THE EFFECT OF NITROSATIVE AND OXIDATIVE STRESS ON *HELICOBACTER PYLORI*

By

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(Under the Direction of Robert Maier)

ABSTRACT

The gastric pathogen *Helicobacter pylori* is able to withstand an onslaught of oxidative and nitrosative stress from the host immune system. One such stress arises from protein tyrosine nitration by peroxynitrite ($ONOO^-$), which is a product of superoxide and nitric oxide released from macrophages. We utilized a proteomics-based approach, combining anti-nitrotyrosine antibody western blots and MALDI-TOF analysis, to identify targets of nitration within *H. pylori*. Through this approach, we have identified several target proteins. Most of the identified proteins are predicted to localize either to the periplasm or associate with the membrane. Further studies on purified catalase showed its activity decreases in a peroxynitrite dose-dependent manner, with a concomitant increase in tyrosine nitration. An additional study was conducted to understand the role of protein L-isoaspartate methyltransferase in *H. pylori* physiology. By immunoblot analysis, we have begun to describe how and where this protein is expressed in *H. pylori*.

INDEX WORDS: *Helicobacter pylori*, Peroxynitrite, Tyrosine nitration, Oxidative stress, Protein L-isoaspartate methyltransferase, Isoaspartate

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CHAPTER 1

LITERATURE REVIEW

Helicobacter pylori

The epsilon proteobacterium *Helicobacter pylori* was first described by Barry Marshall and Robin Warren in 1983 (70). It is a Gram negative microaerophile that colonizes the gastric mucosa of approximately 50% of humans worldwide (14). *H. pylori* can exist in two different morphological forms – spiral and coccoid. The spiral form is considered the primary infective morphology type as it is able to divide and is culturable, whereas the coccoid form is non-culturable and can be induced by a number of environmental factors, such as antibiotics (10) and aging (18). Motility in *H. pylori* is achieved through the presence of 2-6 polar flagella that allow the bacterium to quickly traverse the thick mucus layer of the stomach (48). Optimal conditions for *H. pylori* cultivation, in the laboratory, include a temperature of 37°C, 2-5% O_2, 5-10% carbon dioxide (CO_2). The balance gas typically used is nitrogen.

The first *H. pylori* strain sequenced was strain 26695 in 1997 (66). Decreasing costs and increasing whole-genome sequencing availability have since led to 62 complete *H. pylori* strain sequences deposited in Genbank, with more in the pipeline. The *H. pylori* genome is approximately 1.6 Mb and contains 1,590 predicted genes. The average

G + C content is 39% (66). Other *H. pylori* genomes deposited in Genbank seem to adhere to these characteristics within a few thousand base pairs.

In order to colonize the gastric mucosa and cause disease, *H. pylori* utilizes a number of virulence factors. As mentioned, the presence of flagella allows *H. pylori* to traverse the mucus layer. Strains lacking genes encoding components of the flagellum, *flaA* and/or *flaB*, resulted in decreased colonization of gnotobiotic piglets, when compared to the wild type (19). Throughout the gastric mucosa, *H. pylori* encounters a significant pH gradient ranging from pH of 2-3 on the luminal side to pH of 7 at the epithelial cell surface (58). In order to combat the lower range of this pH gradient, *H. pylori* expresses significant amounts of the enzyme urease, potentially as high as 10% of the total protein (7). Urease has the potential to influence the pH of the microenvironment through its production of ammonia from urea. This is supported by studies showing that *H. pylori* urease mutants are quickly killed at low pH (59) and are unable to colonize gnotobiotic piglets (17).

Beyond colonization, a number of other factors influence *H. pylori* virulence. One of these is a genetic element known as the *cag* pathogenicity island (*cag* PAI), which consists of approximately 30 genes. The *cag* PAI genes include components of a type IV secretion system as well as the *cagA* gene (45). CagA is a 129 kDa protein that is translocated, via the type IV secretion system, into gastric epithelial cells where it becomes phosphorylated by host cell machinery (4). Phosphorylated CagA then works to deregulate host cell proliferation and enhance cell motility (45). It is thought that the genomic presence of *cagA* results in higher incidence of gastric cancer of infected patients (9). In addition to *cagA*, another virulence factor gene *vacA* contributes

significantly to *H. pylori* pathogenesis. The VacA toxin is a secreted protein that was first discovered due to its ability to induce intracellular vacuolization of host cells (36). Further studies have shown that VacA is involved in a number of host cell-disrupting mechanisms including vacuolization, anion channel formation (65), apoptosis, and disruption of cellular pathways (31).

Oxidative stress response of *H. pylori*

Oxidative stress is a challenging factor of life for most organisms. One source of oxidative damage is the Fenton reaction, which is the reaction of oxygen with elemental iron. As a consequence, enzymes containing iron-sulfur [Fe-S] clusters are obvious candidates of oxidative damage. For instance, it has been shown that [Fe-S] containing hydratases are highly susceptible to oxidative inactivation by superoxide anion (21). Other Fe-S hydratases, including *E. coli* aconitase B (22) and fumarases A and B (37), are similarly inactivated by oxidative stress. DNA, too, can be damaged by hydroxyl radicals resulting in nicks and single-stranded breaks, ultimately leading to cell death (29). For these reasons, it is important to note that *H. pylori* possesses a suite of oxidative stress response enzymes to combat the negative effects of oxygen.

One of the most ubiquitous oxidative stress response enzymes is superoxide dismutase (SOD), which is responsible for the dismutation of superoxide to hydrogen peroxide. *H. pylori* produces one iron-containing SOD (64); it has been shown to be important in protection against DNA damage and is required for successful colonization via the mouse model (60). Catalase is another important, and related, enzyme in that it can decompose the hydrogen peroxide produced by SOD to water and oxygen. In *H.*

pylori, catalase is produced in high amounts (4-6% of total protein) (40), has a high isoelectric point (27), and is able to withstand up to 3,000-fold molar excess of hydrogen peroxide (69). Survival of *H. pylori* has been shown to be dependent on catalase when the bacterium is presented to professional phagocytes (54) and macrophages (6).

