A NOVEL ANTIOXIDANT ROLE FOR *HELICOBACTER PYLORI* UREASE AND A NEW APPROACH TO TARGET ITS ACTIVITY

by

ALAN ANTHONY SCHMALSTIG

(Under the Direction of Robert J. Maier)

ABSTRACT

Helicobacter pylori depends upon the Ni^{2+}-dependent metalloenzyme urease for the catalytic hydrolysis of urea into ammonia and bicarbonate for colonization of the human stomach. The produced ammonia acts as a buffer against the low pH of the stomach lumen. After colonization in the gastric mucosa, *H. pylori* is able to persist in the stomach for the lifespan of the host. Urease is the fourth most abundant protein in *H. pylori* and has a relatively high percentage of methionine (Met) residues. Based on these unique characteristics of urease, as well as the activity of methionine sulfoxide reductase (Msr), it was proposed that urease could act in a noncatalytic manner to quench oxidants through a Met residue oxidation-reduction cycle. To test this hypothesis, I constructed site directed mutants that were catalytically inactive due to an inability to bind nickel, but they still produced urease as detected by western blot. These apo-urease mutants were significantly more resistant to hypochlorous acid (HOCl) than a complete urease deletion mutant (Δ*ureAB*). Purified apo- and holo-urease were able to protect *H. pylori* against HOCl in both the wildtype and in a Δ*ureAB* background. To further characterize the role Met residue recycling plays in oxidant quenching, a mass spectrometry (MS) approach

was used. Unoxidized, oxidized, and oxidized and then Msr-repaired urease was analyzed by MS/MS to identify the Met residues susceptible to oxidation and repair. Of the 25 Met residues of urease, 11 were subject to both oxidation and Msr-mediated repair. This provides evidence for a noncatalytic antioxidant role for urease whereby oxidized urease Met residues are reduced to create a recyclable sink for oxidants. This new role for urease could contribute to the extended persistence of *H. pylori* infection. Additionally, I explored possible oxidant quenching in other Met-rich *H. pylori* proteins as well as targeting of Ni metalloenzymes (e.g. hydrogenase and urease) with a nickel chelator as a new antimicrobial mechanism.

INDEX WORDS: *Helicobacter*, urease, methionine sulfoxide reductase, oxidative stress, nickel, metal chelation, antibiotic resistance, hydrogenase

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ALAN ANTHONY SCHMALSTIG

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ALAN ANTHONY SCHMALSTIG

Major Professor:	Robert J. Maier
Committee:	Timothy Hoover
	Duncan Krause
	Vincent Starai

Electronic Version Approved:

Suzanne Barbour
Dean of the Graduate School
The University of Georgia
May 2019

DEDICATION

I dedicate this dissertation to my parents Dennis and Judy. They have set me up for success in both my academic career and life as a whole. Their unending support and advice has been critical to my success.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Helicobacter pylori was first discovered by Robin Warren and Barry Marshall from stomach biopsies in patients with ulcers (1). Although a rod-shaped bacterium was found in patients with stomach ulcers, it had not been cultured and was not considered as a live agent of ulcers (2). To establish a connection between *H. pylori* and gastric ulcers, Barry Marshall drank a *H. pylori* culture. He developed gastritis and was able to reverse the symptoms with a course of tinidazole (3). Now found in almost half of the world's population and considered a class I human carcinogen by the World Health Organization, *H. pylori* is a truly unique pathogen (4, 5). *H. pylori* has been associated with humans for around 100,000 years and has been used as a marker for human migration (6-9). *H. pylori* growth and persistence in the stomach is an excellent example of adaptation in a specialized niche. Once thought to be sterile, the human stomach is now known to be inhabited by both commensals and the gastric pathogen *H. pylori*; the latter often persists in the host for a lifetime (10). Gastric ulcers and gastric cancers are often associated with long-term *H. pylori* infections. Recent studies demonstrate a possible link between *H. pylori* and neurodegenerative disorders such as Alzheimer disease (11, 12).

General characteristics of *Helicobacter pylori*

Morphology. *H. pylori* has two distinct morphologies. Predominately, it is a curved or spiral bacillus; however, coccoid forms have been reported in environmental samples and when grown for extended periods of time in culture (13). There is debate as to whether

this coccoid morphology represents a viable but nonculturable (VBNC) state or an indication of cell death. There are conflicting reports as to whether coccoid cells in a VBNC state can be resuscitated and cultured again (14, 15).

Epidemiology. The average worldwide prevalence of *H. pylori* is 44% of the general population with a higher prevalence in developing countries (51%) compared to developed countries (35%) (4). This distinction between developing and developed countries may be due to the link between *H. pylori* transmission (via the fecal-oral or oral-oral route) and poor sanitation in developing countries, as well as higher antibiotic use in developed countries (16-19).

Treatment and antibiotic resistance. Common treatment of peptic ulcer disease associated with *H. pylori* involves a triple drug therapy involving a proton pump inhibitor (PPI), clarithromycin, and amoxicillin or metronidazole (20). As clarithromycin resistance has become more common, a quadruple drug therapy involving a PPI, bismuth, tetracycline, and a nitroimidazole is often used (20). Biofilm formation at the site of infection may also contribute to antibiotic resistance (21-24). Indeed, an *in vitro* study demonstrated that the minimum inhibitory concentration (MIC) of clarithromycin increased 16-fold against 3-day *H. pylori* biofilms compared to planktonic growth (21). Cells in biofilms also showed an increased expression of resistance-nodulation-cell division (RND) type efflux pumps, which likely contribute to antibiotic resistance (21).

Hydrogen metabolism

H. pylori is able to use hydrogen as an energy source through its membrane-bound [NiFe]-uptake hydrogenase (25, 26). The hydrogenase operon consists of five genes (*hydABCDE*), where *hydABC* are structural genes and *hydDE* are accessory genes.

Additional accessory genes (*hypABCDEF*) are required for enzyme maturation (27, 28). Molecular hydrogen is split into protons and electrons, which can be used to power various mechanisms requiring a proton motive force (PMF) such as ATP synthase and the flagellar motor. Electrons generated by hydrogenase can be shuttled into the electron transport chain, thus also contributing to the generation of a PMF and subsequent ATP production (29, 30). In *Helicobacter hepaticus*, amino acid uptake has been linked to hydrogenase produced energy (i.e. transmembrane potential), as a hydrogenase deletion showed much decreased uptake of a ^{14}C-labelled amino acid mixture (31).

Hydrogenase mutant strains (Δ*hyd*) show attenuated colonization (in mice) and gastric cancer rates (in gerbils) compared to wild-type strains (25, 32). Additionally, a hydrogenase mutant strain was unable to translocate CagA into human gastric epithelial AGS cells (32). Hydrogenase activity and protein produced increased when *H. pylori* was exposed to an atmosphere containing hydrogen, which can reach high levels within the host from normal microflora fermentation reactions (25, 33).

A proteomic approach showed that exposure to molecular hydrogen caused an increase in expression of the biotin carboxylase subunit of acetyl coenzyme A (acetyl-CoA) carboxylase (ACC) (33). Additionally, hydrogen exposure caused an increase in activity of ACC (33), suggesting that *H. pylori* can fix carbon dioxide through a hydrogen oxidation promoted mechanism (33, 34).

Nickel homeostasis

Nickel is a required cofactor for both urease and hydrogenase in *H. pylori* (28, 35). Indeed, urease requires 24 nickel ions per active molecule, urease makes up about 10% of the total protein produced by *H. pylori*, and the large subunit (UreB) is the fourth most

abundant protein in *H. pylori* (35-37). However, high Ni concentrations can be toxic (38). In order to keep the balance of available nickel below toxic levels, *H. pylori* regulates its import and export and has an elaborate nickel homeostasis repertoire (39, 40). Nickel import proteins in the inner membrane include NixA and NiuD, whereas FrpB4 and FecA3 are outer membrane nickel importers (41, 42). As for export, the CznABC system is responsible for nickel, cadmium, and zinc export (43). Nickel is stored in Hpn, Hpn-like, and HspA proteins, apparently so the bacterium has a ready reservoir of the metal that is crucial for survival (44, 45). These proteins have a high percentage of histidine residues to bind Ni^{2+} ions (46). NikR is a nickel-responsive regulator that acts to both induce and repress expression of several genes in *H. pylori* (40, 47-49). Upon binding Ni^{2+}, NikR represses transcription of the gene that encodes it, as well as genes that encode hydrogenase (*hydABC*), and nickel transporters (*nixA* and *fecA3*) (42, 48-52). Nickel-bound NikR also activates expression of genes that encode Ni storage proteins (*hpn*, *hpn-like*, and *hspA*) and urease (*ureAB*) (49, 53).

Pathogenesis

After ingestion, *H. pylori* makes its way from the stomach lumen (pH 1-2) to the gastric mucosa (pH 7) (Figure 1.1). *H. pylori* urease prevents acidification by producing ammonia from urea (54). Urease has been shown to be required for both initial colonization and persistence (55-58). *H. pylori* persists in the gastric mucosa, an environment shown to be pH neutral (59). The requirement for urease provides strong evidence that urease plays a noncatalytic role in persistence. Urease maturation involves the addition of two Ni atoms per structural unit (UreAB) which is accomplished by accessory proteins UreEFGH. UreFGH form a chaperone complex and UreE acts as a metallochaperone that binds Ni^{2+}

ions (60). UreG-mediated GTPase activity powers the insertion of Ni^{2+} from UreE into the active site of apo-urease (60-62). Additionally, UreI is a proton-gated urea channel that allows for a rapid intake of urea as the surrounding pH decreases (63-66). With 12 subunits of UreA and UreB, urease forms a 1.1-MDa dodecamer (35). Neutrophilic *H. pylori* cannot survive long in the acid even with urease. It makes use of lophotrichous flagella and chemotactic signaling to propel itself toward the gastric epithelium. TlpA and TlpD are chemotactic receptor proteins that sense pH (67). *H. pylori* persists in the mucosa with ~20% of cells bound to gastric epithelial cells via adhesins (68-71).

H. pylori is known for its genome heterogeneity (72, 73). Almost all strains cause low levels of gastritis, however only a small percentage of strains cause atrophic gastritis and ulcers, and only 2-3% cause gastric cancer (5, 71). These symptoms are associated with strains that have a *cag* pathogenicity island (*cag* PAI)(74, 75). The island encodes a type IV secretion system (T4SS) which secretes CagA. CagA has various cellular effects, such as cytoskeletal rearrangement, induction of cell scattering, cell-cell dissociation, and interleukin (IL) 8 secretion resulting in neutrophil recruitment (76-81). In addition to the *cag* pathogenicity island, *H. pylori vacA* encodes, a secreted vacuolating cytotoxin that is also associated with more severe disease symptoms (82-84). In addition, VacA has been shown to cause gastric epithelial cell apoptosis (85).

Host immune response and immune modulation by *Helicobacter pylori*

Paradoxically, *H. pylori* both induces and inhibits the host immune response. The host produced ROS and RNS are released to destroy bacterial cells; however, as often the case with infectious disease the immune response to infection causes damage to host tissue (71). This collateral damage could result in the release of nutrients required for *H. pylori*

persistence (86). Thus, *H. pylori* must strike a balance between gastric epithelial cell death and its ability to defend against toxic oxidants. To persist in the hostile environment created by the immune system, *H. pylori* has several ways to circumvent and modulate host defenses. Firstly, it can evade immune detection. Secondly, it induces an inflammatory response. Thirdly, it can protect itself against reactive oxygen species (ROS) through catalytic and noncatalytic mechanisms. Finally, it can repair damaged protein and DNA.

