx2 in conferring resistance to oxidative stress or involvement in any cell process are known. We sought to further compare the antioxidant roles of Trx1 and Trx2 in *H. pylori* (strain ATCC 43504). We approached this by studying the effect of *trx* deletions on the subsequent state of macromolecules in the cell with a focus on biomarker

damage for oxidative stress. We determined the effect of oxygen on cell viability, total protein carbonyls, lipid hydroperoxides, 8-oxoguanine (8-oxoG) content in DNA of the strains, and determined the colonization ability of the strains compared to the wild-type strain in a mouse model. The studies infer an important role for these reductant proteins in maintaining macromolecule integrity and survival of *H. pylori* under oxidative stress conditions, including in vivo.

Materials and Methods

Bacterial strains and growth conditions. *H. pylori* 26695 (21) or ATCC 43504 was used for all in vitro studies and strain X47 (29) was used for the mouse colonization assay. *H. pylori* were routinely grown on *Brucella* agar (Oxoid) plates (BA) containing 10% defibrinated sheep blood (HemoStat Laboratories, Bogart, Ga) and maintained in a 37°C humidified chamber under 5% CO_2 and low levels of oxygen (4%) with N_2 used as the balance. Chloramphenicol was added to the plates at a concentration of 30 μg/mL as needed.

Oxygen stress conditions. *H. pylori* 43504, Δ*trx1* or Δ*trx2* from 2 day grown BA plates were suspended in phosphate-buffered saline (PBS), pH 7.4 and exposed to either atmospheric oxygen (20%) or HOCl. For exposure to atmospheric oxygen (20%), cells were suspended in PBS to an OD_{600} = 2 and placed at 37°C with shaking at 60 RPM for up to 12 h. For viability determination, cells were taken at each 2 h time point; dilutions were made and plated on BA plates. All plates were then incubated at 4% O_2 for 5 days and the number of colonies determined. Cells exposed to HOCl were resuspended in PBS to an OD_{600} = 2, subjected to HOCl treatment (0 or 200 μM), and incubated at 37°C shaking for 1 h at 200 RPM. Cells were then washed with PBS and harvested via centrifugation at 7500 x *g* for 10 min at 4°C.

Construction of mutant strains. *(i) Construction of Δtrx1 mutant.* Primers *trx1*-F and *trx1*-R (Table 1) were used to PCR-amplify an 864-bp fragment containing the *H. pylori trx1* gene (*hp0824*). The PCR fragment was directly cloned into pGEM-T vector (Promega) according to the manufacturer's instruction, to generate pGEM-*trx1*. The host strain used for cloning was DH5α. Subsequently, a chloramphenicol acetyl transferase (CAT) cassette was inserted at the unique *NheI* (nucleotide G130) site within the *trx1* sequence of pGEM-*trx1*. The allelic exchange (chloramphenicol resistant) transformants were isolated by selection of single colonies upon incubation in 4% O_2 partial pressure conditions. The balance of the atmosphere was N_2 (91%) and CO_2 (5%). The disruption of the gene in the genome of the mutant strain was confirmed by PCR using primers *trx1*-1 and *trx1*-4, which lie outside the mutagenized region. The PCR product indicated an increase to the expected size, corresponding to insertion of the CAT cassette. *(ii) Construction of Δtrx2 mutant.* The Δ*trx2* mutant strain was constructed by overlapping PCR. Genomic DNA and primers *trx2*-1 and *trx2*-2cat were used to PCR amplify a 388 bp region that contains part of the *hp1459* gene and part of the *hp1458* and *cat* (Cm^r) genes. Next, primers *trx2*-3 and *trx2*-4 were used to PCR amplify a 409 bp region containing part of the *cat* and *hp1458* genes and *hp1457* gene. After each PCR, the products were gel purified and used for a final PCR step using primers *trx2*-1 and *trx2*-4. The final elongation step yielded a 1.5-kb product that was then used for (natural) transformation into strain 26695, 43504 or X47. The transformants (grown at 4% O_2) were transferred onto BA plates supplemented with 30 μg/mL chloramphenicol, and individual colonies appeared after 3-5 days of incubation at 4% O_2. The mutant strains grow at a similar rate to wild type on BA at 4% O_2. The deletion of Δ*trx2* was confirmed by PCR using genomic DNA from each chosen clone. The PCR product was analyzed on a 0.7% Tris-acetate-EDTA (TAE) agarose gel. The clones were further confirmed via

sequencing at the Georgia Genomics Facility (University of Georgia, Athens, GA, U.S.A). Construction of deletion mutants using overlapping PCR was also confirmed by RT-PCR.

Construction of complemented mutant strains. *E. coli* strain DH5α was used for all plasmid DNA manipulations. The genes *trx1* or *trx2* were PCR-amplified from *H. pylori* genomic DNA using primers *trx1*-F and *trx1*-R or *trx2*-F and *trx2*-R. The PCR products were digested with NdeI and XhoI and ligated into the pPA plasmid digested with the same restriction enzymes. This created pPA*trx1* or pPA*trx2* such that the genes are located downstream of the *ureA* promoter. Digestion of the plasmids with BglII and XhoI released the fragment. The fragments were ligated into pEM39kan, also digested with BglII and XhoI. The plasmid pEM39kan has been shown to efficiently insert DNA by homologous recombination into the region of the chromosome corresponding to the HP0405 site. Disruption of this gene has been shown to have no effect on the cell (30, 31). The plasmid was then naturally transformed into the *trx1* or *trx2* mutant strain and selected on BA containing 30 μg/mL kanamycin and 30 μg/mL chloramphenicol. This results in the insertion of *trx1* or *trx2* along with the *ureA* promoter at the HP0405 site in the chromosome. Successful recombination of *trx1* or *trx2* into the chromosome was confirmed via PCR and functional restoration by determining viability of the complemented strains, lipid peroxide level, carbonylated protein content, and mouse colonization as described previously.

Preparation of cell extracts. *H. pylori* was grown on BA plates for 2 days before harvest. Cells were resuspended in sterile PBS, pH 7.4 to an optical density of 1-2 at 600 nm. Cells were then broken by sonication (Heat Systems Ultrasonics Sonicator) in 3 sets of 10 sec intervals (4 watt output power and 40% duty cycle) and placed on ice. Cell debris was removed by centrifugation at 14,000 *x g* for 10 min at room temperature (25°C).

Quantitative real-time PCR. The Aurum total RNA mini kit (Bio-Rad) was used to extract total RNA from *H. pylori* wild type, Δ*trx1* and Δ*trx2* cells grown under 4% O_2. The Turbo DNA-free kit (Ambion) was used to degrade any remaining DNA. cDNA was then synthesized using the iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer's instructions. The iQ SYBR green supermix (Bio-Rad) kit was used for qPCR according to the manufacturer's instructions. Primers specific for *trx1* (*trx1*-F/R(rt)) or *trx2* (*trx2*-F/R(rt)) were used along with primers specific for the housekeeping gene *gyrA* (Table 1). Relative transcript abundance was calculated using the $2^{-\Delta CT}$ formula (32).

Determination of lipid hydroperoxides. Lipid hydroperoxides were extracted from the sample into chloroform as described in Wang *et al.* 2006 (33) and the total amount of lipid hydroperoxides formed was quantified using a lipid hydroperoxide assay kit (Cayman Chemical, Ann Arbor, MI) following the manufacturers instructions. The chloroform extract was mixed with chloroform-methanol solvent and then combined with reagents 1 and 2 of the kit. Reagent 1 contained ferrous sulfate, which reacts with hydroperoxides to produce ferric ions. The resulting ferric ions react with ammonium thiocyanate, a chromogen that is detected at 500 nm with an extinction coefficient of 16,667 $M^{-1}cm^{-1}$.

Fluorescent staining of cells and quantification of 8-oxoG. *H. pylori* 43504, Δ*trx1* and Δ*trx2* were analyzed for the presence of 8-oxoG by fluorescent staining of the cells as described by Wang *et al.* 2006 (33). 8-oxoG is structurally similar to avidin's natural substrate biotin, so avidin binding to 8-oxoG can be used for detection of 8-oxoG (34). Eight sets of the immunofluorescent images were examined for the luminosity of FITC and PI as well as for cell

morphology, and a representative set of images is shown in Fig. 4. The average ratio of luminosity of FITC/PI was calculated from eight sets of images for the wild-type and the mutant cells.

Isolation of carbonylated proteins. Two-day grown *H. pylori* 43504 from BA plates was exposed to 20% O_2 for 2 or 4 h (as described above) and then washed twice (centrifugation at 10,000 x *g* for 5 min at room temperature) in 50 mM potassium phosphate buffer, pH 6.7 with 1 mM EDTA. Cells were then suspended in this buffer and cell extracts obtained, as described above. Samples were analyzed for contaminating nucleic acids by determining the ratio given by 280/260 nm. The carbonylated proteins can be quantified from cell extracts by reacting them with 2,4-Dinitrophenylhydrazine (DNPH) (35). DNPH (Sigma) at 10 mM dissolved in 2 M HCl was added to the sample tubes and 2 M HCl was added to the control tubes. All samples were incubated for 1 h in the dark with vortexing every 15 min. After incubation with DNPH, 20% Trichloroacetic acid (TCA) (Sigma) was added to each tube. Samples were centrifuged at 10,000 *x g* for 10 min and the pellet was suspended in 10% TCA. Samples were centrifuged again at 10,000 *x g* for 10 min and the pellet was suspended in a 1:1 ethanol/ethyl acetate mixture and washed twice with this mixture. The remaining pellet was suspended in 6 M guanidine hydrochloride (Fisher), centrifuged at 10,000 x *g* for 10 min, and the supernatant transferred to the wells of a microtitre plate. Absorbance was then determined at 340 nm. The supernatant was also used to measure protein concentration of the samples by reading the absorbance at 280 nm and comparing to a BSA standard curve.