Alkyl hydroperoxide reductase (AhpC) is an enzyme produced by *H. pylori* that catalyzes the reduction of lipid hydroperoxides to their corresponding alcohols. It has been reported to have the ability to act upon a broad range of substrates and its recycling depends on NADPH, TrxR, Trx1 reduction system (5). It was originally thought that production of AhpC is essential to *H. pylori* survival; however, mutants can be obtained when grown at very low oxygen concentrations (49). Generation of these mutants gives rise to two different phenotypes; with slightly different protein expression profiles, but both are unable to colonize in the mouse model (50). Furthermore, *H. pylori* AhpC has been shown to have peroxynitrite reductase activity (12).

In addition to detoxification of damaging oxidative molecules, *H. pylori* has the ability to enzymatically repair oxidatively damaged proteins via the enzymatic activity of methionine sulfoxide reductase (MSR). Similar to AhpC, MSR utilizes the NADPH, TrxR, Trx1 reduction system to reduce protein-bound methionine sulfoxide back to methionine and restore enzymatic activity (40). This type of repair activity has been demonstrated, in *H. pylori*, for specific protein targets including catalase (40), the urease accessory protein UreG (33), and AhpC (8). The importance of MSR has been demonstrated by decreased *H. pylori* survival during oxidative stress and mouse colonization deficiency phenotypes of an *msr* knockout mutant (1).

H. pylori pathogenesis

Infection by *H. pylori* results in several human pathologies. For example, it has been shown that *H. pylori* infection is the causative agent of gastritis and duodenal ulcers (67). Furthermore, *H. pylori* infection, is a risk factor for development of gastric adenocarcinoma (26) and mucosa-associated lymphoid tissue lymphoma (51). In fact, *H. pylori* is classified as a human carcinogen by the International Agency for Research on Cancers (CAS No. 059820-43-8) (71).

During *H. pylori* infection, the immune response plays an important role in host defense. In response to *H. pylori* infection, inducible nitric oxide synthase (iNOS) is up-regulated by host cells (57). iNOS produces nitric oxide from arginine in response to pathogen invasion. Furthermore, monocytes in contact with *H. pylori* are stimulated to produce superoxide anion (41). The production of these two metabolites *in vivo* can result in their reaction to form another strong oxidizing and nitrating agent, peroxynitrite (53).

Peroxynitrite chemistry

Peroxynitrite is formed from the reaction between superoxide anion and nitric oxide at a rate of approximately 10^{10} $M^{-1}s^{-1}$ (53). Due to the short half-life of superoxide anion, relative to nitric oxide, formation of peroxynitrite occurs predominantly at the site of superoxide production. This an especially important point to consider since *H. pylori* has been shown to produce superoxide as well (46). Peroxynitrite is in equilibrium with its protonated form, peroxynitrous acid, which has a pK_a of 6.8 (52). The ratio of

peroxynitrite to peroxynitrous acid depends upon the local pH. At a pH of 7.4, approximately 80% of peroxynitrite will exist in its anionic form (53).

The reactivity of peroxynitrite makes it an interesting molecule with regard to biology. One such reaction occurs between peroxynitrite and CO_2. The rate of this is reaction is very fast at $5.7x10^4$ $M^{-1}s^{-1}$ and gives rise to the carbonate (11) and nitrogen dioxide radicals (38). These two radicals can then go on to propagate further radical reactions. Major reaction targets of peroxynitrite within biological systems are typically sulfhydryls, transition metal centers, and CO_2 due to rates and target concentrations (53). Peroxynitrite can access such targets within cells, due to its ability to cross cellular membranes either by passive diffusion in its protonated form, or via anion channels in its anionic form (15).

Tyrosine nitration

An important reaction in peroxynitrite chemistry is the chemical reaction with either free or protein-bound tyrosine. This reaction yields 3-nitrotyrosine via a two-step radical reaction. As mentioned before, two of the breakdown products of peroxynitrite are carbonate and nitrogen dioxide radicals. The carbonate radical can react with tyrosine to yield a tyrosyl radical, which further reacts with nitrogen dioxide, in a radical termination step, yielding 3-nitrotyrosine (63). Due to the nature of the first step, tyrosine nitration yields are generally higher in the presence of CO_2 (63).

The formation of 3-nitrotyrosine within proteins is thought to be a selective process. Evidence for this selectivity is provided by a study of three proteins of similar size, but that vary in degrees of tyrosine content and secondary structure. Revelations of

this study include potential factors of selectivity including location of tyrosine residues with respect to secondary structure and degree of surface exposure. The presence of turn-inducing amino acids in the vicinity of tyrosine also contributes to selectivity (62).

The proposed consequence of nitrated tyrosine residues within proteins is loss of function. One of the first studies linking tyrosine nitration to loss of function was with human manganese superoxide dismutase (Mn-SOD) (39). In this study, the authors show that peroxynitrite-treated Mn-SOD, purified from human renal tissue that had been rejected by the patient, exhibited a dose-dependent loss of activity with a concomitant increase in tyrosine nitration. To further provide evidence that this loss of activity can be attributed specifically to nitration of an individual tyrosine residue, another group genetically engineered Mn-SOD to be incorporated with 3-nitrotyrosine at position 34. This engineered version of Mn-SOD exhibited the same loss of activity (47). In *E. coli*, exposure to peroxynitrite resulted in up-expression of the arginine biosynthetic pathway. Further analysis revealed that tyrosine residues of the negative regulator, ArgR, become nitrated, and with prolonged exposure the protein degrades (44).