Evade immune detection. *H. pylori* is able to evade detection by toll-like receptors (TLRs) through a variety of mechanisms. Bacterial lipopolysaccharides usually elicit a strong immune response after binding to TLR4; however, due to *H. pylori* lipid A composition, the TLR4 response is limited (87-90). TLR5 usually recognizes secreted flagellins; however, *H. pylori* flagellin does not elicit a TLR5-mediated immune response (91, 92). Additionally, the flagellar sheath may prevent TLR5 detection (93, 94). Studies also suggest that *H. pylori* can activate the TLR9 anti-inflammatory response by secreting DNA via the T4SS (95, 96). Also, urease was shown to decrease opsonization (97). Complement protein C3b was able to bind to a urease negative strain at significantly higher rates than the wild-type strain. Along with its vacuolating role, VacA has been shown to inhibit T cell activation, thus weakening the adaptive immune response (98, 99).

Induce inflammatory response. *H. pylori* infection induces a robust inflammatory response. High levels of ROS are produced from gastric cells and phagocytes following *H. pylori* exposure *in vitro* (100, 101). Additionally, patients with *H. pylori* infections had higher amounts of ROS in the gastric mucosa than uninfected patients (102, 103). *H. pylori* contact with epithelial cells causes the secretion of IL8, which induces neutrophil activation and chemotaxis (76-78, 104, 105). Neutrophil activating protein (NapA) and urease have

been shown to cause chemotaxis of neutrophils and monocytes (106-109). Urease was also shown to induce apoptosis in gastric epithelial cells after binding to class II major histocompatibility complex (MHC) receptors (110) and induce proinflammatory cytokines (111). Urease has also been linked to the formation of gastric carcinoma through an ability to promote angiogenesis (112).

Enzymatic protection against ROS. Upon *H. pylori* infection, innate immune cells release ROS including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals ($^{\cdot}OH$), and hypochlorous acid (HOCl) (113, 114). Among the enzymes *H. pylori* uses to defend against these species are alkyl hydroperoxide reductase (AhpC), which reduces organic peroxides to alcohols (115). NapA sequesters iron to protect from oxidative stress caused by Fenton reactions (116). Superoxide dismutase (SodB) converts superoxide to hydrogen peroxide (117). Catalase (KatA) converts hydrogen peroxide to water and oxygen. MdaB is a NADPH dependent quinone reductase which reduces quinone to quinols (118, 119). Individual mutants lacking *sodB, ahpC, katA, mdaB,* or *napA* were more sensitive to oxidative stress *in vitro* and showed inhibited mouse colonization (106, 115, 117, 118, 120-123). NapA mutants only showed inhibited mouse colonization after an initial inoculation with dead wild-type *H. pylori* cells (106). NapA also appears to play a compensatory role in protection against ROS as it was found to be upregulated in mutants lacking *katA*, *sodB*, or *ahpC* (116). Additionally, MdaB was upregulated 3-fold in a *ahpC napA* double mutant strain compared to wild type (116).

The thioredoxin system, comprised of two thioredoxins (TrxA, TrxC) and a thioredoxin reductase (TrxR), reduces oxidized proteins and can act as an electron donor

for peroxidases (124). The system is essential for maintaining a thiol/disulfide balance as *H. pylori* lacks the glutathione-glutaredoxin system found in other bacteria (124, 125).

Noncatalytic protection against ROS. The hypothesis that surface-exposed methionine residues could have an antioxidant mechanism was first proposed by Levine et al. (126). They demonstrated that most surface Met residues of purified *E. coli* glutamine synthetase were oxidized, whereas intact residues were buried in the protein (126). Additionally, a global Met reduced strain of *E. coli* was produced by replacing 40% of the Met residues with norleucine. Norleucine lacks a sulfur atom and is therefore much less susceptible to oxidation than methionine (127, 128). This Met-reduced strain was more susceptible to oxidative stress than wild type (128). *H. pylori* catalase has been shown to have a noncatalytic antioxidant role in protecting whole cells from oxidative challenge (128). Interestingly, noncatalytic catalase *H. pylori* strains were able to colonize the mouse stomach, whereas a strain lacking catalase altogether (Δ*katA*) was largely unable to colonize the stomach (129). This suggests that the ability of *H. pylori* to colonize mice is not dependent upon the catalytic activity of catalase and that noncatalytic catalase is still required for colonization.

Protein repair. Methionine and cysteine are the amino acids most susceptible to oxidation by ROS due to their sulfur-containing ligands (127). Often the oxidation of these amino acids causes the protein to become inactive (130). *H. pylori* produces one methionine sulfoxide reductase (MsrAB), which is often found as two separate proteins in other organisms. MsrA and MsrB each reduce a different isomer Met(S)O and Met(R)O of methionine sulfoxide, respectively, whereas the MsrAB fusion in *H. pylori* only reduces the Met(R)O isomer (131). MsrAB reduces oxidized methionine residues from Met-SO to

Met. The reduction of Met residues by MsrAB has been shown to restore activity of catalase with the addition of GroEL in *H. pylori* (113). Strains lacking *msrAB* or *msrB* are highly sensitive to oxidative stress and are unable to colonize mouse stomachs for extended time periods (132). After Msr reduces methionine residues of a protein it becomes oxidized with the formation of a disulfide bond. TrxA reduces oxidized Msr and TrxR reduces oxidized TrxA (128, 131).

DNA repair. ROS-exposed DNA often results in lesions that prevent proper DNA replication and transcription (133). Single- or double-strand DNA breaks can occur, as well as oxidation of DNA bases. Guanine is highly susceptible to oxidants and forms 8-oxoguanine (134). *H. pylori* possess several mechanisms to repair damaged DNA. These include mechanisms to repair single-stranded and double-stranded DNA breaks, nucleotide excision repair, and base excision repair (86). Mutants lacking these repair mechanisms are reduced in colonization, especially after prolonged infection (86, 135). For example, endonuclease III and MutS are involved in excising oxidized pyrimidines (thymine glycol; 5,6-dihydrothymine) and 8-oxoguanine, respectively (86, 114). Strains lacking endonuclease III or MutS showed decreased mouse colonization (133, 136). Gastric colonization was also decreased in mutants (*addAB, recN, recO, ruvC,* and *recA*) in the recombination repair pathway involved in repair of double-strand DNA breaks (86, 137).

Nickel chelation as an antimicrobial mechanism

Antimicrobial resistance. Multidrug (MDR) and extensive drug resistance (XDR) has increased the possibility of a post-antibiotic era where there are no drug therapies available to treat infection. Antimicrobial resistance causes an estimated 700,000 deaths worldwide each year (138, 139). Several pathogens have been identified as key species to

improve understanding of their antibiotic resistance mechanisms and develop new drug therapies to treat their infections. Known as the ESKAPE pathogens, they include: *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter* species (140). In 2017 the World Health Organization released a ranked list of important antibiotic resistant pathogens (141). Carbapenem-resistant *A. baumannii* was listed as critical, along with carbapenem-resistant, extended spectrum beta-lactamase (ESBL)-producing *Enterobacteriaceae*. Clarithromycin-resistant *H. pylori* was given high priority. As the need for new targets for antibiotics grows, one possibility could be to use Ni chelators to inhibit key bacterial enzymes. There are no known human enzymes that require Ni, whereas several pathogens rely on Ni metalloenzymes such as urease, hydrogenase, and superoxide dismutase for virulence (142).

Bacterial and fungal nickel enzymes

Urease. Several pathogens make use of the catalytic function of urease to convert urea into ammonia and carbon dioxide. The ammonia could be used as a nitrogen source in both *Mycobacterium tuberculosis* and *Klebsiella pneumoniae* (143, 144). During urinary tract infections, the conversion of urea found in urine, by *Proteus mirabilis* urease, into ammonia causes an increase in pH (145). This can cause precipitation of minerals in the urine to form bladder or kidney stones (146). *P. mirabilis* can colonize the stones and becomes much more resistant to antibiotics and can cause chronic infections (147, 148). Mineral deposits and crystalline biofilms can also block urinary catheters (149-151). *Cryptococcus neoformans* and *Coccidioides posadasii* also encode a urease that was shown to be a virulence factor in murine models; urease mutants showed a decrease in host

mortality (152-154). Further work with *C. neoformans* showed that transmigration across the capillary wall into the mouse brain is dependent upon urease expression (155).

Hydrogenase. *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) possesses three uptake [NiFe]-hydrogenases (Hya, Hyb and Hyd) and one evolving type [NiFe]-hydrogenase (Hyc). The uptake hydrogenases (especially Hyb) have been shown to be required for virulence in a mouse model (156, 157). Although *K. pneumoniae*, an ESKAPE pathogen, contains an uptake hydrogenase and two evolving [NiFe]-hydrogenases, little is known about its requirement for virulence. Hydrogenase has been shown to be important for *P. mirabilis* swarming motility (158).

Nickel chelation. Targeting Ni-metalloenzymes such as urease and hydrogenase for inhibition has often been proposed (152, 159-161). In fact, in a process known as nutritional immunity, the host immune system uses chelators such as calprotectin in order to sequester scarce metals (Mn and Zn) away from pathogens (162-164). Metal chelators such as ethylenediaminetetraacetic acid (EDTA) have been used to treat heavy metal poisoning and coronary artery disease. Evidence supporting EDTA therapy for treating coronary artery disease is lacking (165, 166). Adverse side effects for EDTA treatment have been reported, which are most likely due to the chelation of essential metals such as Fe, Zn, Mg, and Ca (166). The nickel chelator diethyldithiocarbamate (DCC) has been used to treat nickel carbonyl poisoning (167, 168).

Dimethylglyoxime (DMG) is a nickel specific chelator that was originally described in 1905 by the Russian chemist Lev Aleksandrovich Chugaev (169, 170). DMG is still used to test for the presence of Ni in environmental samples, consumer products, and on the skin in relation to Ni caused contact dermatitis (171-175). Two molecules of

DMG bind to one Ni^{2+} ion to form a chelated complex which forms a red precipitate (Nickel bis[dimethylglyoximate]) due to its insolubility (176). The amount of Ni originally in solution can be quantitatively determined through colorimetric and gravimetric analysis (177, 178). It is often also used to detect Ni in qualitative spot tests (173). Our lab has previously used DMG to study Ni storage proteins Hpn and Hpn-like (44, 179, 180). Wild-type *H. pylori* X47 cells showed reduced hydrogenase activity after growth on DMG-containing (1 mM) media (179).

Scope of study

When colonizing the gastric mucosa, and especially to persist in an inhospitable environment, *H. pylori* must defend itself against the host immune response. One of the main strategies used to eliminate infection is the creation of reactive oxygen species (ROS) by neutrophils. *H. pylori* has several catalytic mechanisms to defend itself from ROS. It has also been shown that *H. pylori* catalase has a non-catalytic antioxidant effect whereby its methionine residues can quench oxidants. Urease is essential for *H. pylori* initial colonization and persistence in humans (55). Urease is one of the most abundant proteins in *H. pylori* and contains 25 methionine residues. Based on these data, I identified a novel non-catalytic role for urease in protecting *H. pylori* from oxidants which may also contribute to the persistence of *H. pylori*. I also showed that methionine sulfoxide reductase (Msr) can reduce oxidized urease, which could contribute to a recyclable pool of oxidant quenching methionine residues.

Urease and other Ni-metalloenzymes such as hydrogenase contribute to the virulence of several pathogens. The use of nickel chelators should have selective toxicity as there are no known human enzymes that require nickel. I demonstrated *in vitro* inhibition

of *K. pneumoniae* urease activity, inhibition of biofilm formation, and decreased *S.* Typhimurium organ burden in mice via the nickel-specific chelator dimethylglyoxime.