Mouse colonization assay. *H. pylori* X47, Δ*trx1*, Δ*trx2*, *trx1* complement or the *trx2* complement strains were suspended in sterile PBS, pH 7.5 and inoculated into 5-6 week old female C57BL/6NCr mice (NCI, Frederick, MD) via oral gavage with 0.1 mL cell suspension.

The 0.1 mL contained 1.7 x 10^8 viable cells. Three weeks after inoculation, mouse stomachs were removed and homogenized in PBS, pH 7.5 (36). The homogenized samples were then serially diluted and 0.1 mL plated onto BA plates containing 100 μg/mL bacitracin, 10 μg/mL vancomycin, and 10 μg/mL amphotericin. The plates were incubated in a partial pressure O_2 atmosphere (4%) for 5-7 days, the resulting colonies counted and cfu/g stomach calculated. The Institutional Animal Care and Use Committee gave prior approval for all animal work.

Statistical analysis. Results were analyzed using the unpaired *t* test and presented as the mean ± standard deviation (www.graphpad.com). For the mouse colonization assay, the Wilcoxon signed-rank test was used to calculate statistical significance.

Results

Oxygen stress tolerance of non-growing cells. To elucidate the roles of the *trx* genes on the ability of *H. pylori* to survive under oxygen stress, we determined the oxygen sensitivity (i.e. viability) of the *trx* deletion mutants. Non-growing cells were incubated under atmospheric oxygen (20%) for a 12 h period. This condition is considered a stress environment for *H. pylori*, as the bacterium grows optimally under low oxygen (2-10%). As shown in Fig. 1, the wild-type and the complemented strains were only slightly affected by exposure to oxygen during an 8 h period, but Δ*trx1* showed a 100-fold loss in viability after 4 h of atmospheric oxygen exposure (Fig. 1a). The Δ*trx2* mutant also showed a loss of viability as a result of oxygen stress but its sensitivity was not as severe as Δ*trx1*. The Δ*trx2* mutant was 100-fold decreased in viability compared to the wild-type upon 6 h of atmospheric oxygen exposure (Fig. 1b). The viability of both strains was restored to wild-type levels upon complementation with the wild-type version of both *trx1* and *trx2*. This makes conclusions about the roles of these enzymes from mutant strain analysis rigorous and indicates that both Trx1 and Trx2 play important roles in protecting the cell

from oxidative stress. We conclude that *trx1* and *trx2* are essential for optimal growth under oxygen stress in *H. pylori* 43504.

Transcript abundance in the single *trx* gene deletion strains. *trx* transcript abundance was analyzed using quantitative real time PCR (Fig. 2). There was a 6-fold increase in transcript abundance of *trx1* in the Δ*trx2* strain background compared to that in the wild-type (Fig. 2a). However, a 4-fold decrease in transcript abundance of *trx2* was observed in the Δ*trx1* background compared to wild type (Fig. 2b). It is noteworthy that the overall abundance of *trx2* was 1000-fold less than *trx1* in the wild-type. Transcript abundance differences of *trx* have previously been shown in other organisms that possess multiple thioredoxins (25, 37-39).

The amounts of lipid peroxides are greater in the Δ*trx* mutants compared to the wild-type under oxygen stress. Unsaturated phospholipids, glycolipids, and cholesterol in the cell membrane are all targets of oxidant attack. Reactive oxygen-mediated oxidation of these molecules results in the initiation of lipid peroxidation, and subsequent free radical chain reaction, under times of oxidative stress (40). Thus, quantification of LOOH is often used to assess the level of oxidative membrane damage in cells under stress.

H. pylori possesses two peroxiredoxins that are known to reduce such hydroperoxide derivative molecules to their corresponding alcohol; AhpC and bacterioferritin comigratory protein (BCP) (33). Since Trx1 is required for the function of AhpC, we reasoned that the Δ*trx1* strain would contain a greater amount of lipid hydroperoxide compared to the wild-type, especially under oxidative stress. Indeed, upon 200 μM HOCl exposure, we saw a 4-fold greater (over wild type) lipid hydroperoxide level in Δ*trx1* (Fig. 3). The Δ*trx2* strain also had a 4-fold greater level of lipid hydroperoxides compared to the parent strain (Fig. 3). The data from both mutants were shown to be statistically significantly greater than the wild-type with a 99%

confidence level. Importantly, complementation of either of the *trx* deletion strains with the wild-type version of the gene restored lipid peroxide levels to near wild-type levels (Fig. 3). The increase in lipid peroxides in the Δ*trx2* mutant is interesting because Trx2 had previously been ruled out as an electron donor to AhpC (13). The results suggest that both Trx's are involved in preventing lipid peroxidation.

Oxidative stress affects cell morphology and the level of 8-oxoG in *H. pylori* Δ*trx* mutants. Next, we examined the level of 8-oxoG DNA lesions in the mutant cells by using FITC-avidin/PI fluorescence microscopy. As FITC-avidin specifically labels DNA lesions while PI labels all DNA, the intensity ratio of stained FITC to PI reflects the level of 8-oxoG DNA lesions in the cells (41). This technique was successfully used in our lab to examine other oxidative stress-related mutants of *H. pylori* (42, 43).

In this analysis, *H. pylori* wild-type or mutant cells were exposed to air for 4 h before subjecting them to immunofluorescent staining. Fig. 4 shows that 4 h exposure to air had no significant effect on the morphology of the wild-type cells, with ~90% of the cells being in the bacillary form. However, when similarly treated, ~45% of the Δ*trx1* cells and ~90% of the Δ*trx2* cells transformed to the coccoid form or were lysed, suggesting these cells suffer from persistent oxidative stress (44). The ratio of FITC-avidin/PI fluorescence intensity in the Δ*trx1* and Δ*trx2* mutant cells was determined to be 1.7- and 2.4-fold higher, respectively, than that in the wild-type cells (Table 2). These results demonstrated that the Δ*trx* mutant cells exposed to oxidative stress contain considerably higher levels of 8-oxoG DNA lesions, and the Δ*trx2* mutant cells suffered more oxidative damage than the Δ*trx1* mutant cells.

In another experiment, *H. pylori* wild type or Δ*trx* mutant cells were grown under optimum conditions with low O_2 (4% partial pressure in the atmosphere) before conducting the

immunofluorescent staining. No significant difference between wild type and Δ*trx* cells was observed in either cell morphology or in the level of 8-oxoG DNA lesions generated (data not shown).

Oxidative damage to proteins. To further assess the effect of the *trx* deletions on cells under oxidative stress, we estimated the overall oxidative modification of proteins by measuring the presence of carbonyl groups. Protein carbonyls are often used as a biomarker for severe oxidative stress, and the accumulation of carbonylated proteins is linked to *E. coli* cell death (33, 45-50). Although some amino acid residues are precursors to carbonylation and can be repaired, the addition of a carbonyl group is an irreversible post-translational modification (45). Fig. 5 shows that at 20% O_2 exposure for 2 h, the Δ*trx1* and Δ*trx2* mutants contained a 7- and 5-fold greater amount of carbonylated proteins, respectively than the wild-type. At 4 h, however, there was a greater amount of protein carbonyls in Δ*trx2* compared to Δ*trx1*. A 7-fold greater carbonyl level in Δ*trx1* over wild type was observed, while Δ*trx2* was increased 20-fold under 20% O_2. Upon complementation, carbonyl levels were restored to wild type levels. Taken together, these results indicate an increase in protein carbonylation in cells lacking the *trx* genes, and indicate these thioredoxins play roles in limiting protein damage.

The Δ*trx1* and Δ*trx2* mutants are decreased in mouse colonization ability. To determine whether *trx1* or *trx2* had an effect on the ability of *H. pylori* to colonize the stomach, we inoculated mice with all 5 strains (wild type X47, Δ*trx1*, Δ*trx2* or with each complemented strain). Three weeks after inoculation, the mouse stomachs were harvested and colony counts determined. As shown in Fig. 6, we recovered *H. pylori* from all 13 mice inoculated with the wild-type strain at numbers ranging between 10^3-10^6 colony-forming units (cfu) per gram of stomach. Eleven of the 13 mice inoculated with Δ*trx1* were *H. pylori*-positive, but 2 of those 11

showed colonization numbers below 10^3 cfu/g stomach. All 13 mice inoculated with Δ*trx2* were *H. pylori*-negative. Importantly, both the *trx1* and *trx2* complemented strains colonized the stomachs at levels similar to the wild-type. The colonization efficiency of the Δ*trx1* mutant is significantly lower than the wild-type at the 95% confidence level ($P<0.05$), and the Δ*trx2* strain is lower at more than 99% level of confidence. These results suggest that both *trx1* and *trx2* are important for host colonization by the pathogen, but *trx2* is more important.

Discussion

Maintaining the redox balance within *H. pylori* is important for cell viability, growth and metabolism (51). The Trx system essential for survival under oxidative stress, as this is the only disulfide reductase system present in *H. pylori*. The glutathione-glutaredoxin system, present in many other organisms for maintaining the cellular thiol/disulfide balance, is lacking in *H. pylori* (21). In addition, the Trx system is important because it is the electron donor for all three peroxidases, BCP, Tpx, and AhpC (13, 43). *H. pylori* Trx1 contains the typical Trx motif found in most Trx proteins (CGPC) but Trx2 contains an unusual motif (CPDC) (27). It is not known how this affects its activity or its roles. Alignment of the protein sequences of Trx1 and Trx2 shows that the two proteins are only 30% identical. Trx2 has been ruled out as an electron donor for the known oxidative stress-combating reductases tested (AhpC and Msr) (13, 19). The Trx2 of *H. pylori* is, however, still a functional disulfide reductase as it has been shown to successfully reduce insulin in vitro when used as a substrate (13).