There is evidence for two potential pathways of nitrated protein clearance. The first is degradation, as evidenced in the ArgR example, as well as proteolytic degradation of BSA (25), Cu, Zn superoxide dismutase, and tyrosine hydroxylase (61). A second potential clearance mechanism is denitration of affected protein-bound tyrosine residues. This phenomenon was first reported using rat spleen and lung homogenates to seemingly denitrate BSA (32). The data suggests that this is an enzymatic mechanism, due to it not occurring in heat-treated samples, and has since been reported for protein extracts from dog prostate (34) and rat brain and heart (35).

Patients infected with *H. pylori* exhibit increased tyrosine nitration of host epithelial cells (42). It stands to reason that if host cells must contend with tyrosine nitration, *H. pylori* must as well. To date, no studies have attempted to examine the effect of tyrosine nitration on the *H. pylori* proteome. It is the purpose of this study to identify *H. pylori* proteins susceptible to tyrosine nitration by peroxynitrite, and investigate the consequences of this modification.

L-isoaspartate formation

Modifications of amino acids in proteins can result in significant alterations to protein structure and function. One such modification is the spontaneous conversion of asparagine and aspartate residues to L-isoaspartate. The formation of this residue results from the deamidation and/or isomerization of asparagine or aspartyl residues (23). Mechanistically, the deamidation or isomerization of these residues proceeds through a succinimide intermediate, which spontaneously hydrolyzes into a mixture of aspartyl and isoaspartyl residues (23) (Fig. 1.1).

Within proteins, the primary sequence can play a major role in whether or not isoaspartate can form. Adjacent amino acids and fold confirmations that can allow the nitrogen from a peptide bond to interact with a side chain carbonyl will result in increased isoaspartate formation (13). Through the use of synthetic peptides, it has been shown that the attacking peptide-bond nitrogen is first deprotonated allowing for the formation of a nucleophilic anion that can then attack the side chain carbonyl resulting in succinimide intermediate formation (13). The succinimide intermediate then spontaneously hydrolyzes to a mixture of aspartyl and isoaspartyl residues (13) Based on

these types of factors, methods have been developed to predict deamidation rates for proteins *in silico* (56).

Isoaspartate formation in proteins alters both the structure, by introducing a kink in the polypeptide chain, and the charge of the protein. Many proteins have been shown to have significantly decreased activity upon isoaspartate formation. In humans, proteins such as calmodulin (30), human epidermal growth factor (24), and the human antiapoptotic protein Bcl-X_L (16) have all been shown to have decreased activity upon isoaspartate formation. Since such protein damage has been shown to occur, it seems likely that these damaged proteins either have to be degraded or repaired.

Protein L-isoaspartyl methyltransferase

The methyl-transferase protein L-isoaspartyl methyltransferase (PIMT), was first described in 1965 as a methanol-forming enzyme upon incubation with C^{14}-labeled *S*-adenosylmethionine (SAM) (3). It was realized in 1984, that PIMT acts specifically on isoaspartyl residues giving rise to the theory of PIMT-induced protein repair (2). The repair mechanism proceeds in three steps (Fig. 2.1):

1) Transfer of a methyl group from SAM to the isoaspartyl residue by PIMT
2) Spontaneous succinimide intermediate formation
3) Spontaneous succinimide hydrolysis to either aspartyl (15-30%) or isoaspartyl (70-85%) residues (43)

The importance of PIMT has been underscored by its deletion in mice. The deletion resulted in a number of abnormalities including increased brain size, increased occurrence of epileptic seizures (72), modified insulin receptor signaling factor (20), and

premature death (72). Its importance in bacteria seems to be less pronounced. In *E. coli*, deletion of the gene encoding PIMT results in phenotypes primarily related to stress response in aging cells. For example, the mutant had increased susceptibility to environmental stresses in stationary phase (68). An interesting phenotype of aging bacterial cells is that, when mixed with younger cells, they will outcompete and take over the culture within days. This phenotype is known as growth advantage in stationary phase (GASP) (73). The PIMT mutant strain was shown to have a variable GASP phenotype. In some cases it was able to display a phenotype similar to wild-type; however, the majority of the time the mutant was unable to display the GASP phenotype (68). Lastly, an *E. coli* PIMT mutant struggles to recover from long-term stationary phase (28).

Peroxynitrite-induced tyrosine nitration has not been studied in *H. pylori*. The purpose of this study is to determine if *H. pylori* proteins are susceptible to tyrosine nitration and identify potential targets. The effects of tyrosine nitration on activity and structure of target proteins will then be assessed. PIMT has also not been studied in *H. pylori*; therefore, we aim to elucidate its physiological role and identify potential proteins susceptible to isoaspartate formation.