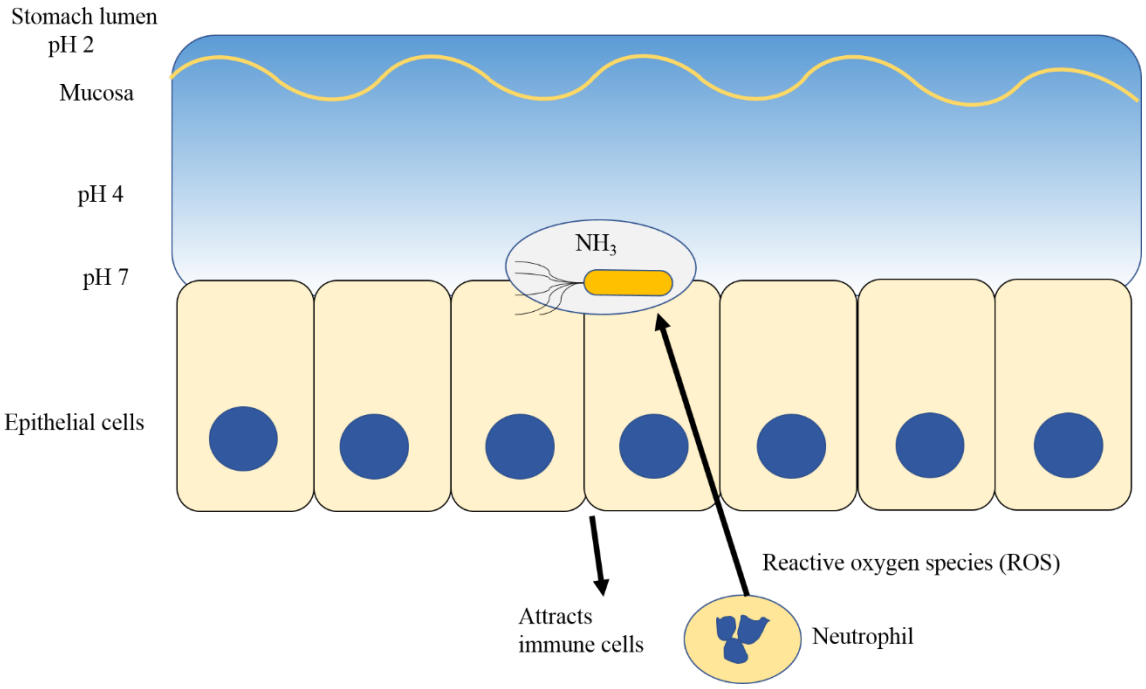

Figure 1.1 *H. pylori* site of infection and immune response. *H. pylori* swims through the acidic stomach lumen with its unipolar flagella and produces abundant urease to neutralize the acid with ammonia production. The bacterium can then burrow into mucosa where some cells bind to gastric epithelial cells via the use of cell surface adhesins. This environment is close to a neutral pH at the epithelial surface. Adhesion and other factors cause the attraction of immune cells such as neutrophils, which produce reactive oxygen species to combat the infection. However, *H. pylori* is able to persist in this state for decades without clearance by the oxidative challenge.

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CHAPTER 2

NONCATALYTIC ANTIOXIDANT ROLE FOR *HELICOBACTER PYLORI* UREASE[1]

[1]Schmalstig, A.A., Benoit, S.L., Misra, S.K., Sharp, J.S., Maier, R.J. 2018. Noncatalytic antioxidant role for *Helicobacter pylori* urease. J. Bacteriol 200:e00124-18. Copyright © 2018, American Society for Microbiology. Reprinted here with permission of publisher.

Abstract

The well-studied catalytic role of urease, the Ni-dependent conversion of urea into carbon dioxide and ammonia, has been shown to protect *Helicobacter pylori* against the low pH environment of the stomach lumen. We hypothesized that the abundantly expressed urease protein can play another noncatalytic role in combating oxidative stress via Met residue-mediated quenching of harmful oxidants. Three catalytically inactive urease mutant strains were constructed by single substitutions of Ni binding residues. The mutant versions synthesize normal levels of urease, and the altered versions retained all methionine residues. The three site-directed urease mutants were able to better withstand a hypochlorous acid (HOCl) challenge than a Δ*ureAB* deletion strain. The capacity of purified urease to protect whole cells via oxidant quenching was assessed by adding urease enzyme to non-growing HOCl-exposed cells. No wild-type cells were recovered with oxidant alone, whereas urease addition significantly aided viability. These results suggest that urease can protect *H. pylori* against oxidative damage and that the protective ability is distinct from the well-characterized catalytic role. To determine the capability of methionine sulfoxide reductase (Msr) to reduce oxidized Met residues in urease, purified *H. pylori* urease was exposed to HOCl and a previously described Msr peptide repair mixture was added. Of the 25 methionine residues in urease, 11 were subject to both oxidation and to Msr-mediated repair, as identified by mass spectrometry (MS) analysis; therefore, the oxidant-quenchable Met pool comprising urease can be recycled by the Msr repair system. Noncatalytic urease appears to play an important role in oxidant protection.

Importance

Chronic *Helicobacter pylori* infection can lead to gastric ulcers and gastric cancers. The enzyme urease contributes to the survival of the bacterium in the harsh environment of the stomach by increasing the local pH. In addition to combating acid, *H. pylori* must survive host-produced reactive oxygen species to persist in the gastric mucosa. We describe a cyclic amino acid-based antioxidant role of urease, whereby oxidized methionine residues can be recycled by methionine sulfoxide reductase to again quench oxidants. This work expands our understanding of the role of an already acknowledged pathogen virulence factor and specifically expands our knowledge of *H. pylori* survival mechanisms.

Introduction

Persistent *Helicobacter pylori* infections can cause chronic gastritis, peptic ulcer disease, and gastric cancer (1-4). Decades of immune response to the infection can lead to chronic inflammation and tissue damage (5, 6). *H. pylori* must first survive the harsh conditions of stomach gastric acid and then a prolonged host immune response after its colonization of the gastric mucosa (5). To combat the low pH of the stomach lumen, *H. pylori* expresses urease, which catalytically converts urea into ammonia and carbon dioxide, the former enabling *H. pylori* to resist the acidic gastric environment (7-9). Urease has been shown to be the most highly expressed protein in *H. pylori*, making up to 10% of the total protein content of the gastric pathogen (10). The enzyme consists of two structural proteins, UreA and UreB, with 12 subunits each that form a 1.1-MDa dodecamer (11). The UreAB heterodimer contains 25 methionine (Met) residues, 3% of the total amino acid content (12). Enzyme activity requires the addition of two nickel atoms per UreB monomer,

and such maturation is facilitated by the urease accessory proteins UreEFGH and the hydrogenase maturation proteins HypAB (10, 13-15).

Although the catalytic activity of urease is required for initial colonization, (10, 16-18) several studies also indicate that non-acid-neutralizing roles may exist for urease. For instance, urease has been shown to be required for persistence in the mouse gastric mucosa, where the pH approaches neutrality (16, 19) and a urease-negative strain was unable to colonize a pH neutral piglet stomach (20). In addition, using a tetracycline-inducible system to turn on or off urease expression, Debowski et al. were able to show that urease-expressing bacteria were selected over time in a murine infection model, indicating that urease is required for chronic infection (16). Additionally, *H. pylori* urease has been shown to play multiple roles in modulating the host immune response. Indeed, it was shown to decrease opsonization (21), stimulate the chemotaxis of neutrophils and monocytes (22), induce apoptosis in gastric epithelial cells after binding to class II major histocompatibility complex (MHC) receptors (23), and induce proinflammatory cytokines (24). Recently, urease has been linked to the formation of gastric carcinoma through an ability to promote angiogenesis (25).

After reaching the gastric epithelium, *H. pylori* triggers responses by the host innate immune cells, which respond to the infection by generating reactive oxygen species (ROS) such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals ($^{\cdot}OH$), and hypochlorous acid (HOCl) (10, 26, 27). Indeed, the exposure of gastric cells (28) or phagocytes (29) to *H. pylori* increases host cell ROS production. Patients with *H. pylori* infections have been shown to have larger amounts of ROS in their gastric mucosa (30). ROS can damage protein, DNA, and lipids (31). With regard to proteins, the amino acids

most susceptible to oxidation are Met and cysteine (Cys) due to their sulfur-containing ligands (32).

H. pylori has many mechanisms to protect itself from, as well as repair damage caused by, oxidative stress. For instance, catalase and superoxide dismutase act to convert H_2O_2 and O_2^- into less harmful products. Catalase was recently shown to protect *H. pylori* against oxidative damage via an oxidant-quenching mechanism of its Met residues (33). The reaction involves methionine sulfoxide reductase (Msr) to reduce Met-SO to Met (34, 35). Msr reduces oxidized methionines of damaged proteins and has been shown to restore function to the damaged protein (36, 37). Cross-linking and direct repair assays showed that *H. pylori* Msr has at least five repair target proteins (34, 38, 39). These include AhpC, UreG, GroEL, catalase, and a site-specific recombinase (SSR), but urease was not observed as a repair target in those studies. In the present study, we describe the evidence for a noncatalytic role for urease, which involves Met-S/Met-SO recycling. This role aids in protecting the pathogen against oxidation mediated cell death.

(A preliminary account of this work was presented at the American Society for Microbiology Microbe, New Orleans, LA, 1 to 5 June 2017 [40].)

Results

Construction of catalytically inactive urease. Strains that synthesize inactive urease (herein referred to as apo-urease) were constructed to determine if the catalytically inactive enzyme is sufficient to quench oxidants. A sucrose-kanamycin selection-counterselection was used to make markerless single-amino-acid substitutions in the UreB subunit of urease. The three single substitutions (His136Ala, His138Ala, and Lys219Ala) were constructed to replace nickel binding residues known to be required for enzyme

activity (41). As expected, the three site-directed strains (*ureB*^{H136A}, *ureB*^{H138A}, and *ureB*^{K219A} strains) lacked urease activity compared to the wild-type (WT) strain 43504 (Table 2.1). Next, we sought to verify that the lack of urease activity was not linked to a decrease or loss of urease, e.g., that the mutant strains were still making apo-urease. Since urease is the most abundant protein in *H. pylori*, both UreA (27 kDa) and UreB (62 kDa) structural subunits can readily be visualized on Coomassie-stained SDS-PAGE gels (Figure 2.1A). UreAB levels in the three site-directed mutants were found to be similar or slightly less than WT levels (Figure 2.1A). This was confirmed by immunoblot, using anti-UreA antiserum (Figure 2.1B). No UreA-specific band was seen in the Δ*ureAB* deletion strain, our negative control. These results indicate that apo-urease is still synthesized in each of the three site-directed mutants.

Catalytically inactive urease confers resistance to HOCl. The three catalytically inactive strains, as well as the WT (positive control) and the Δ*ureAB* deletion strain (negative control), were exposed to HOCl to determine their ability to quench an oxidant and survive. The cells were incubated with HOCl for 1 h, diluted, and the CFU were counted (Figure 2.2). The catalytically inactive mutants were as resistant as the WT and significantly ($P < 0.01$) less sensitive to HOCl oxidative damage than the urease gene deletion strain (Figure 2.2). This suggests that the presence of urease, independent of its catalytic activity, can play a role in preventing cell death due to oxidative damage.

Both holo-urease and apo-urease protect against HOCl-mediated killing. To determine if holo-urease (purified from WT strain 43504) and apo-urease (purified from either strain *ureB*^{H138A} or strain *ureB*^{K219A}) were able to protect against HOCl-mediated oxidative damage, each purified urease was preincubated with the oxidant for 15 min and

the mixture was added to whole nongrowing WT or Δ*ureAB* cells. Cell survival was then monitored and compared to that with a HOCl-only treatment (no protein) (Figure 2.3). While no cells were recovered from the HOCl treatment alone (detection limit, 10^2 CFU/ml), when HOCl was preincubated with purified urease (both holo and apo), the cell recovery levels reached those of cells without HOCl treatment (Figure 2.3). This suggests that purified urease itself can quench HOCl, irrespective of urease activity. Interestingly, the Δ*ureAB* strain, shown to be more sensitive than the WT to HOCl challenge (Figure 2.2), was almost fully protected against HOCl that had been preincubated with purified urease (Figure 2.3). An additional protein, UreE, with a low Met content (1%) was shown to not protect against the HOCl challenge, suggesting that Met content might contribute to the protective effect.

MS/MS identification of oxidized and reduced methionine residues in UreAB.