The O_2-exposure viability assay suggests that both Trxs have independent roles in protecting the cell from oxygen stress (Fig. 1). This is similar to what was concluded based on mutants in other organisms that contain more than one *trx* gene and no GSH reduction system. For example, *L. casei* is also a GSH negative organism and has four *trx* genes. Disruption of

either *trx1* or *trx2* resulted in moderate growth defects under aerobic conditions, and a *trx1trx2* double mutant of *L. casei* had severe growth defects (25). Still, the partial growth ability of the double mutant suggests that the other Trxs function to some extent. Similar results were shown in *B. fragilis*, which contains six *trx* genes. A single deletion strain of one of the *trx* genes did not show any growth defect but deletion of multiple genes in a single strain did reveal a defective growth phenotype (52). A previous study using *H. pylori* 26695 as the parent strain showed a defective growth associated with a Δ*trx1* strain but a less severe defect in the Δ*trx2* strain. A Δ*trx1trx2* double mutant showed the largest growth defect (15). Our result implicates Trx2 as a major player in protecting the cell against oxygen stress and for survival in vivo.

The Δ*trx2* strain contained more 8-oxoG than both the Δ*trx1* strain or wild type cells (Fig. 4). This effect on DNA is a novel finding and has not been demonstrated in any other organism. Trxs are known to have important roles in the reduction of ribonucleotide reductase, an enzyme required for DNA synthesis (53). The function of *H. pylori* Trx2 is not known but it is possible that it could be involved in DNA replication and/or DNA repair. One indication that Trx2 could be involved in DNA replication is based upon the location of *trx2* in the chromosome. The *trx2* gene is located near *dnaE* (825 bp upstream), encoding the DNA polymerase III alpha subunit. This is similar to what was observed regarding the location of the essential *trxA* in *B. fragilis*; *trxA* is located directly downstream of *dnaE*. Phylogenetic analysis of TrxA grouped it with *Campylobacter*, *Helicobacter* and *Porphyromonas gingivalis* indicating that the roles of TrxA and Trxs in these organisms may overlap (52). In addition to DNA synthesis, Trx2 may be involved in DNA repair. Under oxygen stress conditions, DNA can become damaged by guanine oxidation (54). Oxidized guanine can then be incorporated into the genome resulting in mispaired bases. *H. pylori* contains several proteins that function to excise 8-

oxoG; MutT, MutY, and MutS (55). MutY is an iron-sulfur containing glycosylase that excises mispaired adenines from the 8-oxoG (56). The Trx reductase system has been shown to be essential for iron binding in the iron-sulfur cluster protein IscA in *E. coli* (57). It is possible that *H. pylori* Trx2 aids in the iron binding of MutY.

We demonstrated an association between individual Trxs and host colonization ability for the first time in a bacterial system. Previously, a *B. fragilis* Trx reductase (*trxB*) mutant was shown to be essential for survival in an in vivo mouse abscess model (24). This mutant renders the whole system incomplete (the reductase and Trx) so it does not provide insight as to the importance of the Trx alone. We show that the *H. pylori* Δ*trx1* mutant is decreased in ability to colonize the mouse stomach (Fig. 6). This phenotype is not surprising in light of the known functions of Trx1 as the reductant for oxidized Msr and AhpC, and its function as a chaperone for proper protein folding (13, 19, 28). However, the complete lack of colonization by the Δ*trx2* mutant (Fig. 6) was not expected. Based upon this result, it seems that *H. pylori* Trx2 plays a significant role in vivo. It may play a broad role like Trx proteins that function to provide electrons to DsbD located in the cytoplasmic membrane (58). *H. pylori* contains several Dsb (disulfide bond) protein oxidases that allow for disulfide bond formation in the periplasm (59-62). This mechanism has been well characterized in *E. coli*, but less so in *H. pylori* (63). In *E. coli*, DsbD functions as the acceptor of electrons from Trx and then transfers them to various periplasmic oxidoreductases and a cytochrome synthesis protein (DsbB, DsbA, DsbC, and CcmG). DsbD receives the electrons from cytoplasmic Trx proteins and has been shown to reduce electrons to proteins involved in disulfide bond isomerization, cytochrome c maturation, and possibly other processes (64). In *H. pylori*, homologues of DsbG, DsbC, DsbB (DsbI), and DsbD (CcdA) have been identified (60, 61). DscC and DsbG both have the Trx CXXC motif and

DsbG has been shown to have reductase activity. The reductase activity combined with a Trx motif indicates that DsbG functions as an oxidoreductase in the periplasm (59).

There are many virulence proteins located in the periplasm of *H. pylori* that if damaged, would likely result in a lack of host colonization. While Trx maintains the methionine repair enzyme Msr reduced in the *H. pylori* cytoplasm, the players carrying out this role in the periplasm (where Msr is also known to function) are not known (19, 65). A membrane-bound Trx-like protein PilB provides electrons to Msr in the *N. gonorrhoeae* periplasm, and the *N. gonorrhoeae* and *H. pylori* Msr's are highly homologous to each other (18, 66). Catalase is also an important periplasmic virulence protein in *H. pylori* (67). Catalase has been shown to be susceptible to oxidative inactivation and subsequent repair by Msr. Failure to repair a methionine sulfoxide-containing catalase could certainly result in a decrease in host colonization ability (68). It is noteworthy that during long term colonization, a Δ*msr* mutant could not be recovered from the mouse stomach, indicating the importance of Msr in host colonization (18).

A proteomic analysis of *E. coli* Trx-targeted proteins revealed Trx to be involved in at least 26 different cellular processes including transcriptional regulation, cell division, energy transduction, and several biosynthetic pathways (69). Trx is likely to be involved in multiple processes in *H. pylori* as well. Further work is needed to ascertain if any new protein-protein interactions occur that would aid in revealing new Trx roles, ones perhaps aiding peroxidase activity or macromolecule repair processes. While the specific roles of Trx2 are not known, the importance of this protein in survival is now revealed. Both Trxs studied herein aid macromolecule integrity and homeostasis.

Acknowledgement

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Table 3.1 List of primers used in this study.

Primer[a]	Sequence (5' to 3')
trx1-1	TTGAAATGGTGGAGAGGAAC
trx1-4	CCGGTAGCGATAATCACGC
trx1-F	ATTTATGCGATCACGCCGAG
trx1-R	AAGTCTTGCCATCTTCTGCC
trx2-1	CGCGATGCAAGAGGGCTTG
trx2-2	ATCCACTTTTCAATCTATATCAATCATT
trx2-3	CCCAGTTTGTCGCACTGATAACGTTAT
trx2-4	CGCTTGAGCAACCCACC
trx2-F	ACACCATATGTCAGAAATGATTAACGG
trx2-R	ACACCTCGAGCAATAACGCTTTTAGAG
trx1-F(rt)	GTGGGCCTTGTAAGATG
trx1-R(rt)	CTGATACCAATTTTGCGCTC
trx2-F(rt)	GGATTGCAGAAAGATTGAGC
trx2-R(rt)	CTTGCGGATGCCTAAG
gyrA-F(rt)	GCTAGGATCGTGGGTGATGT
gyrA-R(rt)	TGGCTTCAGTGTAACGCATC

[a] All primers were obtained from Integrated DNA Technologies

Table 3.2 Cell morphology and the level of 8-oxo guanine in *H. pylori* cells. [a] Cell morphology was examined by fluorescent microscopy, and the numbers are estimated percentage of damaged cells including coccoid and broken cells in the whole cell population. [b] 8-oxoG level is expressed as mean intensity ratio of FITC/PI, with standard deviation. According to statistical analysis with Student's *t* test, the data of each mutant strain are significantly different from that of the wild type ($P<0.001$), n=3.

Strains	Cell morphology [a] (% damaged cells)	8-oxoG level [b] (FITC/PI intensity ratio)
WT	~ 10	0.86 + 0.05
trx1	~ 45	1.45 + 0.06
trx2	~ 85	2.05 + 0.13

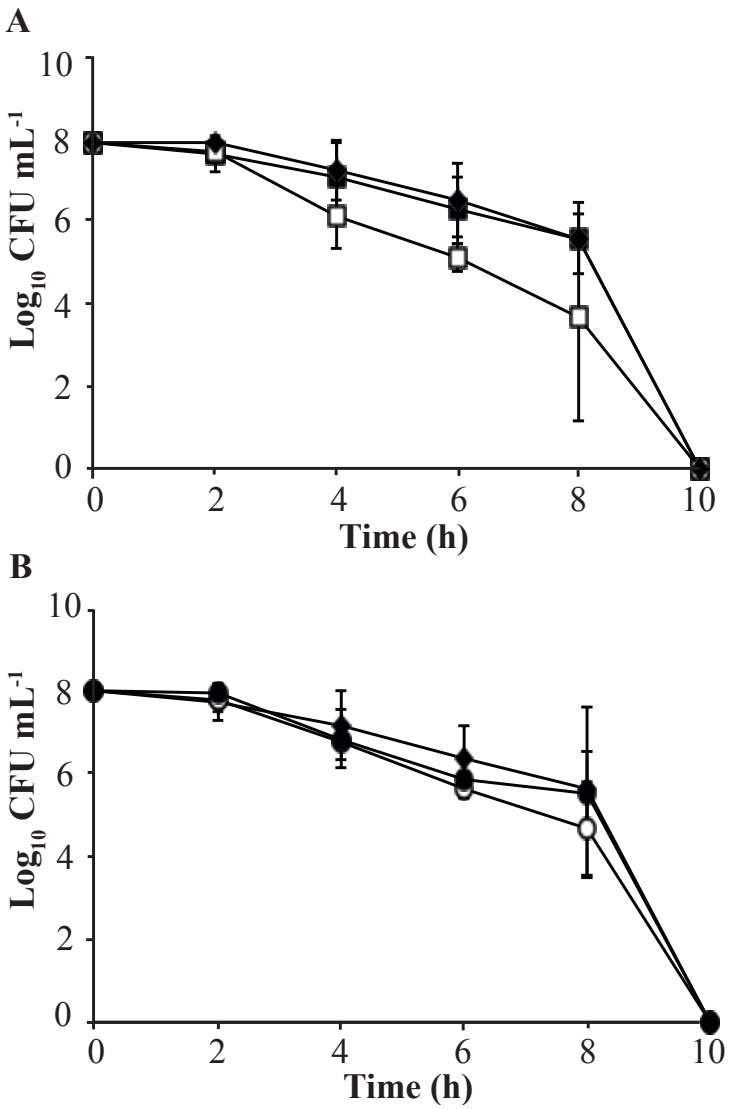

Figure 3.1. Survival of non-growing cells incubated under atmospheric oxygen. A) Wild type (closed diamond), Δ*trx1* (open square), and Δ*trx1* comp. (filled square). B) Wild type (filled diamond), Δ*trx2* (open circle), and Δ*trx2* comp. (filled circle). All strains were resupended in sterile PBS and incubated under atmospheric oxygen for a 12-hour period. Samples were taken at 2 h intervals, diluted, and plated onto BA plates. Dilutions were then incubated in a 4% partial pressure O_2 atmosphere and colony counts determined. The mean and standard deviation from 4 independent observations are plotted.