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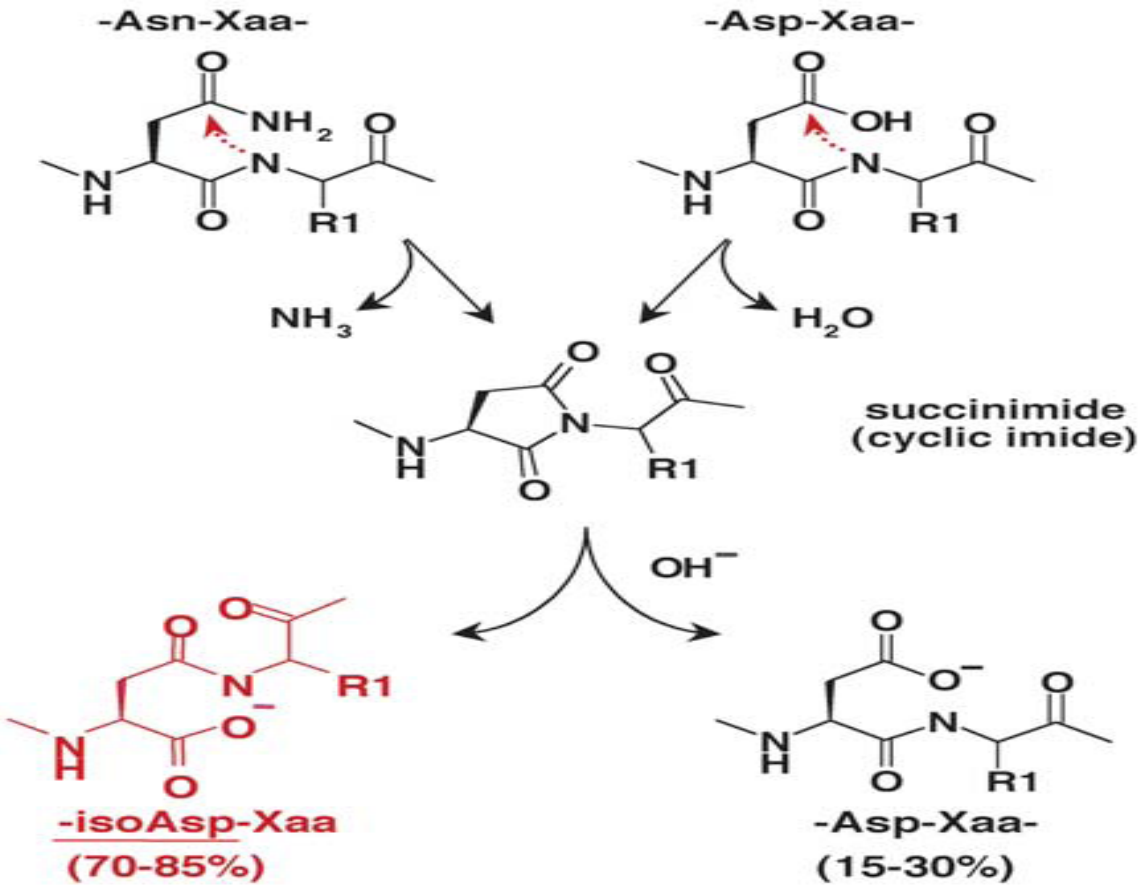

Figure 1.1. Formation of isoaspartate (55). Aspartyl and asparagine residues undergo a nucleophilic attack by the peptide-bond nitrogen, resulting in formation of a succinimide intermediate. The succinimide then spontaneously hydrolyzes to a mixture of aspartyl and isoaspartyl residues.

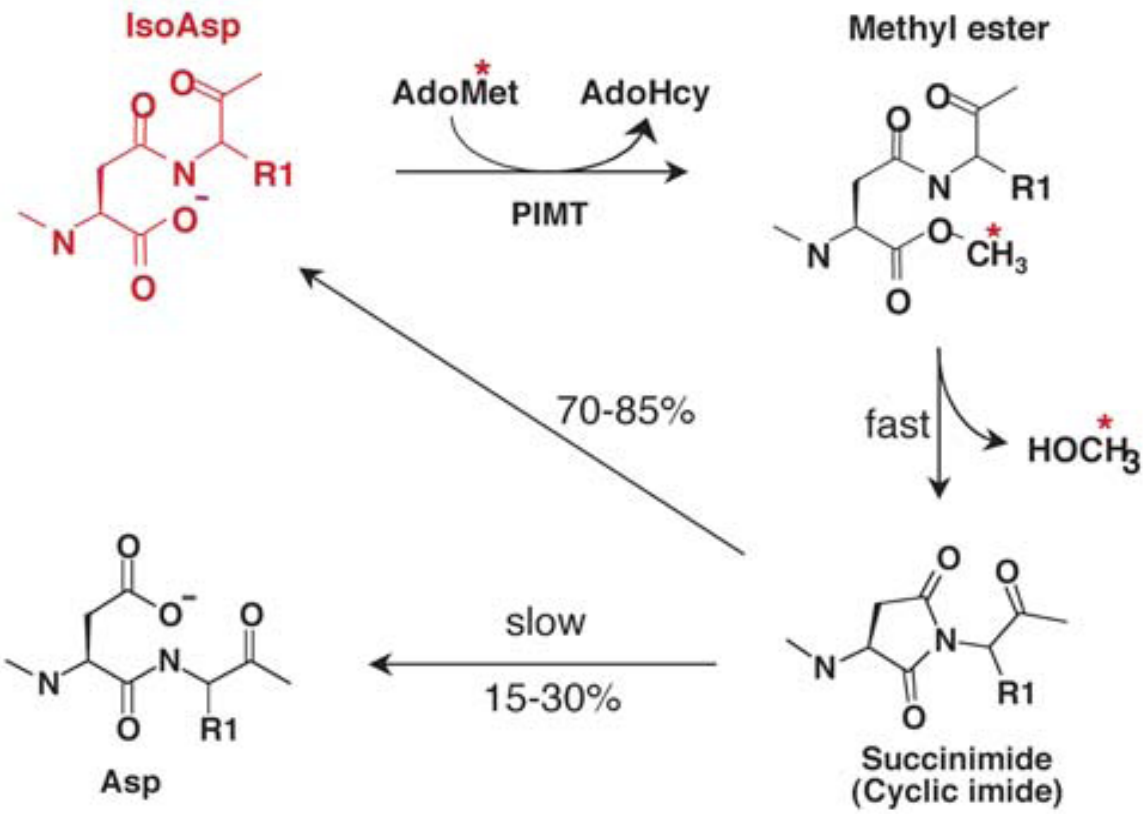

Figure 1.2. PIMT-induced isoaspartate repair (55). PIMT transfers a methyl group from *S*-adenosylmethionine (star denotes either tritiated or C^{14}-labeled SAM) to the isoaspartyl residue forming a methyl ester. Methanol is quickly lost converting the methyl ester to a succinimide intermediate, which spontaneously hydrolyzes to a mixture of isoaspartyl and aspartyl residues.