Urease is abundantly expressed, is found outside cells as well as in the cytosol, and contains many Met residues (25 of 807 residues, accounting for 3% of the total residues) (10, 42, 43). To identify which Met residues of urease (UreAB) are oxidized and repaired, we used liquid chromatography-tandem mass spectrometry (LC-MS/MS). The urease holoenzyme was first oxidized with HOCl, and then a sample of oxidized urease was subsequently incubated with an Msr repair mixture. Oxidized, oxidized and repaired, and untreated samples were analyzed by LC-MS/MS. Of the 25 methionine residues in urease, 19 were detected by LC-MS/MS (Figure 2.4). UreA Met^1 and UreB Met^1, Met^{104}, Met^{182}, Met^{191}, and Met^{478} were either not detected or could not be quantified reliably. An examination of the protein sequence shows that four of these methionines reside in peptides containing multiple basic residues that were digested by trypsin resulting in small

hydrophilic fragments, making their detection by LC-MS/MS highly unlikely. A fifth methionine-containing peptide was not included in the data analysis, as the signal-to-noise ratio was far too low for reliable quantitation. Most of the detected Met residues in the unoxidized samples had low levels of oxidation (less than 0.15 average oxidation per peptide) except UreA Met^{71}, Met^{77}, and Met^{86} and UreB Met^{262}. HOCl-treated Met residues UreA Met^{44}, Met^{71}, Met^{77}, and Met^{86} and UreB Met^{12}, Met^{262}, Met^{315}, Met^{317}, Met^{319}, Met^{353}, and Met^{366} had greater than 0.8 average oxidation per peptide. UreA Met^{12} and UreB Met^{448} showed approximately 0.5 average oxidation per peptide, and all other residues showed less than 0.5 average oxidation per peptide. After HOCl-oxidized urease was treated with an Msr repair mixture, most Met residues were reduced to levels similar to that observed for the unoxidized sample, with the exception of UreB Met^{12}, Met^{262}, Met^{353}, and Met^{366}. Most of the digested peptides contained a single Met residue, the exceptions being UreA peptide 63 to 92 and UreB peptide 289 to 326, which contain three Met residues each (Met^{71}, Met^{77}, and Met^{86} and Met^{315}, Met^{317}, and Met^{319}, respectively). Likewise, UreB peptide 339 to 368 contains two Met residues (Met^{353} and Met^{366}). For peptides with more than one Met residue, the oxidation of each Met cannot be accurately quantified by collision-induced dissociation (CID) MS/MS (44, 45). In summary, the Msr repair mixture reduced 11 of the oxidized Met residues to close to the baseline non-oxidant-treated levels. This suggests that oxidized urease can be reduced by Msr and thus probably recycles the oxidant-quenchable pool of urease.

Discussion

Previous studies have focused on the catalytic by-products of urea hydrolysis by urease (NH_3 and CO_2) and their contribution to pathogen virulence. The ammonia produced

by the enzyme supplements nitrogen metabolism, promotes alkalinization, and contributes to damage of host cell tissue (46). Additionally, carbon dioxide has been shown to protect the bacterium against highly toxic peroxynitrite during *H. pylori* infection (47). Now, there is growing evidence that urease plays noncatalytic roles in *H. pylori* infection. Indeed, urease is required for persistence in the mouse gastric epithelium, where the pH approaches neutrality (16, 19). Urease has been shown to play roles in modulating the immune response to infection (21–24), and *ureA* was found to be upregulated in response to oxidative stress (48).

We demonstrate that urease has a previously undescribed role as an antioxidant, thus adding to the growing number of noncatalytic roles that urease can play. Indeed, some of the observations cited above (e.g., urease requirement for persistence even at neutrality) may be due to an antioxidant role. This role depends at least in part on the primary sequence of the protein (e.g., Met residues). Although gastric acid exposure is an impressive obstacle to overcome, *H. pylori* also survives a barrage of host-produced ROS, including HOCl, which can reach levels as high as 5 mM at inflamed sites (49). *H. pylori* survives this via multiple mechanisms of detoxification and repair, enabling the infection to oftentimes persist throughout the life span of the host. Nickel-deficient mutants that lacked catalytic activity were as resistant as the WT to HOCl-mediated oxidative stress. In contrast, a urease deletion mutant was much more sensitive than the WT to HOCl-mediated oxidative stress. Thus, catalytic activity is not required for combating the oxidant. Rather, we propose that the Met residues of urease can quench oxidants, forming Met-SO in the process. Catalase, another abundant protein, was similarly shown to quench oxidants to aid bacterial survival, and the mechanism was independent of catalytic activity (33). A similar antioxidant

mechanism of relying on Met residues was assigned to pure *E. coli* glutamine synthetase; however, the system was not shown to confer oxidative stress resistance to cells (50).

We predict that Met-SO residues of urease could be reduced by Msr and then used again to quench oxidants. Indeed, Msr was previously shown to reduce and repair several *H. pylori* proteins such as AhpC, UreG, GroEL, catalase, and site-specific recombinase (SSR) (34, 38, 39). However, it was previously unknown whether Msr could reduce urease Met residues. Through mass spectrometry analysis, we found that of the 25 Met residues in urease, 11 were shown to be susceptible to oxidation and were reduced (i.e., repaired) by Msr. Thus, urease can be added to the list of Msr repair targets. Upon PDBePISA analysis of surface accessibility, using the X-ray crystal structure of *H. pylori* urease (Protein Data Bank code 1E9Z), 9 of the 11 Met residues that were oxidized and then subsequently reduced are surface exposed (11, 51). This is consistent with previous reports of surface methionine residues being subject to oxidation and subsequent repair by Msr (26, 38). *H. pylori* Msr deletion mutants showed lowered urease activity when exposed to oxidative stress (21% O_2) (38). This was attributed to the inability of the strain to repair UreG, a urease accessory protein required for urease activity; however, we can now hypothesize the lowered activity could also be due to the lack of reduction of oxidized Met residues of the structural proteins of urease (UreAB).

An Msr deletion mutant has been shown to be more sensitive to HOCl challenge than its parent strain (SS1) (39). Here, we show a high HOCl sensitivity of a strain lacking one of the described repair targets of Msr, namely, urease. Conclusions based on comparing the phenotypes of the Δ*ureAB* strain to that of the Δ*msr* strain (39) are not possible due to

the use of different parent strains. Comparisons are further complicated by the fact that Msr has multiple repair targets besides urease.

While urease lacks a described secretion pathway in *H. pylori*, it has been found outside the cell, possibly due to cell lysis (42, 52). We show that purified urease added exogenously and whole cells expressing urease quench HOCl and survive HOCl challenge, respectively. Regardless of location, urease activity is not required for the described oxidant quenching effect. Seemingly, both extracellular and intracellular ureases likely act to quench oxidants. However, intracellular urease may play a larger role in protecting against oxidants because of its Met turnover—Msr is unlikely to be active extracellularly. Although this study focused on the role of Met residues in urease oxidant quenching, the Cys residues of urease might also contribute to the antioxidant ability we describe. While Cys residues are very sensitive to oxidation, the UreAB heterodimer only contains 4 Cys residues compared to 25 Met residues. Oxidized Cys residues could be reduced enzymatically through the thioredoxin/thioredoxin reductase (Trx/TrxR) system (32). *H. pylori* contains two thioredoxins (Trx1 and Trx2), which have been shown to play a role in preventing macromolecule damage, and one TrxR; however, it is not known if the Trx system can repair oxidized Cys residues in *H. pylori* (53, 54).

Future site-directed mutagenesis studies of individual Met residues in urease could elucidate the role of individual residues in the quenching. Also, the use of the mouse model to gauge colonization by the catalytically inactive strains is likely to be a promising approach to assess *in vivo* function, although the acid barrier would have to be minimized to observe colonization by urease-activity-negative strains. Catalytically inactive urease

may contribute to the persistence of *H. pylori* infections of the host gastric epithelium, where the pathogen is exposed to multiple oxidants (30).

Although the role of urease in *H. pylori* pathogenesis has been extensively studied, urease is also a virulence factor for several pathogens, including *Proteus mirabilis* and *Klebsiella pneumoniae* (55). During *P. mirabilis* infection of the urinary tract, urease is required for mineral precipitation to form urinary stones, which impede the elimination of the infection (56). During mice gastrointestinal tract infections, urease-negative strains of *K. pneumoniae* were outcompeted by WT strains, and so it was suggested that the ammonia produced by urease serves as a supplementary nitrogen source (57). *K. pneumoniae* and *P. mirabilis* must also defend against HOCl produced by neutrophils as a part of the innate immune response to infection (56, 58). These species also contain Msr, enabling the possibility that a similar method for urease methionine oxidation and repair by Msr would be found in additional pathogens. Additionally, other highly expressed proteins that are Met rich might play a similar antioxidant role. Indeed, catalase, a Met-rich and abundant *H. pylori* protein, has been shown to have similar noncatalytic antioxidant properties (33).

Urease and Msr can be found in plants, fungi, archaea, and bacteria (59, 60). Throughout the three domains of life, urease is increasingly seen as a "moonlighting protein" with a variety of noncatalytic roles, including fungicidal, insecticidal, and proinflammatory effects (61). For *H. pylori*, an antioxidant mechanism based on urease Met residue recycling is expected to explain at least part of the *in vivo* requirement for this important enzyme.

Materials and Methods

Growth conditions. *Escherichia coli* cells were cultured at 37°C aerobically in Luria-Bertani (LB) broth or on agar LB plates with 100 µg/ml ampicillin and 30 µg/ml chloramphenicol as needed. *H. pylori* cells (ATCC 43504) were routinely grown at 37°C on brucella agar plates supplemented with 10% defibrinated sheep blood (BA) under microaerophilic conditions (4% O_2, 5% CO_2, and 91% N_2). As needed, *H. pylori* was grown on BA supplemented with 5 or 10 µM $NiCl_2$, 5% sucrose, 20 µg/ml kanamycin, or 30 µg/ml chloramphenicol. For urease protein purification, *H. pylori* cells were grown on BA plates supplemented 5 µM $NiCl_2$.

Strain construction. The *E. coli* and *H. pylori* strains and plasmids used in this study are listed in Table 2.2. Site-directed mutants were constructed using a kanamycin-sucrose selection-counterselection system described previously (33, 39, 62). Briefly, a 1,400-bp DNA fragment containing a partial *ureB* gene was amplified by PCR using primers UreBF1 and UreBR1 (Table 2.3) from the chromosomal DNA of *H. pylori* 26695 (12) and cloned into pBluescript KS(+) to obtain pKS-*ureB*. The Kan^r-*sacB*-P_{flaA} (KSF) cassette (62) was inserted into *ureB* resulting in pKS-*ureB*::KSF, which was then naturally transformed into WT strain 43504, thereby replacing parent *ureB* with *ureB*::KSF. The resulting strain (Δ*ureB*::KSF strain) was found to be urease negative and kanamycin resistant. Site-directed mutations were introduced to pKS-*ureB* by overlapping PCR using internal primers UreB:H136AF, UreB:H136AR, UreB:H138AF, UreB:H138AR, UreB:K219AF, and UreB:K219AR and external primers UreBF1 and UreBR1 (Table 2.3). The resulting plasmids (pKS-*ureB*^{H136A}, pKS-*ureB*^{H138A}, and pKS-*ureB*^{K219A}) were introduced into the Δ*ureB*::KSF strain by natural transformation. UreB site-directed

mutants were selected on BA plates containing 5% sucrose. The resulting strains were sucrose resistant and urease negative. The Δ*ureAB* strain was constructed as described previously (15). All plasmids and PCR products were sequenced at the Georgia Genomics Facility, University of Georgia, Athens, GA, and compared with DNA sequences from strain 26695 (12) to ensure that no error had been introduced following PCR amplification, as well as to verify the presence of engineered site-directed mutations within *ureB*.

Urease assays. Whole cells grown on BA plates under microaerophilic conditions at 37°C were resuspended in 50 mM HEPES (pH 7.5), lysed by sonication (Heat Systems Ultrasonics sonicator: 30 s at 4-W output power and 50% duty cycle), and centrifuged at 21,100 x *g* for 5 min. The cell-free supernatant was assayed for urease activity following the phenol-hypochlorite (Weatherburn) method (63). Protein concentration was determined using the bicinchoninic acid (BCA) kit (Thermo Scientific Pierce).