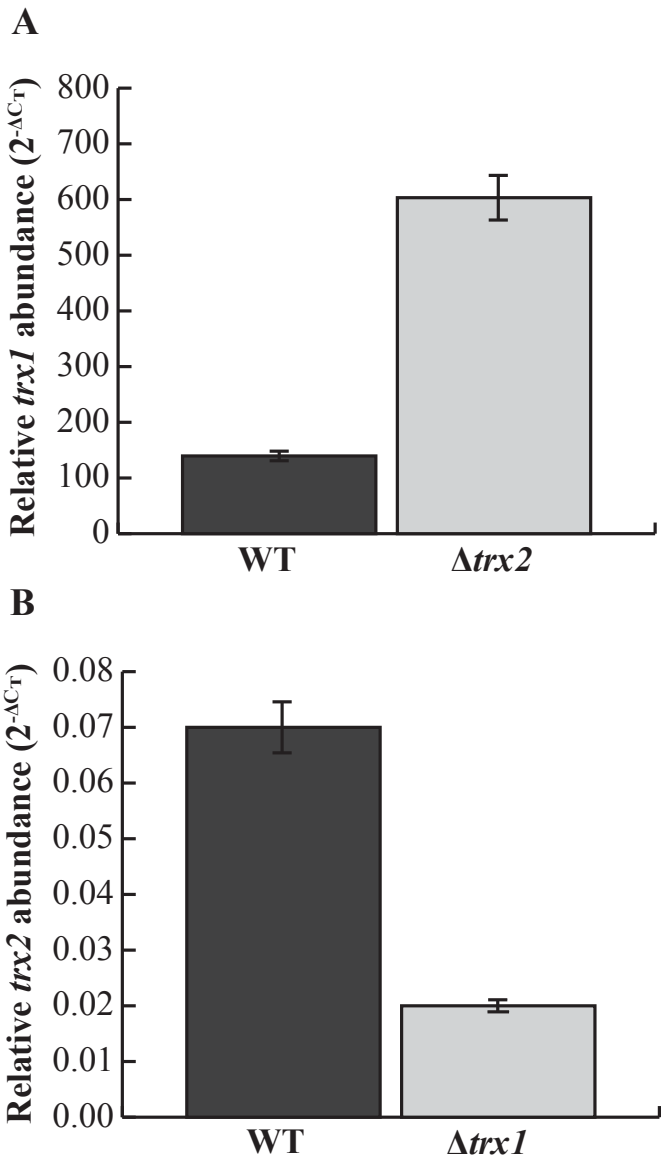

Figure 3.2. Relative *trx* transcript levels. A) *trx1* abundance in the wild-type and Δ*trx2* strains. B) *trx2* abundance in the wild-type and Δ*trx1* strains. Transcript abundance was determined by quantitative real time PCR after growing cells under conditions favorable for microaerophilic growth (4% O_2). The *gyrA* housekeeping gene was used as an internal control. Results shown are from two separate experiments sampled in triplicate. The experiment was repeated again with similar results (data not shown).

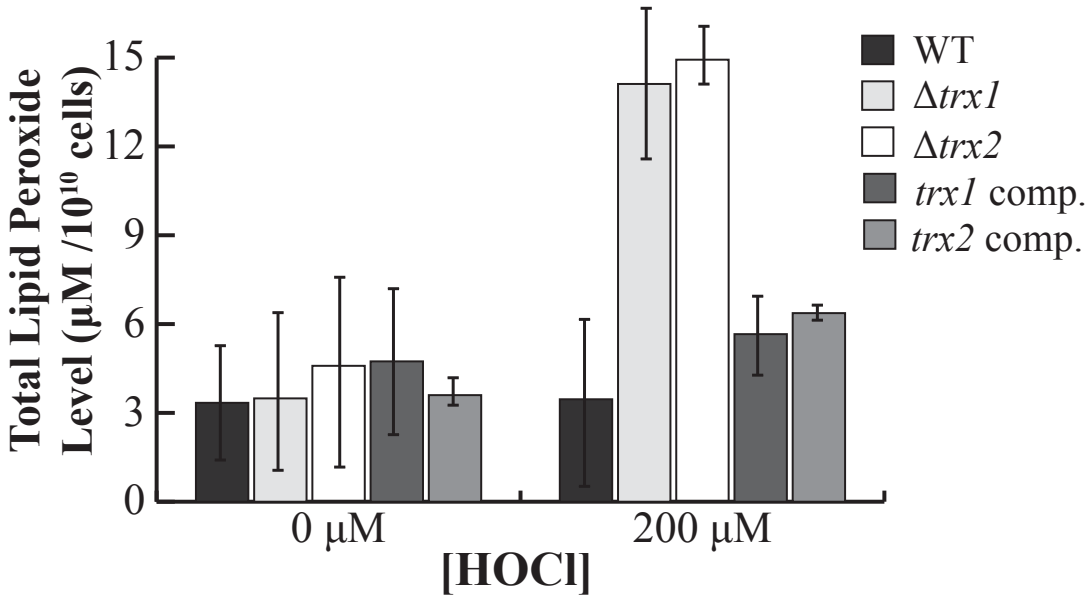

Figure 3.3. Lipid peroxide level in *H. pylori trx* mutants exposed to hypochlorous acid (HOCl) stress. *H. pylori* were grown on BA plates for 2 days, exposed to oxidant (or not), and cell extracts obtained. The total amount of lipid peroxides was determined and the hydroperoxide concentration in the sample was calculated. Data are presented as the average of three independent experiments. Based on the Student's *t* test, the Δ*trx1* and Δ*trx2* strains exposed to HOCl contain significantly greater amounts of hydroperoxide than the wild-type at the 99% level of confidence.

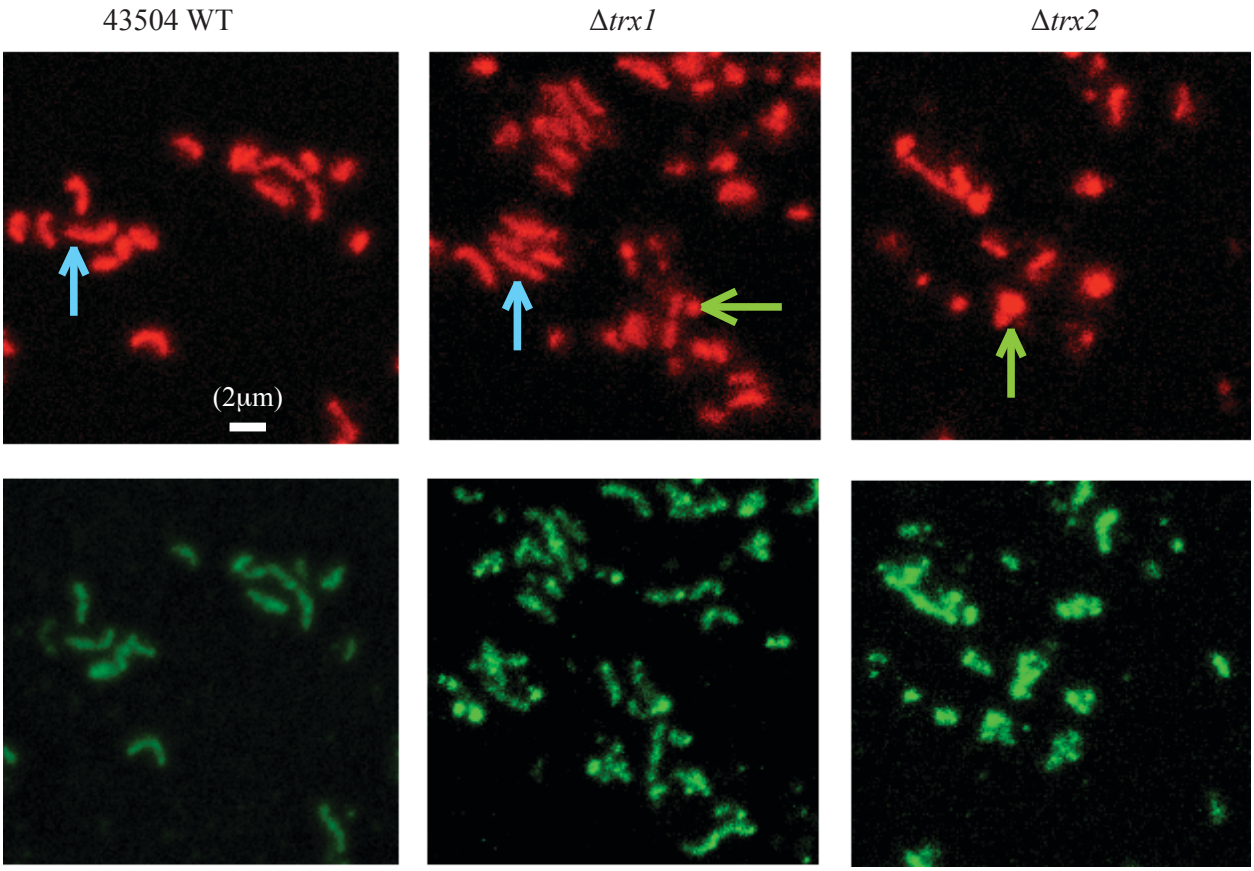

Figure 3.4. Detection of 8-oxoG by immunofluorescent staining. *H. pylori* WT, Δ*trx1* and Δ*trx2* mutant cells were fixed on the glass slide and stained with 8-oxoG -specific avidin-FITC conjugate (lower panel) and propidium iodide (upper panel) followed by examination via fluorescence microscopy. The contrast adjustment was normalized for all the images, and a representative set of images is shown here. Blue arrows point to some examples of bacillary cells in WT and Dtrx1 strains, and green arrows highlight some coccoid or broken cells in D*trx1* and D*trx2* strains. A 2 μm bar is given as a size scale.