CHAPTER 2

EFFECTS OF PEROXYNITRITE ON THE *HELICOBACTER PYLORI* PROTEOME

Introduction

Helicobacter pylori is a human gastric pathogen that infects approximately one-half of the world's population and persists for the lifetime of the host unless treated (5). The organism is a Gram-negative, microaerophilic bacterium with a 1.6 Mb genome (23). Effects of *H. pylori* infection in the host include mucous membrane inflammation, peptic ulcers, and even gastric cancer (16). In fact, *H. pylori* is classified as a human carcinogen by the International Agency for Research on Cancers (CAS No. 059820-43-8) (25).

Throughout infection, *H. pylori* induces an inflammatory response by the host and, as a result, increased expression of inducible nitric oxide synthase (iNOS), which in combination with superoxide results in formation of peroxynitrite (19). Peroxynitrite is a potent oxidative and nitrosative stress agent, produced through the reaction of nitric oxide and superoxide (2). Peroxynitrite exhibits bactericidal effects on *H. pylori* and has been shown to alter cell morphology (22). Urease can contribute to a certain level of resistance to $ONOO^-$ through its production of CO_2, which readily reacts with, and neutralizes, peroxynitrite (9).

Upon formation, peroxynitrite is able to cross the membrane barrier and affect a number of biomolecules, including lipids and proteins (8, 18). One of the effects of the

interaction between peroxynitrite and proteins is nitration of tyrosine residues. The results of protein tyrosine nitration vary, but can include loss of function and degradation. For example, peroxynitrite-induced nitration of Tyr34 of human Mn superoxide dismutase (Mn-SOD) has been shown to abolish activity (11). In fact, by substituting 3-nitrotyrosine for the native tyrosine at Tyr34, Mn-SOD activity was decreased by 97%, suggesting that abolished activity is due entirely to tyrosine nitration (15). Similar results were obtained for human glutathione reductase (20). A study in *Escherichia coli* identified the negative regulator of arginine biosynthesis, ArgR, as a target of nitration by peroxynitrite. Upon exposure to peroxynitrite and subsequent tyrosine nitration, ArgR is degraded thereby resulting in increased expression of the arginine biosynthesis machinery (14).

Tyrosine nitration by peroxynitrite is thought to be selective in both the target protein and modified tyrosine residue. Factors proposed to be involved in selection of target proteins and tyrosine residues include degree of solvent exposure, location of tyrosine residues in loop structures, and the presence of acidic amino acids adjacent to the target residue (21). However, a consensus sequence has not, at this time, been identified.

H. pylori is well known for its ability to cause persistent infection. In order to infect the host and withstand the immune response, the organism expresses an impressive array of oxidative and nitrosative stress-combating enzymes. Two of the most prominent enzymes responsible for combating oxidative stress are catalase (KatA) and the peroxiredoxin AhpC. *H. pylori* AhpC was shown to exhibit peroxynitrite reductase activity *in vitro* (3). AhpC combats oxidative stress when reduced by a thioredoxin system. *H. pylori* contains two thioredoxins (Trx1 and Trx2) as well as one thioredoxin

reductase (TrxR). Deletion mutants of *trx1* and *trx2* were both susceptible to nitrosative stress, with the *trx1* mutant being more susceptible (4). Catalase from bovine liver as well as from *Salmonella enterica* serovar Typhimurium and *Mycobacterium tuberculosis* was also shown to have peroxynitrase activity (6, 13, 24). *H. pylori* possesses other enzymes involved in combating oxidative stress including MdaB, NapA, two other peroxiredoxins, Bcp and Tpx, and DNA repair enzymes that repair oxidative damage to DNA. It is possible that any or all of these are involved in the *H. pylori* response to peroxynitrite stress.

To date, the effect of protein tyrosine nitration, overall or on specific proteins, has not been examined in *H. pylori*, nor has the contribution of the various oxidative stress-combating enzymes to peroxynitrite resistance been determined. For these reasons, we sought to identify proteins susceptible to tyrosine nitration and examine the effect of nitration on these protein targets.

Materials and Methods

Reagents and bacterial strains

Commercial peroxynitrite used in experiments was purchased from Calbiochem. Anti-nitrotyrosine monoclonal antibodies were purchased from Biotem. Wild-type *H. pylori* strains SS1 (10) and 26695 (23) were used throughout the course of this study. Strains were cultured on brucella agar (BA) plates supplemented with 10% defibrinated sheep blood. Incubation occurred at 37°C with 4% O_2, 5% CO_2, and balanced with N_2.

H. pylori peroxynitrite exposure

H. pylori was grown 24-48 hours on BA plates, and then resuspended in phosphate buffered saline (PBS), pH 7.0 to an OD_{600} of 1.0-1.1. Cell suspensions were incubated in PBS (control) or PBS supplemented with 250 µM peroxynitrite, at room temperature for 30 minutes while shaking (50 RPM). Cells were then centrifuged at 6,000 RPM for 5 minutes and the resultant pellet was washed twice with PBS. Pellets could then be stored at -80°C or immediately used for further experiments.