SDS-PAGE and immunoblotting. Whole cells grown on BA plates supplemented with 10 μM $NiCl_2$ were resuspended in phosphate-buffered saline (PBS) and treated as above to obtain cell-free extracts (CFE). Total protein from CFE (0.4 μg protein per lane) was loaded onto 12.5% SDS-PAGE gels and stained with Coomassie brilliant blue G-250 or transferred to a nitrocellulose membrane for immunoblotting as previously described with modifications (33, 38). Briefly, the nitrocellulose membrane was blocked with 3% gelatin in Tris-buffered saline (TBS; 50 mM Tris [pH 7.6] and 150 mM NaCl [pH 7.6]). After blocking, the membrane was incubated with anti-UreA antibody (rabbit polyclonal; Santa Cruz Biotechnology) diluted 1:2,000 in TBS with 0.1% Tween 20 (TTBS) containing 1% gelatin for 1 h. The membrane was then washed with TTBS and incubated with goat anti-rabbit IgG conjugated to alkaline phosphatase (Bio-Rad) diluted 1:2,000 in TTBS with

1% gelatin. After 1 h of incubation, the blot was developed with Nitro Blue Tetrazolium-5-bromo-4-chloro-3-indolyl phosphate in 10 mM Tris-HCl (pH 9.5) and 150 mM NaCl.

Whole-cell HOCl tolerance assay. Whole-cell HOCl tolerance assays were performed as previously described (33) with the following modification. Briefly, cells grown on BA plates with 10 μM $NiCl_2$ were resuspended in PBS to an optical density at 600 nm (OD600) of approximately 1.1 and then mixed with either PBS or PBS with NaOCl (100 μM final concentration, 10 to 15% available chlorine [referred to as HOCl]) for 1 h at 37°C. The HOCl-challenged cells were diluted in PBS and plated on BA plates, and the CFU were counted after 72 h of incubation at 37°C under microaerophilic conditions.

Purified protein HOCl tolerance assay. HOCl tolerance assays with purified proteins were performed as previously described (33). Briefly, wild-type and Δ*ureAB* 43504 cells (OD600 of 1.3) were incubated for 1 h in PBS, PBS with 200 μM HOCl, or PBS with HOCl that had been previously incubated with 0.25 μM UreAB or UreE for 15 min at 37°C. After the PBS or HOCl challenge, the cells were plated on BA plates and the CFU were counted after 72 h of incubation at 37°C under microaerophilic conditions.

Protein purification. *H. pylori* 43504 cells were grown on BA plates supplemented with 5 μM $NiCl_2$ under microaerophilic conditions (4% O_2, 5% CO_2, 91% N_2) at 37°C and broken by passage through a French pressure cell at 18,000 lb/in2 or by cell disruption at 20,000 lb/in^2 (Constant Systems One Shot). Cell debris was removed by centrifugation at 4,500 x *g*. Urease was purified from the resulting supernatant (cell-free extract) using fast protein liquid chromatography (AKTA; GE Healthcare). Cell-free extract was loaded onto a Q-Sepharose HiTrap column that had been equilibrated with 50 mM NaPO4 (pH 7.2) and 25 mM NaCl (buffer A). The protein was eluted with a linear

gradient of 0.025 to 1 M NaCl in buffer A. Urease-containing fractions, as determined by the phenol red urease assay (64), were concentrated with Amicon Ultra-4 devices with a 10-kDa molecular mass cutoff (Merck Millipore). The concentrated urease was further purified with a HiLoad 16/60 Superdex 75 column in buffer A with 0.35 M NaCl. Urease-containing fractions were identified and concentrated as above, and the final protein concentration was determined using the BCA kit (ThermoScientific Pierce). UreAB proteins were purified to near homogeneity (>90%), as determined by SDS-PAGE. UreE was purified as previously described (65).

Urease oxidation and Msr repair. Urease oxidation and subsequent Msr repair were performed as previously described with modifications (26). Briefly, purified urease (10 µM) was incubated with a 60-fold molar excess of HOCl for 15 min at room temperature in the dark. HOCl was quenched with excess Met (15 mM final) for 30 min on ice. Excess Met and HOCl were removed via dialysis (Spectra/Por; molecular weight cutoff [MWCO], 12,000 to 14,000) in 50 mM sodium phosphate buffer. Oxidized and quenched urease was incubated with 6.2 µM Msr, 5 µM Trx1, 0.5 µM TrxR, 400 µM NADPH, and 100 µM dithiothreitol (DTT) for 2 h at 37°C. The reaction was stopped by incubating at 95°C for 5 min.

Sample preparation and mass spectrometry analysis. Concentrated ammonium bicarbonate buffer was added to the protein to adjust to a final buffer concentration of 50 mM (pH 8.0). The samples were denatured by heating at 95°C for 20 min in the presence of 10 mM DTT. After cooling the sample, 20 mM iodoacetamide was added and the sample was kept at room temperature in dark for 45 min. Next, 20 mM DTT was added to quench the excess iodoacetamide and the mixture was incubated for 20 min at room temperature.

Finally, the samples were digested with sequencing-grade trypsin at a 1:20 ratio of protease to protein by incubating at 37°C overnight with rotation. The samples, after trypsin digestion, were acidified with 0.1% formic acid and spun briefly, and the supernatant was transferred to sample vials. An Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific) coupled to an Ultimate 3000 high-performance liquid chromatography (HPLC) system was used for the nano-LC-MS/MS analysis. The samples were first loaded onto a trap cartridge containing C_{18} stationary phase to desalt the samples. After 4 min, the samples were back-eluted from the trapping cartridge onto a nano PepMap column (75-µm inner diameter, 150-mm length, 3-µm particle size). Mobile phase A consisted of water with 0.1% formic acid, and mobile phase B consisted of acetonitrile with 0.1% formic acid. The peptide separation was achieved with a linear binary gradient from 0 to 40% phase B for 25 min at 0.3 µl/min. The peptides were fragmented by collision-induced dissociation, and the normalized collision energy was set to 35%. The MS scan range was *m/z* 150 to 2,000, and the top five peaks were selected in precursor scan for the data-dependent CID fragmentation in each cycle. The data files were searched against the protein sequences of UreA and UreB of *H. pylori* strain 26695. The peak intensities of the unoxidized peptides and their corresponding methionine oxidation products observed in LC-MS were used to calculate the average oxidation events per peptide in the sample. The ion intensity of the oxidized peptides was multiplied by the number of oxidation events required for the mass shift (e.g., one oxidation on methionine for +16, two events for +32) and then divided by the sum of the ion intensities of all unoxidized and oxidized peptide masses as represented by the equation below.

$P = [I(+16)\text{oxidized} \times 1 + I(+32)\text{oxidized} \times 2 + I(+48)\text{oxidized} \times 3 + \ldots /$

$[I\text{unoxidized} + I(+16)\text{oxidized} + I(+32)\text{oxidized} + I(+48)\text{oxidized} \ldots]$

In this equation, P represents the average oxidation level of the peptide and I represents the peak intensities of oxidized and methionine unoxidized peptides.

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Table 2.1 Urease activity of *H. pylori* wild-type, urease deletion, and site-directed mutant strains

Strain	Urease activity (μmol/min/mg)[a]
43504 (wild type)	4.65 ± 0.96[a]
Δ*ureAB* strain	0.06 ± 0.01
ureB^{H136A} strain	0.07 ± 0.02
ureB^{H138A} strain	0.07 ± 0.01
ureB^{K219A} strain	0.08 ± 0.01

[a]Urease activity expressed in micromoles of NH_3 produced per minute per milligram of total protein. Results shown are the means ± SDs from 3 to 8 independent experiments with assays performed in triplicate.

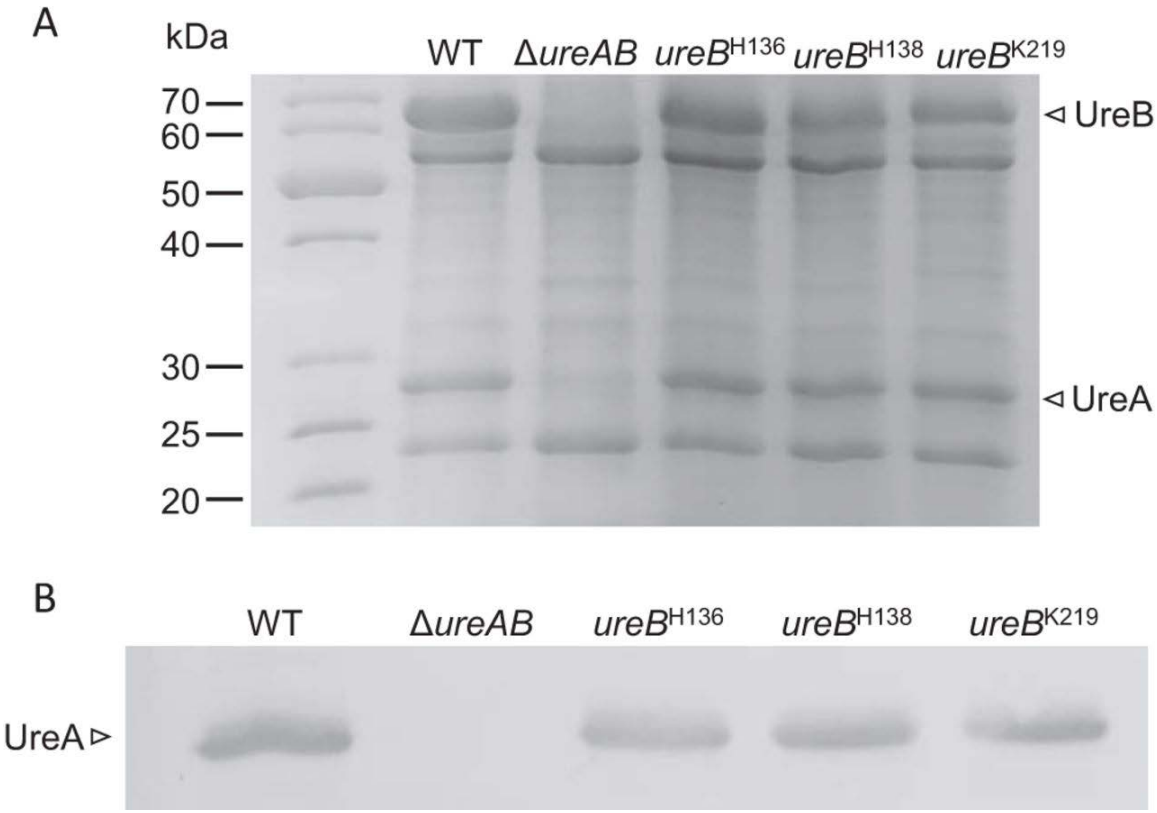

Figure 2.1 Urease in cell-free and whole-cell extracts of WT and *ureAB* mutants. (A) SDS-PAGE (12.5%) analysis of cell-free extract. (B) Immunoblot analysis. Whole-cell extracts were separated via 12.5% SDS-PAGE, transferred to a nitrocellulose membrane, and blotted with anti-UreA antibodies. Strains are indicated above each lane. UreA and UreB are indicated.