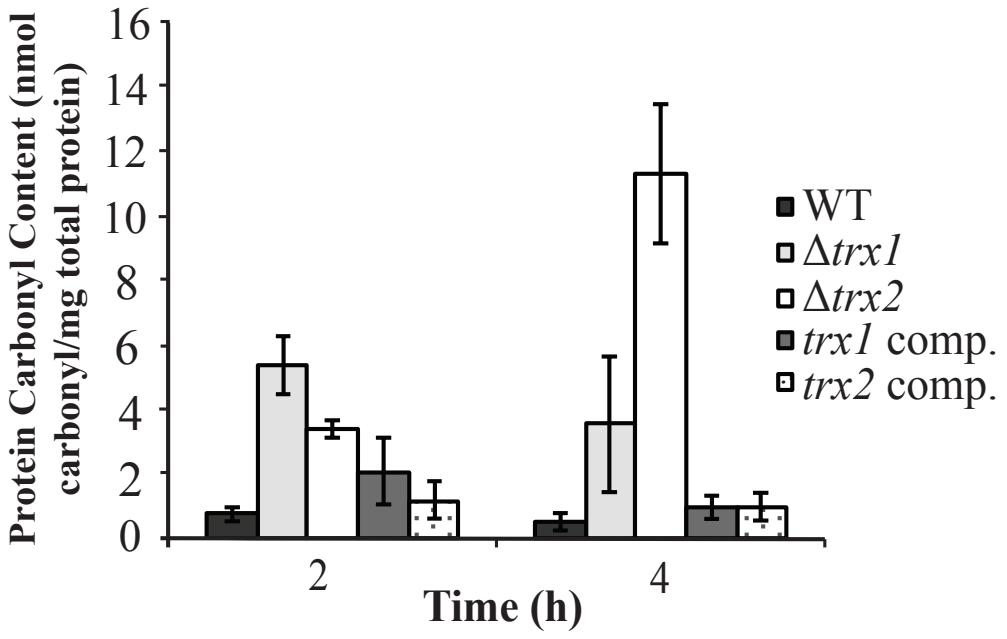

Figure 3.5. Protein carbonyl content in cells exposed to oxygen stress (20%) for 2 or 4 h.WT, D*trx1*, D*trx2*, *trx1* comp. or *trx2* comp. were exposed to 20% O_2 for 2 or 4 h and extracts reacted with DNPH. The carbonyl content was determined by measuring the absorbance at 340 nm. Total carbonyl content is expressed as nmol carbonyl/mg total protein. Results shown are the average of three independent experiments. The carbonyl content of the both deletion strains was significantly greater than the wild-type at both 2 and 4 h time points (P<0.01). In low O_2 (i.e. 4%), the carbonyl content of the mutant strains was not significantly greater than the wild-type (data not shown).

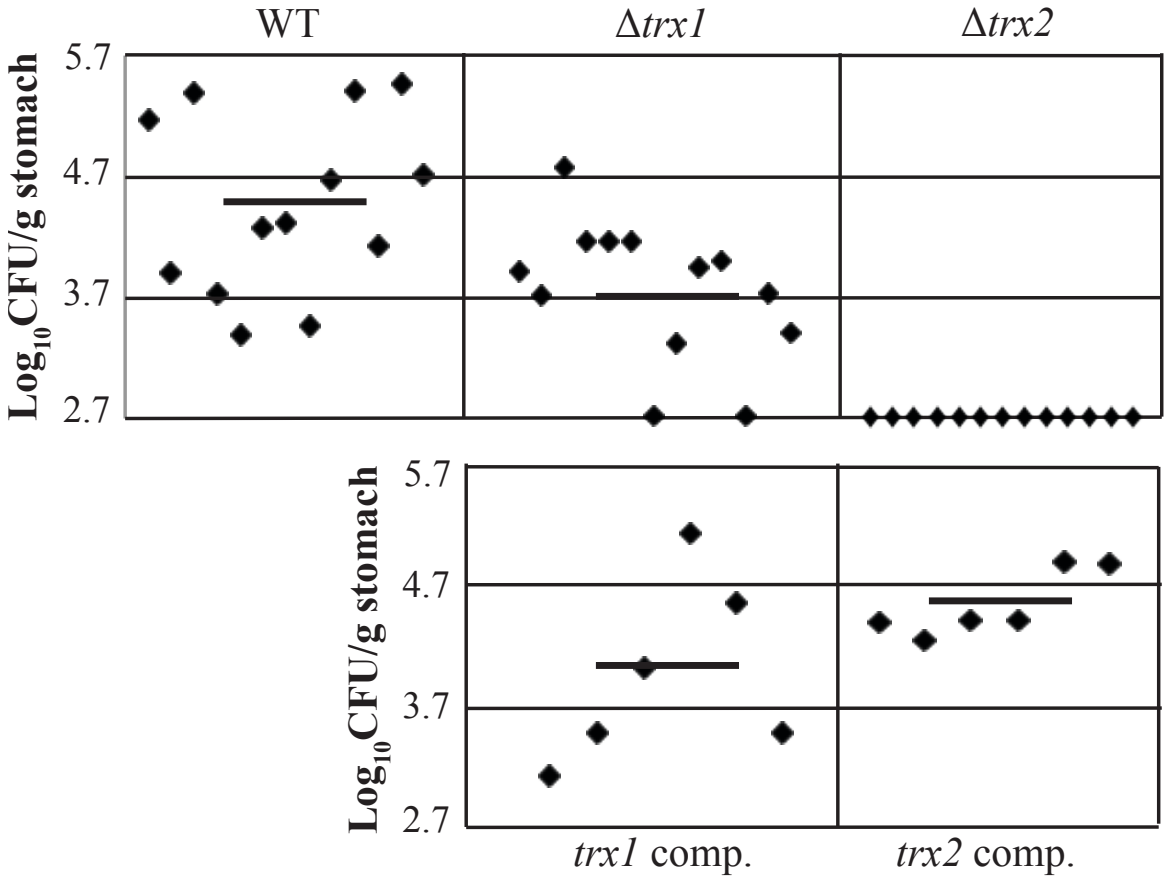

Figure 3.6. Mouse colonization assay of *H. pylori* X47 and *trx* strains. The mice were inoculated with a dose of 1.7 x 10^8 viable cells. Colonization of the mouse stomachs was determined 3 weeks post-inoculation. Mouse stomachs were homogenized and serial dilutions were plated. Data are presented as a scatter plot of colony forming units per gram of stomach as determined by plate counts. Each point represents CFU count for one stomach, and the solid horizontal line represents the mean colonization for each strain. The base line [$\log_{10}$(CFU g^{-1})=2.7] is the detection limit of the assay, which represents a count below 500 CFU g^{-1} stomach. The experiment was repeated with similar results to those shown (data not shown). Horizontal bars represent the average $\log_{10}$ CFU/g stomach. Wild-type and trx1 significant at P<0.05. Wild-type and trx2 significant at P<0.01.

References

1. **Stadtman ER, Levine RL.** 2000. Protein oxidation. Ann N Y Acad Sci **899:**191-208.

2. **Bagchi D, Bhattacharya G, Stohs SJ.** 1996. Production of reactive oxygen species by gastric cells in association with *Helicobacter pylori*. Free Radic Res **24:**439-450.

3. **Ramarao N, Gray-Owen SD, Meyer TF.** 2000. *Helicobacter pylori* induces but survives the extracellular release of oxygen radicals from professional phagocytes using its catalase activity. Mol Microbiol **38:**103-113.

4. **Davies GR, Simmonds NJ, Stevens TR, Sheaff MT, Banatvala N, Laurenson IF, Blake DR, Rampton DS.** 1994. *Helicobacter pylori* stimulates antral mucosal reactive oxygen metabolite production in vivo. Gut **35:**179-185.

5. **Baik SC, Youn HS, Chung MH, Lee WK, Cho MJ, Ko GH, Park CK, Kasai H, Rhee KH.** 1996. Increased oxidative DNA damage in *Helicobacter pylori*-infected human gastric mucosa. Cancer Res **56:**1279-1282.

6. **Nardone G, Rocco A, Malfertheiner P.** 2004. Review article: *Helicobacter pylori* and molecular events in precancerous gastric lesions. Aliment Pharmacol Ther **20:**261-270.
7. **Stent A, Every AL, Sutton P.** 2012. *Helicobacter pylori* defense against oxidative attack. Am J Physiol Gastrointest Liver Physiol **302:**G579-587.