Identification of nitrated proteins

Following peroxynitrite treatment, the cells were washed twice with PBS, pH 7.0. The final pellet was resuspended in 25 mM sodium phosphate pH 7.6, 50 mM NaCl, lysed by French press, and then applied to a HiTrap SP HP, 5 mL cation exchange column (GE Healthcare). The flow through was collected and pooled and applied to a HiTrap Q HP 5 mL anion exchange column (GE Healthcare). Proteins were eluted in one step by application of 25 mM sodium phosphate, pH 7.6, 1 M NaCl. Fractions containing proteins were pooled and concentrated using Amicon Ultra-4 10 kDa molecular weight cutoff centrifugal filters (Millipore). Protein concentrations were quantified by BCA assay kit (Thermo Scientific). 20 µg of protein was loaded onto a Criterion^{TM} Tris-HCl 18-well 10% polyacrylamide gel (Bio-Rad). The gel was loaded such that after running it could be cut into two identical halves. One half was used for an immunoblot with anti-nitrotyrosine antibodies while the other was stained with Coomassie. Proteins that showed reactivity with the anti-nitrotyrosine immunoblot were excised from the

corresponding Coomassie-stained gel and sent for identification by MALDI mass fingerprinting at the University of Georgia PAMS facility.

Immunoblotting

Following SDS-PAGE, samples were electrophoretically transferred to a 0.2 µm nitrocellulose membrane (Bio-Rad). The membrane was blocked by incubating in PBS, pH 7.0 supplemented with 5% dry milk and 0.1% Tween-20. The blocking step was followed by incubation with 1:10,000 dilution of anti-nitrotyrosine monoclonal antibody in blocking buffer for 1-4 hours. The membrane was washed three times with PBS, pH 7.0 plus 0.1% Tween-20, and then incubated with goat anti-rabbit secondary antibody conjugated to alkaline phosphatase (1:1000) in blocking buffer for 1 hour. Again, the membrane was washed three times followed by detection of bound antibodies with nitro blue tetrazolium (0.25 mg ml^{-1}) and 5-bromo-4-chloro-3-indolyl phosphate (0.125 mg ml^{-1}) (Sigma). Immunoblots against catalase utilized a 1:1000 dilution of anti-catalase serum followed by washing and incubation with 1:1000 goat anti-rabbit antibodies conjugated to horseradish peroxidase (Bio-Rad). Bound antibodies were detected by Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare) and images developed using a FluorChem E imager (ProteinSimple).

Catalase purification

Catalase was purified natively from *H. pylori* as described previously (12). Briefly, *H. pylori* SS1 was grown on BA plates, and then suspended in 25 mM sodium phosphate, pH 7.6, 50 mM NaCl. Cells were lysed by French press and then centrifuged to remove

cellular debris. The cell-free extract was loaded onto a HiTrap SP HP, 5 mL cation exchange column (GE Healthcare) and proteins were eluted with a linear gradient of 25 mM sodium phosphate, pH 7.6, 1M NaCl. Fractions containing catalase were identified by adding 5 µL of each fraction to 1 mL of 3% H_2O_2 and looking for oxygen evolution. These samples were then collected and pooled. Catalase was further purified by gel filtration on a HiLoad 16/60 Superdex 75 column (GE Healthcare). The gel filtration buffer was 20 mM sodium phosphate, pH 7.6, 150 mM NaCl. Fractions containing catalase were collected, pooled, and concentrated by centrifugal concentration in Amicon Ultra-4 10 kDa molecular weight cutoff centrifugal filters (Millipore).

Exposure of pure catalase to peroxynitrite

Peroxynitrite was diluted in 0.1 M NaOH and its concentration determined by measuring absorbance at 302 nm (molar extinction coefficient of 1670 M^{-1} cm^{-1}). The pure catalase concentration was normalized to 10 µM and incubated with various fold molar excesses of peroxynitrite, as indicated in the figures. Incubations took place at room temperature for 30 minutes. Following the incubation period, 5 mM (final) L-methionine was added to quench the reaction and then samples were dialyzed against 2 L cold PBS, pH 7.0 to remove excess peroxynitrite.

Catalase activity assay

Catalase activity was assessed according to (7). Briefly, 0.1 µg of catalase was mixed with 15 mM H_2O_2 and the disappearance of H_2O_2 was followed spectrophotometrically at an absorbance of 240 nm. The molar extinction used for H_2O_2 was 43.48 M^{-1} cm^{-1} and

one unit of catalase activity was defined as 1 µmol $H_2 0_2$ decomposed/min/µg catalase. To measure catalase activity in cell-free extracts, 1 µg of extract was added to 15 mM H_2O_2 and its disappearance followed as described above.

Results

Tyrosine nitration in H. pylori

To determine if peroxynitrite can nitrate tyrosine residues of *H. pylori* proteins, *H. pylori* strain SS1 was grown for 24 hours, then exposed to varying concentrations of peroxynitrite and the tyrosine nitration profile was assessed by immunoblot with anti-nitrotyrosine antibodies (Fig. 2.1). As shown in Figure 2.1, increasing concentrations of peroxynitrite resulted in increased levels of tyrosine nitration. Importantly, it appeared that not every protein was targeted for nitration, suggesting that specific proteins were susceptible to this modification. It should be noted that the peroxynitrite concentrations used were arbitrarily chosen due to the fact that it is unknown at what concentration peroxynitrite exists *in vivo*.

Identification of putative nitrated proteins

Tyrosine nitration of proteins can lead to devastating effects on the target protein and, as a result, the organism. In order to determine the effects of tyrosine nitration in *H. pylori*, a proteomic-based approach was used to identify putative protein targets of tyrosine nitration by peroxynitrite. Peroxynitrite-treated whole cells were lysed, fractionated by ion-exchange column chromatography, and the fractions subjected to SDS-PAGE followed by an immunoblot with anti-nitrotyrosine antibodies. Figure 2.2 is a

representative example of the immunoblots from the cation exchange fraction. Using the immunoblots, bands were excised from a Coomassie-stained gel of the same sample run simultaneously to the immunoblot and then sent for identification by MALDI mass fingerprinting. Three independent experiments were run. Table 2.1 is a representative list of proteins identified as putative targets of peroxynitrite-mediated tyrosine nitration. Of the identified proteins, catalase appeared in all three trials of the experiment; whereas, none of the other putative targets were identified more than once.