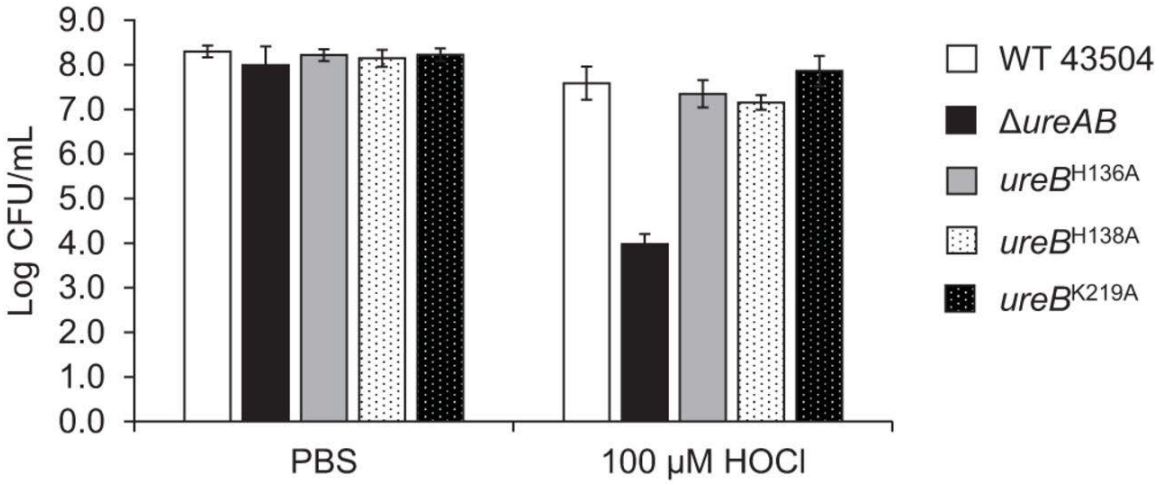

Figure 2.2 Apo-urease confers resistance to HOCl. Cells grown on BA plates with 10 µM $NiCl_2$ were incubated with PBS or with PBS supplemented with 100 µM HOCl for 1 h at 37°C, diluted, and plated. CFU were counted after 72 h at 37°C. Error bars indicate standard deviations from 4 to 10 independent experiments.

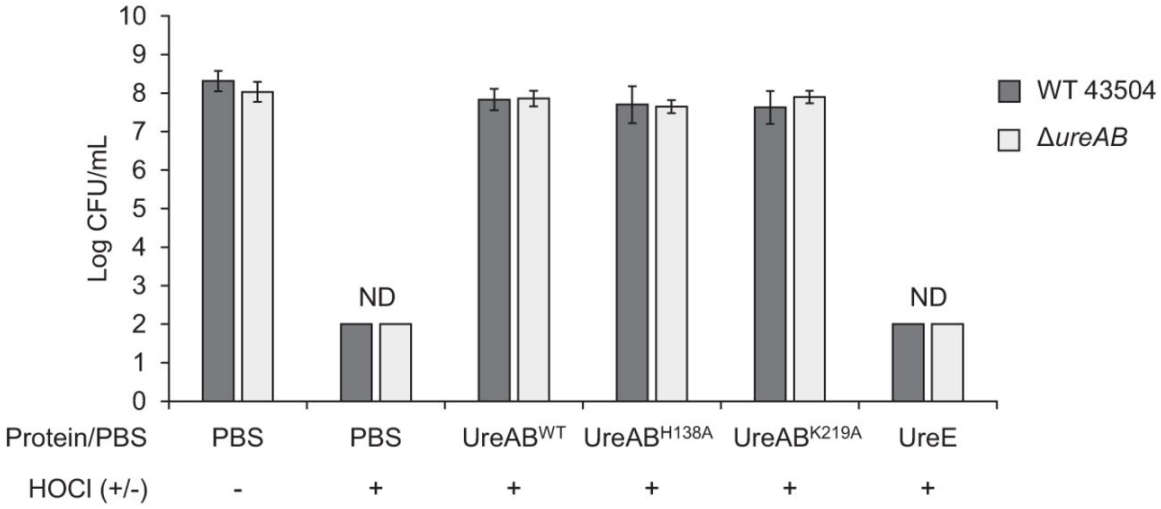

Figure 2.3 Holo- and apo-urease proteins protect against HOCl. Wild-type and Δ*ureAB* 43504 cells were incubated for 1 h in PBS, PBS with HOCl, or PBS with HOCl that had been previously incubated for 15 min at 37°C with either purified $UreAB^{WT}$, $UreAB^{H138A}$, $UreAB^{K219A}$, or UreE as indicated below each column. Final protein concentration was 0.25 µM, and final HOCl concentration was 200 µM in each reaction. Error bars indicate standard deviations from 3 to 9 independent experiments. ND, no CFU could be detected (detection limit, 10^2 CFU/ml).

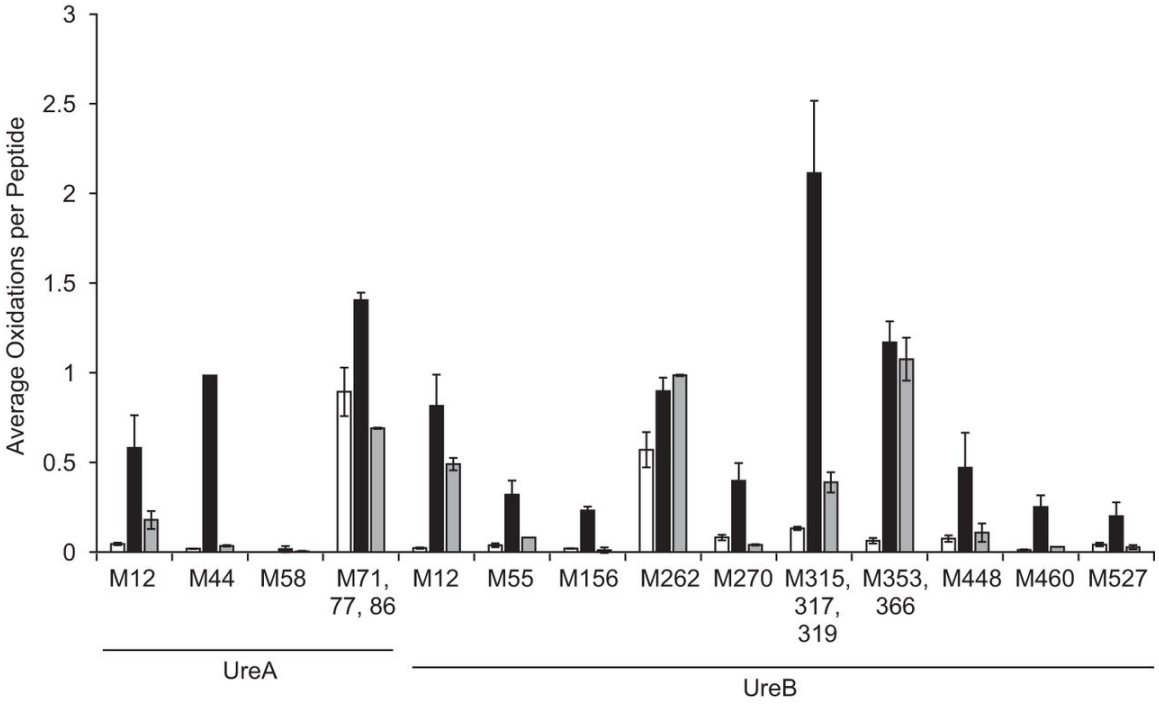

Figure 2.4 LC-MS/MS analysis of oxidized and Msr-repaired urease. Purified urease was incubated with a 60-fold molar excess of HOCl for 15 min. Excess HOCl was quenched with 15 mM Met and removed via dialysis. After dialysis, oxidized urease was incubated with purified Msr, Trx1, TrxR, NADPH, and DTT for 2 h at 37°C. Urease samples were digested by trypsin, and oxidation levels of methionine residues were quantified by LC-MS/MS. The oxidation level for the untreated sample was below the limit of detection for UreA Met[58]. White bars, untreated; black bars, HOCl treated; gray bars, treated with HOCl plus Msr repair mixture. Error bars indicate standard deviations from 2 independent experiments with 3 replicates each.

Table 2.2 Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)[a]	Source or reference
E. coli TOP10	Cloning strain	Invitrogen
H. pylori		
43504	Parental strain	ATCC[b]
Δ*ureB* mutant	Δ*ureB*::KSF Kan^r Suc^s	This study
Δ*ureAB* mutant	Δ*ureAB*::cat Cm^r	15
ureB^{H136A} strain	Codon for His136 replaced by Ala	This study
ureB^{H138A} strain	Codon for His138 replaced by Ala	This study
ureB^{K219A} strain	Codon for Lys219 replaced by Ala	This study
Plasmids		
pBluescript KS(+)	Cloning vector; Ap^r	Stratagene
pKS-*ureB*	*ureB* cloned into the KpnI-SacI site of pBluescript KS(+)	This study
pKS-*ureB*::KSF	KSF cassette cloned into the BamHI site of pKS-*ureB*	This study
pKS-*ureB*^{H136A}	pKS-*ureB* with His136 replaced by Ala	This study
pKS-*ureB*^{H138A}	pKS-*ureB* with His138 replaced by Ala	This study
pKS-*ureB*K^{219A}	pKS-*ureB* with Lys219 replaced by Ala	This study

[a]Cm, chloramphenicol; Ap, ampicillin; Kan, kanamycin; Suc, sucrose; KSF, Kan^r-*sacB*-P_{flaA} (62).
[b]American Type Culture Collection, Manassas, VA.

Table 2.3 Primers used in this paper

Name	Sequence (5′→3′)[a]	Restriction site
UreBF1	GCTTCGGTACCCCTGAGAGAAGGCATGAG	KpnI
UreBR1	GCATCGAGCTCCCGGCAACACTTGTCT	SacI
UreB:H136AF	GTGGTATTGACACAGCTATCCACTTCATTTCAC	
UreB:H136AR	GTGAAATGAAGTGGATAGCTGTGTCAATACCAC	
UreB:H138AF	ATTGACACACACATCGCCTTCATTTCACCCC	
UreB:H138AR	GGGGTGAAATGAAGGCCATGTGTGTGTCAAT	
UreB:K219AF	GGTGCGATTGGCTTTGCAATCCACGAAGACTGG	
UreB:K219AR	CCAGTCTTCGTGGATTGCAAAGCCAATCGCACC	

[a]Primers were purchased from Integrated DNA Technology, Coralville, IA.

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CHAPTER 3

CONCLUSIONS AND FUTURE DIRECTIONS

Helicobacter pylori infections can cause a variety of symptoms, including gastritis, gastric ulcer, and gastric cancer (1). Gastric ulcers and gastric cancers are rarer and often occur after the host has been chronically infected for several decades (2-4). In order to survive the low pH of the stomach, *H. pylori* makes use of powerful flagella and uses urease to increase the pH of its periplasm through ammonia production. While persisting in the gastric mucosa, *H. pylori* faces a barrage of reactive oxygen species which it combats with several enzymes such as superoxide dismutase (SodB) and catalase (KatA). Both superoxide dismutase and catalase *H. pylori* mutant strains has been shown to be hypersensitive to oxidative stress and show decreased mouse colonization (5-7).

There is mounting evidence that *H. pylori* urease also plays a role in persistence in addition to its well-studied critical role in initial colonization. An elegant study makes use of a conditional *H. pylori* urease mutant with urease expression regulated by tetracycline (8). This allows for the controlled expression of urease during initial infection and to measure the effect of urease on persistent infection (8). Debowski et al. found that urease was required for both initial infection and chronic infection, as after blocking urease expression, established infections were eradicated (8).

Urease could play a role as an antioxidant that is required for persistence via a methionine (Met) redox mechanism. Urease Met residues could be oxidized and then repaired by methionine sulfoxide reductase (Msr). Oxidized Msr is reduced by thioredoxin

(Trx) which itself becomes oxidized. The oxidized Trx is reduced by thioredoxin reductase (TrxR) with NADPH as an electron donor (9, 10). In order to maintain sufficient reducing power during persistence, a possible source of energy could be hydrogen gas, as *H. pylori* hydrogenase can utilize molecular hydrogen to generate a proton motive force (11). Reducing power for Msr can come from a number of sources, but electrons from H_2 would be expected to be a major etiological source. The average H_2 gas concentration in the gastric mucosa of mice is 43 µM, whereas the K_M of *H. pylori* hydrogenase for H_2 is 1.8 µM (11). Therefore, we would expect the hydrogenase and the H_2 oxidizing electron transport chain to be saturated under many, if not most, *in vivo* conditions.