8. **Bagchi D, McGinn TR, Ye X, Bagchi M, Krohn RL, Chatterjee A, Stohs SJ.** 2002. *Helicobacter pylori*-induced oxidative stress and DNA damage in a primary culture of human gastric mucosal cells. Dig Dis Sci **47:**1405-1412.

9. **Chaturvedi R, Asim M, Romero-Gallo J, Barry DP, Hoge S, de Sablet T, Delgado AG, Wroblewski LE, Piazuelo MB, Yan F, Israel DA, Casero RA, Jr., Correa P, Gobert AP, Polk DB, Peek RM, Jr., Wilson KT.** 2011. Spermine oxidase mediates the gastric cancer risk associated with *Helicobacter pylori* CagA. Gastroenterology **141:**1696-1708 e1691-1692.

10. **Briggs RT, Drath DB, Karnovsky ML, Karnovsky MJ.** 1975. Localization of NADH oxidase on the surface of human polymorphonuclear leukocytes by a new cytochemical method. J Cell Biol **67:**566-586.

11. **Spiegelhalder C, Gerstenecker B, Kersten A, Schiltz E, Kist M.** 1993. Purification of *Helicobacter pylori* superoxide dismutase and cloning and sequencing of the gene. Infect Immun **61:**5315-5325.

12. **Hazell SL, Evans DJ, Jr., Graham DY.** 1991. *Helicobacter pylori* catalase. J Gen Microbiol **137:**57-61.
13. **Baker LM, Raudonikiene A, Hoffman PS, Poole LB.** 2001. Essential thioredoxin-dependent peroxiredoxin system from *Helicobacter pylori*: genetic and kinetic characterization. J Bacteriol **183:**1961-1973.

14. **Zhou Y, Wan XY, Wang HL, Yan ZY, Hou YD, Jin DY.** 1997. Bacterial scavengase p20 is structurally and functionally related to peroxiredoxins. Biochem Biophys Res Commun **233:**848-852.

15. **Comtois SL, Gidley MD, Kelly DJ.** 2003. Role of the thioredoxin system and the thiol-peroxidases Tpx and Bcp in mediating resistance to oxidative and nitrosative stress in *Helicobacter pylori*. Microbiology **149:**121-129.

16. **Olczak AA, Wang G, Maier RJ.** 2005. Up-expression of NapA and other oxidative stress proteins is a compensatory response to loss of major *Helicobacter pylori* stress resistance factors. Free Radic Res **39:**1173-1182.

17. **Wang G, Olczak AA, Walton JP, Maier RJ.** 2005. Contribution of the *Helicobacter pylori* thiol peroxidase bacterioferritin comigratory protein to oxidative stress resistance and host colonization. Infect Immun **73:**378-384.

18. **Alamuri P, Maier RJ.** 2004. Methionine sulphoxide reductase is an important antioxidant enzyme in the gastric pathogen *Helicobacter pylori*. Mol Microbiol **53:**1397-1406.

19. **Alamuri P, Maier RJ.** 2006. Methionine sulfoxide reductase in *Helicobacter pylori*: interaction with methionine-rich proteins and stress-induced expression. J Bacteriol **188:**5839-5850.

20. **Pesci EC, Pickett CL.** 1994. Genetic organization and enzymatic activity of a superoxide dismutase from the microaerophilic human pathogen, *Helicobacter pylori*. Gene **143:**111-116.

21. **Tomb JF, White O, Kerlavage AR, Clayton RA, Sutton GG, Fleischmann RD, Ketchum KA, Klenk HP, Gill S, Dougherty BA, Nelson K, Quackenbush J, Zhou L, Kirkness EF, Peterson S, Loftus B, Richardson D, Dodson R, Khalak HG, Glodek A, McKenney K, Fitzegerald LM, Lee N, Adams MD, Hickey EK, Berg DE, Gocayne JD, Utterback TR, Peterson JD, Kelley JM, Cotton MD, Weidman JM, Fujii C, Bowman C, Watthey L, Wallin E, Hayes WS, Borodovsky M, Karp PD, Smith HO, Fraser CM, Venter JC.** 1997. The complete genome sequence of the gastric pathogen *Helicobacter pylori*. Nature **388:**539-547.

22. **Lu J, Holmgren A.** 2014. The thioredoxin antioxidant system. Free Radic Biol Med **66:**75-87.

23. **Newton GL, Arnold K, Price MS, Sherrill C, Delcardayre SB, Aharonowitz Y, Cohen G, Davies J, Fahey RC, Davis C.** 1996. Distribution of thiols in microorganisms: mycothiol is a major thiol in most actinomycetes. J Bacteriol **178:**1990-1995.

24. **Rocha ER, Tzianabos AO, Smith CJ.** 2007. Thioredoxin reductase is essential for thiol/disulfide redox control and oxidative stress survival of the anaerobe *Bacteroides fragilis*. J Bacteriol **189:**8015-8023.

25. **Serata M, Iino T, Yasuda E, Sako T.** 2012. Roles of thioredoxin and thioredoxin reductase in the resistance to oxidative stress in *Lactobacillus casei*. Microbiology **158:**953-962.

26. **Fahey RC, Brown WC, Adams WB, Worsham MB.** 1978. Occurrence of glutathione in bacteria. J Bacteriol **133:**1126-1129.

27. **Windle HJ, Fox A, Ni Eidhin D, Kelleher D.** 2000. The thioredoxin system of *Helicobacter pylori*. J Biol Chem **275:**5081-5089.

28. **McGee DJ, Kumar S, Viator RJ, Bolland JR, Ruiz J, Spadafora D, Testerman TL, Kelly DJ, Pannell LK, Windle HJ.** 2006. *Helicobacter pylori* thioredoxin is an arginase chaperone and guardian against oxidative and nitrosative stresses. J Biol Chem **281:**3290-3296.

29. **Veyrier FJ, Ecobichon C, Boneca IG.** 2013. Draft Genome Sequence of Strain X47-2AL, a Feline *Helicobacter pylori* Isolate. Genome Announc **1**.

30. **Olson JW, Mehta NS, Maier RJ.** 2001. Requirement of nickel metabolism proteins HypA and HypB for full activity of both hydrogenase and urease in *Helicobacter pylori*. Mol Microbiol **39:**176-182.

31. **Olson JW, Agar JN, Johnson MK, Maier RJ.** 2000. Characterization of the NifU and NifS Fe-S cluster formation proteins essential for viability in *Helicobacter pylori*. Biochemistry **39:**16213-16219.

32. **Livak KJ, Schmittgen TD.** 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods **25:**402-408.

33. **Wang G, Hong Y, Johnson MK, Maier RJ.** 2006. Lipid peroxidation as a source of oxidative damage in *Helicobacter pylori*: protective roles of peroxiredoxins. Biochim Biophys Acta **1760:**1596-1603.

34. **Struthers L, Patel R, Clark J, Thomas S.** 1998. Direct detection of 8-oxodeoxyguanosine and 8-oxoguanine by avidin and its analogues. Anal Biochem **255:**20-31.

35. **Levine RL, Garland D, Oliver CN, Amici A, Climent I, Lenz AG, Ahn BW, Shaltiel S, Stadtman ER.** 1990. Determination of carbonyl content in oxidatively modified proteins. Methods Enzymol **186:**464-478.

36. **Seyler RW, Jr., Olson JW, Maier RJ.** 2001. Superoxide dismutase-deficient mutants of *Helicobacter pylori* are hypersensitive to oxidative stress and defective in host colonization. Infect Immun **69:**4034-4040.

37. **Gustafsson TN, Sahlin M, Lu J, Sjoberg BM, Holmgren A.** 2012. *Bacillus anthracis* thioredoxin systems, characterization and role as electron donors for ribonucleotide reductase. J Biol Chem **287:**39686-39697.

38. **Lillig CH, Prior A, Schwenn JD, Aslund F, Ritz D, Vlamis-Gardikas A, Holmgren A.** 1999. New thioredoxins and glutaredoxins as electron donors of 3'-phosphoadenylylsulfate reductase. J Biol Chem **274:**7695-7698.

39. **Miranda-Vizuete A, Damdimopoulos AE, Gustafsson J, Spyrou G.** 1997. Cloning, expression, and characterization of a novel *Escherichia coli* thioredoxin. J Biol Chem **272:**30841-30847.

40. **Girotti AW.** 1998. Lipid hydroperoxide generation, turnover, and effector action in biological systems. J Lipid Res **39:**1529-1542.

41. **Chen SK, Tsai MH, Hwang JJ, Chang WP.** 2001. Determination of 8-oxoguanine in individual cell nucleus of gamma-irradiated mammalian cells. Radiat Res **155:**832-836.

42. **Wang G, Alamuri P, Humayun MZ, Taylor DE, Maier RJ.** 2005. The *Helicobacter pylori* MutS protein confers protection from oxidative DNA damage. Mol Microbiol **58:**166-176.

43. **Wang G, Conover RC, Olczak AA, Alamuri P, Johnson MK, Maier RJ.** 2005. Oxidative stress defense mechanisms to counter iron-promoted DNA damage in *Helicobacter pylori*. Free Radic Res **39:**1183-1191.

44. **Park AM, Li Q, Nagata K, Tamura T, Shimono K, Sato EF, Inoue M.** 2004. Oxygen tension regulates reactive oxygen generation and mutation of *Helicobacter pylori*. Free Radic Biol Med **36:**1126-1133.

45. **Madian AG, Regnier FE.** 2010. Proteomic identification of carbonylated proteins and their oxidation sites. J Proteome Res **9:**3766-3780.

46. **Dalle-Donne I, Rossi R, Giustarini D, Milzani A, Colombo R.** 2003. Protein carbonyl groups as biomarkers of oxidative stress. Clin Chim Acta **329:**23-38.

47. **Suzuki YJ, Carini M, Butterfield DA.** 2010. Protein carbonylation. Antioxid Redox Signal **12:**323-325.