Tyrosine nitration and oligomerization of pure catalase

Catalase was purified from *H. pylori* and exposed to various concentrations of peroxynitrite. Pure catalase displayed increasing levels of tyrosine nitration with increasing concentrations of peroxynitrite exposure (Fig. 2.3). In addition to increased tyrosine nitration, there also appeared to be an increasing amount of oligomerization, and perhaps even breakdown products. In order to investigate the appearance of oligomers, peroxynitrite-treated catalase was analyzed by SDS-PAGE and stained with Coomassie blue (Fig. 2.4). Additionally, an anti-catalase immunoblot was performed to examine the effect of peroxynitrite treatment (Fig. 2.5). The polyacrylamide gel displayed increasing levels of catalase oligomerization with increasing peroxynitrite concentration. In fact, at high peroxynitrite concentrations, most of the protein did not leave the well. Similarly, the catalase immunoblot showed the same oligomerization. Interestingly, the catalase sample treated with the highest level of peroxynitrite (200x molar excess) appeared to not be recognized by the catalase antibodies. This seems to suggest that the structure of catalase is severely damaged upon exposure to excessive amounts of peroxynitrite.

Impaired peroxynitrite-treated catalase activity

Activity of peroxynitrite-treated pure catalase was assessed to determine the effect of peroxynitrite exposure. Figure 2.6 shows decreasing catalase activity with a concomitant increase in peroxynitrite concentration. Exposure of catalase to 200x molar excess of peroxynitrite resulted in complete abolition of catalase activity. This phenomenon however, proved to be irreproducible. Subsequent attempts at repeating peroxynitrite-impaired catalase activity gave mixed results. Some attempts resulted in loss of activity; whereas, other attempts did not (Fig. 2.7). This was irrespective of the use of newly purchased peroxynitrite or peroxynitrite synthesized in-house. It is possible that this is due to a misidentification of catalase as a target of tyrosine nitration by peroxynitrite. Lack of reproducibility could also be attributed to contaminants in the peroxynitrite that either inhibit the effectiveness of peroxynitrite or are the source of the catalase activity inhibition.

Discussion

Peroxynitrite is a potent oxidizing and nitrating agent. *Helicobacter pylori* also encounters the precursors to peroxynitrite, nitric oxide and superoxide, during infection. The effect of peroxynitrite-mediated tyrosine nitration has not been examined in *H. pylori*; therefore, we set out to determine which proteins are affected and how this modification affects their activity. We have shown that *H. pylori* exposure to peroxynitrite resulted in tyrosine nitration of multiple proteins; however, not every protein was susceptible to modification (Fig. 2.1). This led us to conclude that the

specificity of the modification would allow for the determination of individual protein targets.

We sought to identify which proteins can be modified by peroxynitrite through a proteomics-based approach (Table 2.1). This approach resulted in the identification of multiple proteins with the caveat that only catalase was identified in more than one trial. The identification of catalase as a putative nitration target of host peroxynitrite appears to make sense from a host-defense standpoint as the peroxide-dissipating activity is critical to the gastric survival of *H. pylori* in the presence of host defenses (1, 17).

To further investigate the effects of peroxynitrite on catalase, we purified the enzyme from *H. pylori* and subjected it to exposure to peroxynitrite. Immunoblots against nitrotyrosine showed that tyrosine residues of catalase are, indeed, nitrated and the nitration levels increase with increasing levels of peroxynitrite exposure (Fig. 2.3). Additional effects include oligomerization (Figs. 2.4, 2.5) and, potentially, degradation. However, the effects on catalase activity appeared to be mixed. In some cases we observed significant loss of catalase activity when exposed to peroxynitrite (Fig. 2.6), and in other trials catalase activity was not inhibited at all (Fig. 2.7). Use of freshly purchased peroxynitrite or even in-house synthesized peroxynitrite exhibited the same variable observations. It is unknown exactly why the catalase activity assays were varied; however, it is possible that catalase is not negatively affected by tyrosine nitration. It is also possible that a contaminant in the peroxynitrite was the source of decreased catalase activity or interfered with the effectiveness of peroxynitrite.

These studies suggest that the *H. pylori* proteome is, in fact, susceptible to tyrosine nitration by peroxynitrite. However, identifying the individual proteins that are

most susceptible proved to be a difficult task. Reproducibility of the identifications was a significant problem throughout this study. In addition, the only protein that seemed to be a true target displayed varying degrees of activity inhibition, suggesting it may have been an artifact during the identification experiments. This is not altogether surprising considering the amount of catalase produced by *H. pylori*. It has been shown that catalase contributes up to 4-5% of the total protein in *H. pylori* (12). Identification of proteins via one-dimensional SDS-PAGE suffers from the fact that multiple proteins can exist in a single band and sufficient separation between protein bands is often difficult to achieve. This can often lead to false positives, as seems to have been the case here.