A mechanism with Met residues as having an antioxidant role was first described in purified *E. coli* glutamine synthetase; however, this study did not address the role of oxidized Met residues in protecting whole cells from oxidants (12). Benoit et al. demonstrated that *H. pylori* catalase, another abundant protein, was able to quench oxidants through a noncatalytic method that aids bacterial survival (13). In order to determine if urease plays a similar noncatalytic role, I constructed site directed mutants that produced apo-urease, such that they lacked urease activity but still produced a noncatalytic urease protein (Chapter 2). Though lacking urease activity, these mutants showed the same resistance to hypochlorous acid (HOCl) as wildtype, whereas the urease deletion (Δ*ureAB*) was highly sensitive to the HOCl challenge. These results are similar to what was seen with *H. pylori* strains expressing noncatalytic catalase compared to the complete *katA* deletion strain (13). It would seem that a strain lacking two major Msr-dependent targets (i.e. a urease and catalase double mutant) would be expected to be extremely sensitive to oxidative stress. Such a strain may not even be recoverable upon mutagenesis, albeit double

mutant strain phenotypes can be complicated. For instance, there is strong evidence that upon deletion of one or more stress-combating factor(s) in *H. pylori*, other stress-combating enzymes are up-expressed, presumably to augment bacterial survival (14).

Purified holo- and apo-urease was able to protect both the wildtype and notably the Δ*ureAB* strain from HOCl challenge. Msr had been previously shown to bind several *H. pylori* proteins through a coimmunoprecipitation approach (9). Notably, some of these repair targets are themselves oxidative stress combating enzymes. Msr was shown to restore activity to oxidized *H. pylori* proteins such as catalase, UreG, and alkyl hydroperoxide C (AhpC) (15-17). Urease had not been shown to interact with Msr previously and there were no data on reduction of oxidized methionine residues in urease (9). I demonstrated that oxidized urease can be reduced by a Msr repair mixture containing Msr, thioredoxin (Trx1), thioredoxin reductase (TrxR), NADPH, and dithiothreitol (DTT). Of the 25 Met residues in urease, 11 were shown to be oxidized and then subsequently reduced by Msr. Surface exposed Met residues have been shown to be readily oxidized and then reduced by Msr (12, 15, 17). Indeed, 9 of the 11 Met residues I found to be oxidized and reduced in urease were surface exposed, based on PDBePISA analysis of the X-ray crystal structure of *H. pylori* urease (Protein Data Bankcode 1E9Z) (18, 19). Efforts to restore enzymatic activity to oxidized and then subsequently Msr reduced urease were unsuccessful. This could be due to the complex dodecameric structure of urease, as well as the complex array of urease accessory proteins required for urease maturation and Ni insertion into the active site. Regardless of activity, Msr can reduce oxidized Met residues of urease, thus conferring to the enzyme a role as a recyclable sink for oxidants.

In order to determine if specific methionine residues are responsible for the observed oxidant quenching phenotype, I constructed a site directed *H. pylori* X47 mutant (*ureAB*-M12L, M44L, M315L, M319V, M448L). These residues were selected based on their susecptibility to oxidation as seen in MS analysis (Figure 2.4). The 5 Met residues were replaced with leucine or valine in order to minimize inhibitory effects on urease activity. Indeed, the mutant had similar urease activity as both wildtype 43504 and X47 strains. When challenged with HOCl, the 5 Met mutant strain yielded variable results, such that firm conclusions about the cumulative role of the methionine residues could not be made. Overall it appears that changes to 5 Met residues is not enough to cause complete susceptibility to HOCl as compared to a Δ*ureAB* strain. As the construction of consecutive site directed mutants is time consuming, further work on this aspect could make use of synthetically constructed genes. This would allow for all of the Met residues to be altered simultaneously. Alternatively, use of previously described substitution of norleucine for methionine could be used (20, 21). This method could be used in two ways. First, since *H. pylori* is a methionine auxotroph it could be grown in a defined media containing norleucine instead of methionine. This would replace Met residues globally and could provide insights into the role of methionine as antioxidant as shown previously in *E. coli* (20). A second approach would be to express recombinant *H. pylori* urease in a Met auxotroph *E. coli* strain and supplement the growth medium with norleucine instead of methionine, as previously described with a Met deficient cytochrome P450 BM-3 heme domain (21). Purified urease could then be assessed for its ability to quench oxidants and protect cells from HOCl challenge.

Another approach to evaluate the role of noncatalytic urease in *H. pylori* persistence is measuring levels of mouse colonization. However, overcoming the acid barrier of the stomach is a difficult challenge. Use of proton pump inhibitors (PPI) such as omeprazole to raise the pH of the stomach are problematic as they have been found to alter the distribution of *H. pylori* in the stomach and alter the orientation of cells in the gastric mucosa (22, 23). Finding a concentration of PPI that would increase the pH of the stomach but not inhibit *H. pylori* colonization would be difficult. Another factor is that the gut microbiome has been shown to be altered by PPIs (24). Although the gastric microbiome is less well studied, there might be a similar affect caused by PPIs. In studying the importance of urease in persistence, Debowski et al. constructed conditional urease mutants (8). One possibility would be to construct a *H. pylori* strain that can be separately regulated to express holo-urease and apo-urease *in vivo*. Thus, holo-urease could be expressed during initial colonization to overcome the acidic passage through the lumen. Then after colonization, apo-urease could be expressed and colonization rates could be monitored. If apo-urease is only expressed during persistent infection I would expect the strain to be able to persist at almost the same levels as wildtype.

Efforts to identify other abundant, Met-rich *H. pylori* proteins that play a similar antioxidant role were unsuccessful. A screen identified several potential targets; however, when deletion strains were exposed to HOCl *in vitro* they were just as susceptible as wildtype *H. pylori* (Appendix A). This lack of success could be due to the lack of abundance (e.g. specific protein level) of target proteins compared to urease. Urease also forms a large 1.1 MDa dodecameric structure with 300 Met residues (19). Although the

targeted proteins have a high percentage of Met residues, their overall Met residue count is much lower than for urease.

Antibiotic resistance has become a significant threat to public health. Cases of untreatable infectious diseases have become more prevalent. There is a great need to discover alternative pathways for antimicrobial development. As urease is important for virulence in several pathogenic bacteria and fungi it has long been considered an attractive therapeutic target (25-27). Urease inhibitors fall into several classes: sulfur compounds, hydroxamic acids, phosphorus compounds, boric and boronic acids, citrate, fluoride, quinones, heavy metals, and polyphenols (28). A common problem with urease inhibitors is toxicity. For example, one of the more popular inhibitors, hydroxamic acids, have shown severe side effects (29, 30).

One method for urease inhibition that has been largely unexplored is direct nickel chelation. There are no known human enzymes that require nickel, so if the chelator is Ni-specific or Ni-preferred, one would expect limited host toxicity. Indeed, work from our lab has shown that dimethylglyoxime (DMG), a nickel specific chelator, has no toxicity in mice and wax moth larvae (*Galleria mellonella*) at high (mM) DMG levels (unpublished data). I demonstrated that urease activity of a multidrug resistant strain of *Klebsiella pneumoniae* could be inhibited *in vitro* with DMG treatment (Appendix B).

Another Ni requiring enzyme is [NiFe] hydrogenase. [NiFe] hydrogenases are found in several pathogens such as *Salmonella* Typhimurium, *Proteus mirabilis*, *K. pneumoniae*, and *Cryptococcus neoformans*. In collaboration with other lab members I demonstrated that *S.* Typhimurium organ burden can be reduced in mice after treatment with DMG (Appendix B). Additionally, I found that DMG has antibiofilm properties

against *Helicobacter pylori*, *Salmonella* Typhimurium, and *K. pneumoniae* (Appendix B). Future work to define DMG antibiofilm properties could compare the viability of the various biofilms before and after DMG treatment. The use of dyes, both fluorescent and redox sensitive, could be used to determine viability. The BacLight Live/Dead assay could be used to fluorescently label live and dead cells for enumeration (31). Triphenyl tetrazolium chloride (TTC) is a dye that changes color after it is metabolized by a viable cell (32). In order to improve DMG Ni specificity and lower the minimum inhibitory concentration of DMG, several analogs could be synthesized and screened. As of yet we have not shown whether the inhibitory mechanism of DMG is Ni chelation based or some other mechanism, albeit it does inhibit expression or activity of two critical enzymes (hydrogenase and urease). A DMG analog that is unable to bind Ni could be useful in determining the extent of a non Ni-chelation mechanism of action against bacteria.

Urease is found in all domains of life: plants fungi, archaea, and bacteria, where it plays important catalytic roles (33). More recently, the noncatalytic nature of urease has been investigated (34). Described as a moonlighting protein, urease has been identified as a fungicide, insecticide, and it has proinflammatory effects (35). The role of urease as a noncatalytic antioxidant adds to this growing list of functions and expands our knowledge on the role of enzymes beyond their strict catalytic function.

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APPENDIX A

UNPUBLISHED DATA FOR NONCATALYTIC ROLES OF METHIONINE RICH PROTEINS

Screen for Met rich proteins. After characterizing the non-catalytic antioxidant role of urease (see Chapter 2) I sought to determine if other methionine rich *H. pylori* proteins could play a similar role. I used a bioinformatic approach to screen for potential candidate proteins with the following conditions: a methionine percentage of ≥3%, high abundance, and encoded by nonessential genes (Table A.1). I also highlighted proteins that where shown or predicted to be secreted extracellularly. From the screen results I selected three uncharacterized or poorly characterized proteins of interest HP0305, HP0596, and HP0721. I also included HP1286 (Met content 2.7%) because of its YceI domain. YceI proteins are a part of a larger group of bacterial lipocalins which were recently shown to bind to antibiotics extracellularly in a *Burkholderia cenocepacia* model (1).

Evaluation of oxidant sensitivity. I constructed three gene deletion strains: Δ*hp1286*::cat, Δ*hp0305*::cat, and Δ*hp0596*::cat and assessed their susceptibility to hypochlorous acid (HOCl) as described in Chapter 2 with the Δ*ureAB* strain. All three strains showed similar susceptibility as the wild type 43504 strain. Next, I expressed HP1286 in *E. coli* (using the pET expression system) and purified it using Ni-NTA affinity chromatography. Using purified HP1286 (without N-terminal signal sequence) I determined it was not able to quench HOCl and protect *H. pylori* cells. Thus, these proteins do not seem to be playing an antioxidant role in *H. pylori*.

Antibiotic sensitivity in YceI mutants. Both HP0305 and HP1286 have a predicted YceI domain. *B. cenocepacia*, encodes two YceI genes (*bcnA, bcnB*). *bcnA* has been shown to bind extracellular antibiotics and the Δ*bcnA* strain has increased antibiotic susceptibility (1). Using E-test strips and a disk diffusion assay, I evaluated Δ*hp1286*::cat and Δ*hp0305*::cat for antibiotic sensitivity compared to wildtype 43504. Both strains showed the same susceptibility to ciprofloxacin, polymyxin B, and rifampicin as wildtype. Additionally, I evaluated the ability of purified HP1286 to chelate antibiotics and protect *H. pylori* 43504 and *Salmonella* Typhimurium JSG210 from rifampicin and polymyxin B. *H. pylori* and *S.* Typhimurium showed the same sensitivity to the antibiotic whether it had been preincubated with purified HP1286 or a PBS only control. Thus, although HP1286 and HP0305 are predicted to have a YceI domain they do not seem to play an antibiotic binding role as seen in *B. cenocepacia* (1).