48. **Nystrom T.** 2005. Role of oxidative carbonylation in protein quality control and senescence. EMBO J **24:**1311-1317.

49. **Gonidakis S, Finkel SE, Longo VD.** 2010. *E. coli* hypoxia-inducible factor ArcA mediates lifespan extension in a lipoic acid synthase mutant by suppressing acetyl-CoA synthetase. Biol Chem **391:**1139-1147.

50. **Dukan S, Nystrom T.** 1999. Oxidative stress defense and deterioration of growth-arrested *Escherichia coli* cells. J Biol Chem **274:**26027-26032.

51. **Kaakoush NO, Sterzenbach T, Miller WG, Suerbaum S, Mendz GL.** 2007. Identification of disulfide reductases in Campylobacterales: a bioinformatics investigation. Antonie van Leeuwenhoek **92:**429-441.

52. **Reott MA, Parker AC, Rocha ER, Smith CJ.** 2009. Thioredoxins in redox maintenance and survival during oxidative stress of *Bacteroides fragilis*. J bacteriol **191:**3384-3391.

53. **Holmgren A.** 1985. Thioredoxin. Annu Rev Biochem **54:**237-271.

54. **Fowler RG, White SJ, Koyama C, Moore SC, Dunn RL, Schaaper RM.** 2003. Interactions among the *Escherichia coli mutT*, *mutM*, and *mutY* damage prevention pathways. DNA repair **2:**159-173.

55. **Hsieh P.** 2001. Molecular mechanisms of DNA mismatch repair. Mutat Res **486:**71-87.

56. **Au KG, Clark S, Miller JH, Modrich P.** 1989. *Escherichia coli mutY* gene encodes an adenine glycosylase active on G-A mispairs. Proc Natl Acad Sci U S A **86:**8877-8881.

57. **Ding H, Harrison K, Lu J.** 2005. Thioredoxin reductase system mediates iron binding in IscA and iron delivery for the iron-sulfur cluster assembly in IscU. J Biol Chem **280:**30432-30437.

58. **Rietsch A, Bessette P, Georgiou G, Beckwith J.** 1997. Reduction of the periplasmic disulfide bond isomerase, DsbC, occurs by passage of electrons from cytoplasmic thioredoxin. J Bacteriol **179:**6602-6608.

59. **Yoon JY, Kim J, Lee SJ, Kim HS, Im HN, Yoon HJ, Kim KH, Kim SJ, Han BW, Suh SW.** 2011. Structural and functional characterization of *Helicobacter pylori* DsbG. FEBS Lett **585:**3862-3867.

60. **Kaakoush NO, Kovach Z, Mendz GL.** 2007. Potential role of thiol:disulfide oxidoreductases in the pathogenesis of *Helicobacter pylori*. FEMS Immunol Med Microbiol **50:**177-183.

61. **Raczko AM, Bujnicki JM, Pawlowski M, Godlewska R, Lewandowska M, Jagusztyn-Krynicka EK.** 2005. Characterization of new DsbB-like thiol-

oxidoreductases of *Campylobacter jejuni* and *Helicobacter pylori* and classification of the DsbB family based on phylogenomic, structural and functional criteria. Microbiology **151:**219-231.

62. **Godlewska R, Dzwonek A, Mikula M, Ostrowski J, Pawlowski M, Bujnicki JM, Jagusztyn-Krynicka EK.** 2006. *Helicobacter pylori* protein oxidation influences the colonization process. Int J Med Microbiol **296:**321-324.

63. **Kadokura H, Katzen F, Beckwith J.** 2003. Protein disulfide bond formation in prokaryotes. Annu Rev Biochem **72:**111-135.

64. **Ritz D, Beckwith J.** 2001. Roles of thiol-redox pathways in bacteria. Annu Rev Microbiol **55:**21-48.

65. **Gonzalez Porqué P, Baldesten A, P. R.** 1970. The involvement of the thioredoxin system in the reduction of methionine sulfoxide and sulfate. J Biol Chem **245:**2371-2374.

66. **Brot N, Collet JF, Johnson LC, Jonsson TJ, Weissbach H, Lowther WT.** 2006. The thioredoxin domain of *Neisseria gonorrhoeae* PilB can use electrons from DsbD to reduce downstream methionine sulfoxide reductases. J Biol Chem **281:**32668-32675.

67. **Harris AG, Hazell SL.** 2003. Localisation of *Helicobacter pylori* catalase in both the periplasm and cytoplasm, and its dependence on the twin-arginine target protein, KapA, for activity. FEMS Microbiol Lett **229:**283-289.

68. **Mahawar M, Tran V, Sharp JS, Maier RJ.** 2011. Synergistic roles of *Helicobacter pylori* methionine sulfoxide reductase and GroEL in repairing oxidant-damaged catalase. J Biol Chem **286:**19159-19169.

69. **Kumar JK, Tabor S, Richardson CC.** 2004. Proteomic analysis of thioredoxin-targeted proteins in *Escherichia coli*. Proc Natl Acad Sci U S A **101:**3759-3764.

Chapter 4

Conclusions and future directions

H. pylori is the etiological agent of peptic ulcer disease, chronic gastritis, and gastric cancer. It is a gram-negative microaerophilic spiral-shaped pathogen that has the remarkable ability to be able to withstand very harsh conditions when colonizing the host stomach region. *H. pylori* can colonize the host stomach for the host's life, so it has evolved mechanisms that allow it to be resistant to both very low pH and to a constant attack by the host immune response. It is able to buffer the surrounding environment due to the production of the urease enzyme (1). It also produces a battery of antioxidant enzymes that function to protect the cell from ROS. My work has contributed to the knowledge of how *H. pylori* is able to maintain infection in harsh gastric conditions.

Methionine sulfoxide reductase (Msr) was first discovered in extracts of *E. coli*, where it was discovered that an enzyme had the ability to reduce oxidized methionine resides. The first protein found to be enzymatically reduced by the extracts was the ribosomal protein L12 (2). Since its discovery, the mechanism of Msr repair has been well characterized but the entirety of its physiological functions still remains an area of investigation. The extent of known interacting proteins with Msr in *H. pylori* includes GroEL, catalase, AhpC, Trx1, site-specific recombinase, and UreG (3-5). More targets for repair have been identified in *H. pylori* than in all other organisms combined. The identification of the domains recognized in known targets along with bioinformatics approaches will likely lead to identification of even more *H. pylori* targets. This

approach would benefit by studying repair of small synthetic peptides that contain domains of small protein targets AhpC or UreG.

Since urease comprises a large percentage of the total protein content of *H. pylori*, it seems likely that this enzyme would be affected by oxidative stress. Although urease is not high in methionine content, the urease maturation protein UreG contains a higher than average methionine content (4.5%). In addition, tandem affinity chromatography using UreG as a bait protein identified Msr as a putative interacting partner (6). Therefore, I decided to explore the role of Msr in urease maturation by determining the effect of oxidant stress on UreG (Chapter 2). I concluded that the activity of UreG was damaged when exposed to oxidants, but this activity could be restored by incubation with pure Msr. Mass spectrometry analysis showed that each methionine was oxidized after exposure to H_2O_2, and each one was repaired after exposing the damaged protein to Msr. This, together with other data described in Chapter 2, led to the conclusion that urease maturation was affected during oxidant stress. However, Msr ensures that urease maturation proceeds, by maintaining a functional UreG. This conclusion is very important for *H. pylori* pathogenesis and further explains the importance for Msr in virulence.

The identification of UreG as a Msr-target protein aids in expanding the known Msr-interacting proteins, but we still do not know the complete set of target proteins recognized, the mechanisms of Msr-mediated repair, or the entirety of physiological roles assigned to Msr. Identifying the complete set of Msr-interacting proteins in vitro has been a challenge, as the interaction between Msr and its repair targets is transient in nature. However, we can identify the mechanism by which Msr repairs the Met-SO in oxidized proteins. For example, it is possible that Msr recognizes either a linear, primary sequence within the repaired protein or a tertiary fold that forms around the Met-SO, or a combination of the two. To test for the recognition of a

primary sequence, we can synthesize small peptides identical to the sites of oxidation in the target proteins and determine the ability of Msr to repair these small peptides. If Msr recognizes a specific fold, we will identify this via hydroxyl radical protein footprinting or multidimensional NMR measurements. These methods will allow us to determine the sites of protection from Msr. During hydroxyl radical protein footprinting, Msr alone, MetSO-UreG alone, or a Msr-UreG repair complex will be exposed to a burst of hydroxyl radicals in solution. The oxidation sites of MetSO-UreG can then be compared to those of the Msr-UreG complex and the protection site will then be determined. Once we have determined the mechanism of Msr interaction, we can compare the interacting sites on each of the five Msr-repair target proteins identified in *H. pylori* and determine any similarities. This will allow us to use a bioinformatics approach to determine even more Msr-interacting proteins.

To address the physiological roles of Msr in *H. pylori*, one could study the ROS scavenging ability of several proteins. For example, catalase and urease are synthesized in high amounts in *H. pylori*. It has been suggested that Met-containing proteins could serve as a sink for ROS due to the Msr oxidation-reduction cycle (7). Since the Met-SO's in these proteins are either repaired (catalase), or not affected by oxidation (urease), it is possible that they could serve as mini-antioxidants. The role of these MetSO's can be studied by constructing mutant catalase strains that retain repairable Met residues, but lack enzyme activity. One approach would be to disrupt one of the heme-binding ligands of catalase, and compare the stress sensitivity of the strain to the parent. Another way to address the physiological roles of Msr is to study the binding affinities to the target proteins. The rationale is that the greater Msr affinity to a target protein, the more important the interaction may be to cell survival. For example, UreG is induced under elevated acid and nickel conditions and catalase is expressed under high oxygen;

these may be "preferred" targets. It should be possible to express Msr on an IPTG-inducible plasmid in a Δ*msr* strain to control for the amount of Msr under these conditions. The cells would then be subjected to in vivo crosslinking and then the Msr-complex harvested via immunoaffinity and quantitated by SDS-PAGE. The complexes can be identified by tandem MS/MS. Whichever Msr-target complex is highest under each condition will determine the priority of Msr-mediated repair and suggest the most important role for Msr under each condition.