Table A.1 A bioinformatic screen of abundant, essential, and Met rich *H. pylori* proteins

Protein	Gene	Rank abundance	Met content (%)	Secreted
60 kDa chaperonin (GroEL protein) (Heat shock protein 60) (Protein Cpn60)	HP0010	2	3.7	N
Alkyl hydroperoxide reductase C	HP1563	3	3.0	N
Urease subunit beta	HP0072	4	3.2	Y
Elongation factor Tu (EF-Tu)	HP1205	14	3.3	N
N-methylhydantoinase	HP0696	21	3.4	N
Uncharacterized protein	HP0697	28	3.6	N
Uncharacterized protein	HP1242	29	3.9	N
Uncharacterized protein	HP0721	35	4.6	Y
Cytochrome c-553 (Cytochrome c553)	HP1227	37	7.3	Y
Isocitrate dehydrogenase [NADP]	HP0027	43	4.0	N
Nucleoside diphosphate kinase	HP0198	60	3.6	N
Pyruvate ferredoxin oxidoreductase, gamma subunit	HP1108	62	4.8	N
Uncharacterized protein (TipAlpha)	HP0596	71	4.2	N
Elongation factor G (EF-G)	HP1195	76	3.6	N
Fructose-bisphosphate aldolase	HP0176	84	3.3	N
Ss-DNA binding protein 12RNP2	HP0827	93	3.7	Y
3-ketoacyl-acyl carrier protein reductase (FabG)	HP0561	94	3.6	N
Hydrogenase/urease maturation factor HypB	HP0900	97	4.5	N
Biotin carboxyl carrier protein of acetyl-CoA carboxylase	HP0371	101	4.5	N
Uncharacterized protein	HP0305	106	3.3	Y
Neuraminyllactose-binding hemagglutinin (Flagellar sheath adhesin)	HP0410	107	3.2	Y
Pyruvate ferredoxin oxidoreductase, delta subunit	HP1109	109	3.1	N
Gamma-glutamyltranspeptidase (Ggt)	HP1118	114	3.0	Y
Uncharacterized protein	HP0097	122	6.8	Y
3-oxoacyl-[acyl-carrier-protein] synthase 2	HP0558	134	3.9	N
Oxygen-insensitive NADPH nitroreductase	HP0954	135	4.8	N
Urease accessory protein UreG	HP0068	144	4.5	N
Elongation factor P (EF-P)	HP0177	152	3.2	N
Protein VdlD	HP0891	156	3.4	N

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APPENDIX B

UNPUBLISHED DATA FOR ANTIMICROBIAL NICKEL CHELATION

Dimethylglyoxime mediated urease inhibition. Dimethylglyoxime (DMG) is a nickel specific chelator that could be adapted to use as an antimicrobial drug (See Chapter 1). There are no known human enzymes that require nickel (Ni) to function; however, several pathogens rely upon Ni-metalloenzymes for virulence. Ureases require 2 Ni^{2+} ions to hydrolyze urea into ammonia and carbon dioxide. It has been suggested that urease-produced ammonia enhances *K. pneumoniae* colonization after a urease deletion strain was outcompeted by wildtype in a mouse infection model (1). In order to determine if DMG could inhibit urease activity, I incubated *K. pneumoniae* with and without DMG during overnight growth. Next, I isolated cell free extracts from a multidrug resistant strain of *K. pneumoniae* (BAA2472) via a freeze/thaw and bead vortexing method. Cell free extract was then assayed for urease activity. Urease activity was inhibited 70% when cells were grown with 1 mM DMG compared to no DMG (Table B.1). No urease activity was detected after treatment with 5 mM DMG (Table B.1). Thus, DMG is able to inhibit urease activity of a multidrug resistant strain of *K. pneumoniae*. DMG-mediated urease inhibition may provide an alternative to current antibiotics by inhibiting enzymes important for virulence.

***Salmonella* Typhimurium organ burden in DMG treated mice.** In order to determine if DMG could inhibit the organ burden of *S.* Typhimurium, mice were infected with *S.* Typhimurium (10^6 cells) and treated with DMG. After 96 hrs of treatment (4 doses of 3 mg DMG) mice were euthanized and livers and spleens were removed. I

homogenized organs which were diluted and plated on bismuth sulfite agar for colony forming units (CFU) counts. For both the liver and spleen, the DMG-treated mice showed a significant decrease in CFU/mL (Figure B.1). Thus, DMG is able to inhibit organ burden in mice.

DMG antibiofilm properties. In order to assess antibiofilm properties of DMG, I used two variations of the well established 96-well crystal violet assay. First, I incubated *H. pylori* 43504, *S.* Typhimurium 700408, and *K. pneumoniae* BAA2472 with DMG in 96 well plates for 18 hrs (*S.* Typhimurium, *K. pneumoniae* in LB) or 48hrs (*H. pylori* in brain heart infusion (BHI) with 0.4% β-cyclodextrin). Then I removed the supernatant and gently washed the wells. The cells were then heat fixed at 60^{o}C for 1 hr. After heat fixing the cells, I stained them with 0.5% crystal violet, washed to remove excess crystal violet, and dissolved remaining crystal violet with a 33% solution of acetic acid. The optical density (OD_{600}) of the wells were recorded. Second, I let *H. pylori* cells incubate with just media for 48hrs, removed the supernatant, added DMG, and incubated for a further 24hrs. I then repeated the above crystal violet assay. These methods evaluate the antibiofilm and biofilm inhibition properties of DMG, respectively. In all three species tested DMG was able to inhibit biofilm formation (Figure B.2). DMG was able to weaken an established (48hrs of growth) *H. pylori* biofilm (Figure B.3). These preliminary experiments provide evidence for promising antibiofilm properties of DMG. Additional independent replications are needed. Also experiments to quantify live cells should be performed as the crystal violet experiments are limited by the fact that the dye binds to all cells, regardless of viability. Using fluorescent probes, the BacLight LIVE/DEAD assay could be used to count the number of living and dead cells after exposure to DMG.

Alternatively, a redox sensitive dye such as triphenyl tetrazolium chloride (TTC) could be used. Once metabolized by living cells, TTC forms a pigmented formazan which can be quantified by optical density.

Table B.1. Effect of DMG chelation on urease activity in *K. pneumoniae* BAA2472

DMG (mM)[a]	Urease activity[b]
0	0.17 ± 0.03
1	0.05 ± 0.01
2	0.03 ± 0.01
5	ND[c]

[a]DMG was added to LB broth, cells were grown overnight and urease assays were performed on cell-free extracts using the phenol-hypochlorite method of Weatherburn (2).

[b]Urease activity is expressed in µmoles of NH_3 produced per min per mg of total protein.

[c]ND, not detected (< 0.001)

Values shown are the mean ± standard deviation for at least three independent biological replicates, with assays done in triplicate. Urease activities measured for all DMG-supplemented conditions are significantly lower compared to the no-DMG control (P<0.01%, Student's t-test).

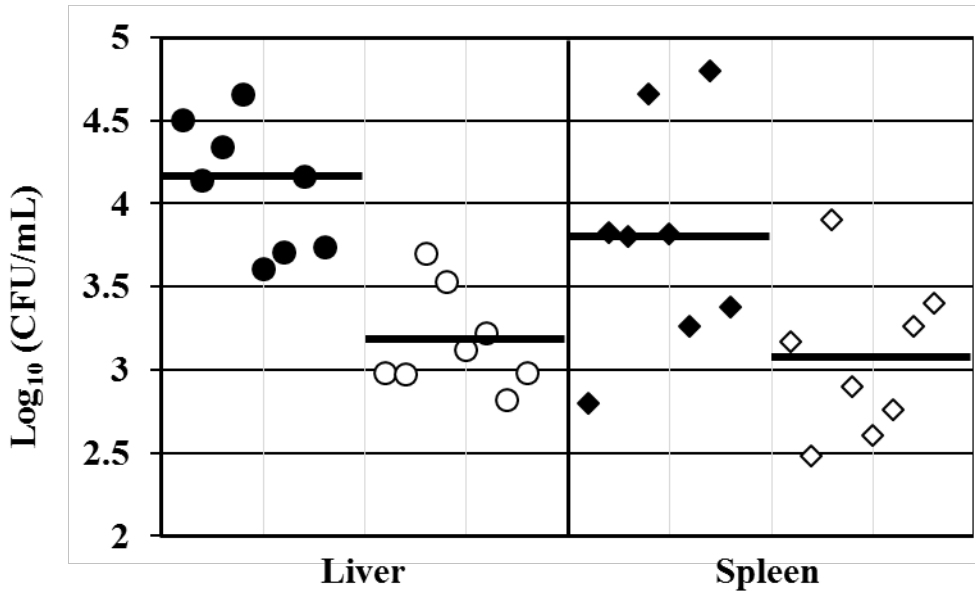

Figure B.1 DMG treatment decreases *S.* Typhimurium organ burden in mice. Organ colonization of *S.* Typhimurium strain 14028 in the livers (circles) and spleens (diamonds) of infected mice, after treatment with DMG (white symbols) or no DMG treatment (black symbols). Each symbol represents the numbers of S. Typhimurium cells (Log_{10} CFU/mL) recovered after organ homogenization, serial dilutions and plating on selective medium. S. Typhimurium colonization is significantly lower in livers ($P<0.01$) and spleens ($P<0.025$, Student's t-test, for livers and spleens, respectively) in the DMG-treated mice compared to the no-DMG group (black symbols).

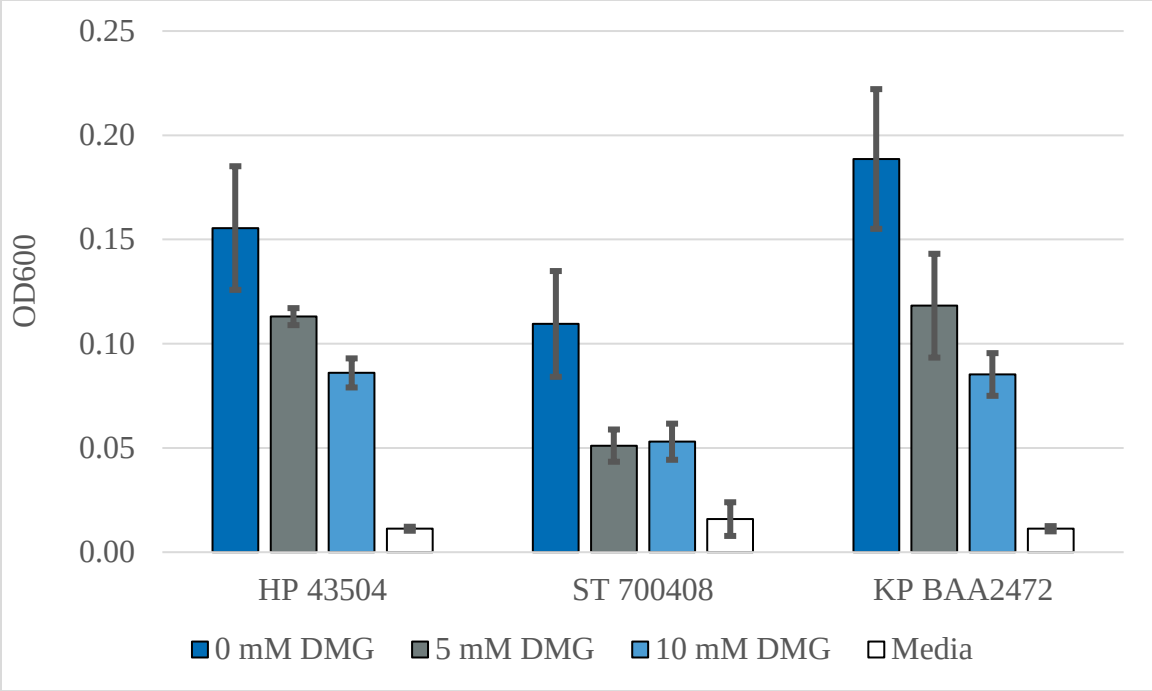

Figure B.2 Biofilm inhibition by DMG. *H. pylori* 43504, *S.* Typhimurium 700408, and *K. pneumoniae* BAA2472 cells were incubated with DMG in 96 well plates for 48 hrs (*H. pylori* 43504) or 16 hrs (*S.* Typhimurium 700408, and *K. pneumoniae* BAA2472). Media only control contained only BHI-0.4% β-cyclodextrin or LB. Determination of biofilm formation was measured by crystal violet staining. Error bars indicate standard deviation from 1 independent experiment with 3-8 replicates per condition.

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