H. pylori possesses many antioxidant enzymes but it does not contain many small compounds to maintain the redox balance of the cell. Other organisms rely on both the thioredoxin and glutaredoxin systems to maintain a disulfide in the cell. *H. pylori*, however, does not contain glutaredoxin or glutaredoxin reductase. It does possess two genes that encode for two separate thioredoxin proteins but these proteins have not been well described in *H. pylori*. A previous study has demonstrated that mutants in either *trx1* or *trx2* cause the cell to become more sensitive to oxidative stress, indicating a protective role of the two proteins (8). Trx1 has been described as the electron donor to Msr and AhpC, but this role is specific to Trx1 alone (3, 9). There has been no discovery of a role for Trx2. Therefore, I decided to study the comparative role of both Trx proteins in *H. pylori* specifically to attempt to begin to narrow down a defined role for Trx2. As shown in Chapter 3, I further described a protective role of these two proteins by determining damage to the macromolecules of the cell and for the first time described an in vivo role of the proteins. To my surprise, Trx2 plays a significant role in protecting the macromolecules from damage after oxidant stress and is essential for colonization of the mouse stomach.

Work remains to determine the specific role that Trx2 plays in the cell. During the 8-oxoG studies, I observed that the Δ*trx2* mutant contained more damaged DNA compared to the wild-type and Δ*trx1*. This prompted me to study more about the repair of 8-oxoG in *H. pylori*, and I discovered that MutY could benefit by using an oxidation-reduction protein to maintain a reduced state. MutY is a DNA glycosylase that recognizes and removes mispaired adenine from 8-oxoG (10). It also contains an iron sulfur cluster that is coordinated by cysteine residues (11). It is possible that Trx2 maintains these cysteines in a reduced state so the Fe-S cluster can properly bind. Preliminary evidence shows that MutY and Trx2, but not MutY and Trx1 interact via crosslinking (Figure 4.1). Other experiments such as assaying the spontaneous mutation rate of both *trx* mutants compared to the wild-type, and *trx2* site-change mutant strains that fail to recognize MutY could be performed to further investigate the roles of Trx2 in MutY-mediated 8-oxoG repair.

In addition to studying the direct interaction between Trx2 and MutY, it would be beneficial to perform protein pull-down assays to find other Trx2-interacting proteins. It would be interesting to try the Sulfo-SBED method (Chapter 2) to identify Trx2 interacting partners. This method uses the Sulfo-SBED compound to label Trx2 and it will then be used as the "bait" protein. Extracts from cells exposed to either elevated oxygen levels or normal oxygen conditions (4% O_2) will be incubated with the Sulfo-SBED labeled Trx2. Any protein that interacts with Trx2 could then be identified via western blot with streptavidin-HRP and identified following trypsin digestion and mass spectrometry. Overall, my work on the thioredoxins of *H. pylori* further demonstrates the cleaver survival strategies that the organism uses to persist in a harsh niche.

Overall, studying macromolecule oxidative stress demonstrates the battle between the host and pathogen and provides insight into the molecular mechanisms behind survival of *H. pylori* during colonization. *H. pylori* induces the host immune response, but the ability to persist long-term suggests the host response is not effective in eliminating the infection. This is partly due to upon induction of the immune response, the ROS generated from the host cells not only contact *H. pylori*, but it also results in damage of the host cells. This could provide an additional advantage for *H. pylori* in colonizing where the host has become weakened. In addition, *H. pylori* is equipped with many antioxidant enzymes that protect it from the damaging effects of ROS (12). I have demonstrated the importance of three of these antioxidant proteins (Msr, Trx1 and Trx2) and their role in protecting the cell from ROS. The function of these proteins provides an advantage to *H. pylori* over the host, and allows it to evade ROS and survive under all conditions. Understanding these proteins is essential to move forward with respect to developing strategies to eliminate infections that have been established. *H. pylori* relies primarily on the proteins that I have described for survival and lacks any compensatory system to overcome the absence of these proteins. Thus, characterizing these systems is essential to begin the development of treatment of this long-term infection.

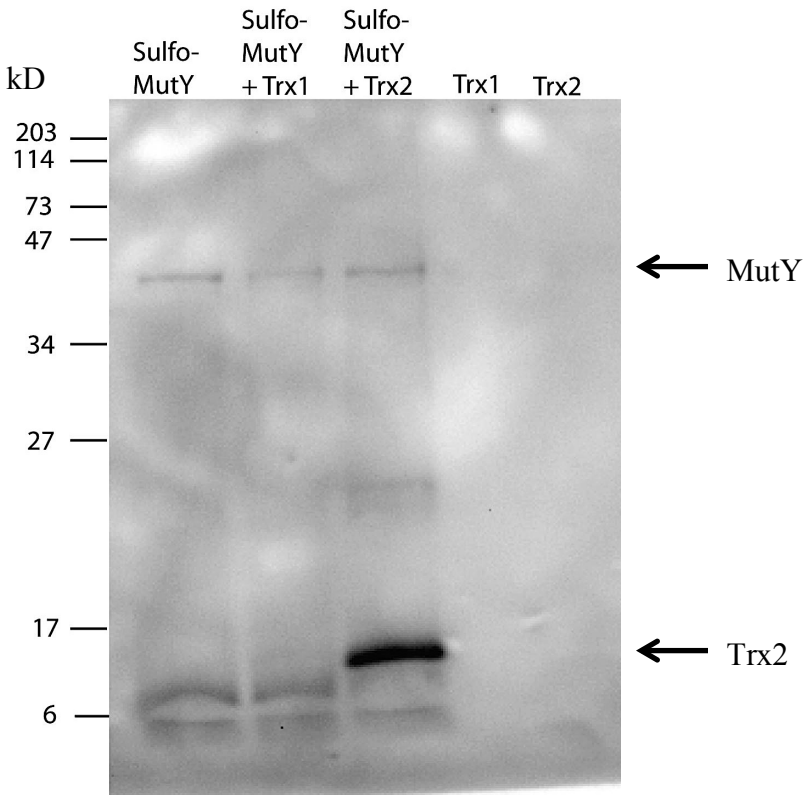

Figure 4.1. Interaction between Trx2 and MutY demonstrated by crosslinking with Sulfo-SBED. MutY was labeled with Sulfo-SBED crosslinking reagent (crosslinking process described in Chapter 2) and after labeling, it was incubated with either Trx1 or Trx2. The mixture was then subjected to UV light to activate crosslinking and the samples were reducted with 0.5 M DTT. The proteins were resolved via SDS-PAGE, transferred to a nitrocellulose membrane, and probed with streptavidin-horseradish peroxidase. Arrows to the right indicate Sulfo-MutY at 42 kD and Trx2 at 11.7 kD.

References

1. **Eaton KA, Brooks CL, Morgan DR, Krakowka S.** 1991. Essential role of urease in pathogenesis of gastritis induced by *Helicobacter pylori* in gnotobiotic piglets. Infect Immun **59:**2470-2475.

2. **Brot N, Weissbach L, Werth J, Weissbach H.** 1981. Enzymatic reduction of protein-bound methionine sulfoxide. Proc Natl Acad Sci U S A **78:**2155-2158.

3. **Alamuri P, Maier RJ.** 2006. Methionine sulfoxide reductase in *Helicobacter pylori*: interaction with methionine-rich proteins and stress-induced expression. J Bacteriol **188:**5839-5850.

4. **Kuhns LG, Mahawar M, Sharp JS, Benoit S, Maier RJ.** 2013. Role of *Helicobacter pylori* methionine sulfoxide reductase in urease maturation. Biochem J **450:**141-148.

5. **Mahawar M, Tran V, Sharp JS, Maier RJ.** 2011. Synergistic roles of *Helicobacter pylori* methionine sulfoxide reductase and GroEL in repairing oxidant-damaged catalase. J Biol Chem **286:**19159-19169.

6. **Stingl K, Schauer K, Ecobichon C, Labigne A, Lenormand P, Rousselle JC, Namane A, de Reuse H.** 2008. In vivo interactome of *Helicobacter pylori* urease revealed by tandem affinity purification. Mol Cell Proteomics **7:**2429-2441.

7. **Levine RL, Mosoni L, Berlett BS, Stadtman ER.** 1996. Methionine residues as endogenous antioxidants in proteins. Proc Natl Acad Sci U S A **93:**15036-15040.

8. **Comtois SL, Gidley MD, Kelly DJ.** 2003. Role of the thioredoxin system and the thiol-peroxidases Tpx and Bcp in mediating resistance to oxidative and nitrosative stress in *Helicobacter pylori*. Microbiology **149:**121-129.

9. **Baker LM, Raudonikiene A, Hoffman PS, Poole LB.** 2001. Essential thioredoxin-dependent peroxiredoxin system from *Helicobacter pylori*: genetic and kinetic characterization. J Bacteriol **183:**1961-1973.

10. **Eutsey R, Wang G, Maier RJ.** 2007. Role of a MutY DNA glycosylase in combating oxidative DNA damage in *Helicobacter pylori*. DNA repair **6:**19-26.

11. **Michaels ML, Pham L, Nghiem Y, Cruz C, Miller JH.** 1990. MutY, an adenine glycosylase active on G-A mispairs, has homology to endonuclease III. Nucleic Acids Res **18:**3841-3845.

12. **Suarez G, Reyes VE, Beswick EJ.** 2006. Immune Response to *H. pylori*. World J Gastroenterol **12:** 5593-5598.