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Table 2.1. Putative nitrated protein targets

Band #	Protein I.D.	Expect Value	MASCOT Score	Predicted Cellular Location	Tyrosine Content (%)
1	SecA	4.6e-27	335	Cytosol/ Cytoplasmic membrane	3.7
2	FecA	2.9e-6	127	Outer membrane	7.2
3	Unidentified	--	--	--	--
4	Catalase	3.7e-15	216	Periplasm	5.0
5	CeuE	3.7e-7	136	Periplasm	2.7
6	RplF	7.4e-5	113	Cytsol	3.4

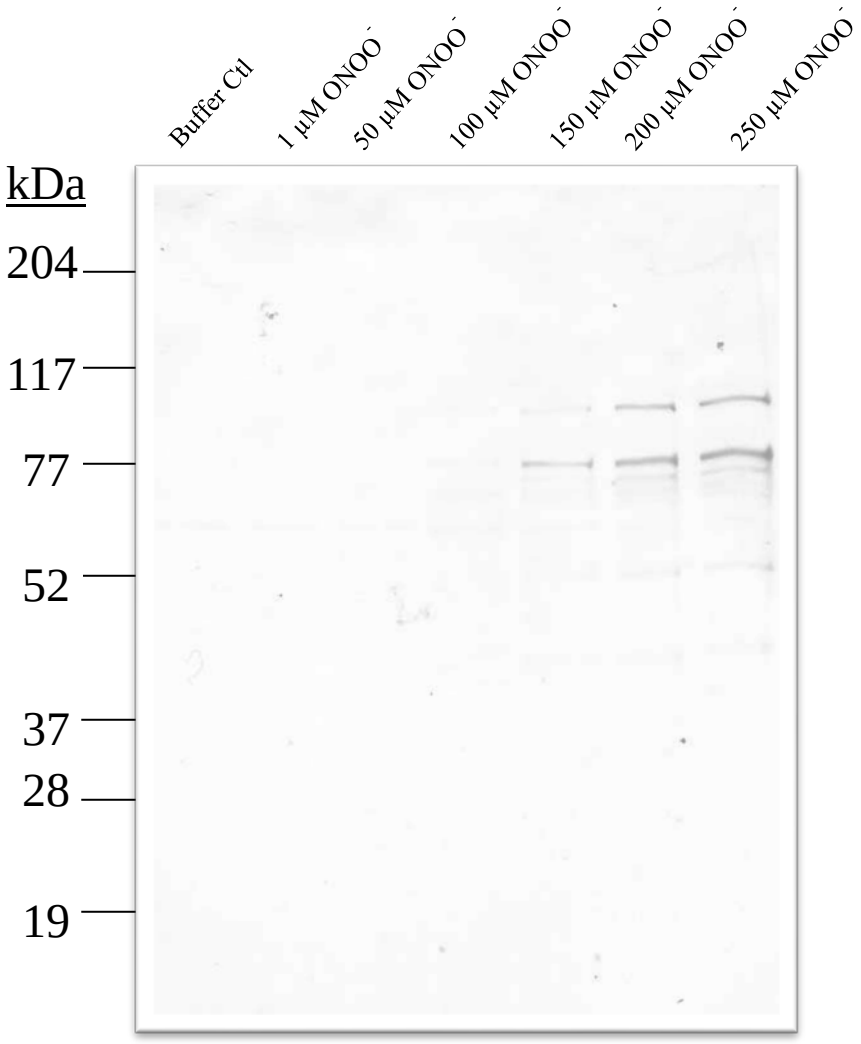

Figure 2.1. Nitration profile of the *H. pylori* proteome. *H. pylori* cells were exposed to varying concentrations of peroxynitrite for 30 minutes. The cells were washed, lysed, and the protein concentration of the resulting extract estimated by BCA assay. A total of 5 µg was loaded onto a gel to be analyzed by anti-nitrotyrosine immunoblot.

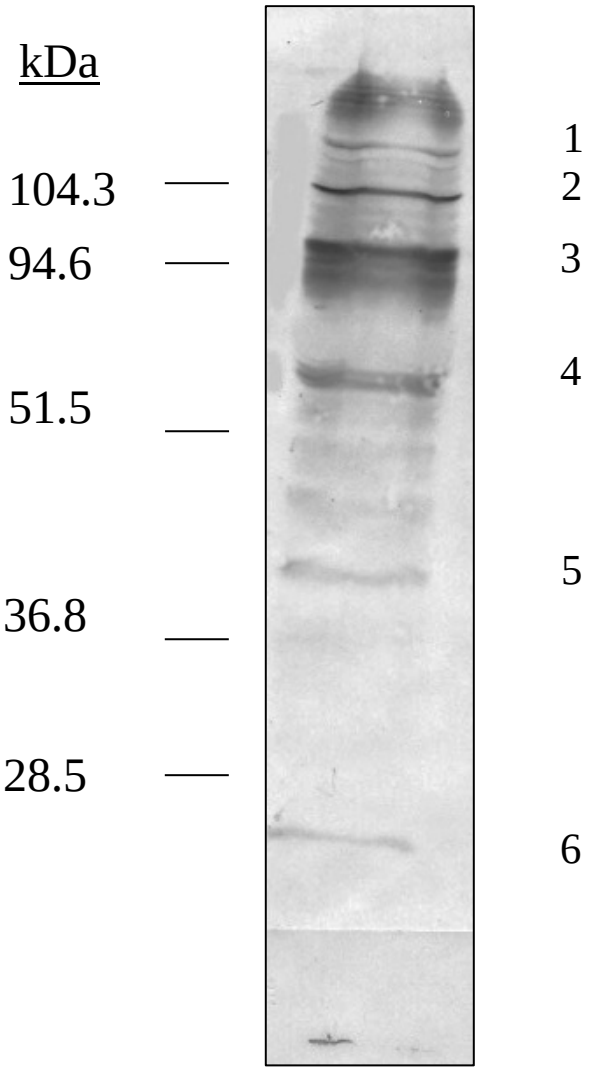

Figure 2.2. Anti-nitrotyrosine immunoblot of cation exchange fraction. *H. pylori* was exposed to 250 µM peroxynitrite, proteins fractionated on a cation exchange column, and 20 µg of resulting fractionation was analyzed by immunoblot for the presence of nitrotyrosine. Numbers along the right correspond to proteins excised from the corresponding Coomassie-stained gel and identifications in Table 1.

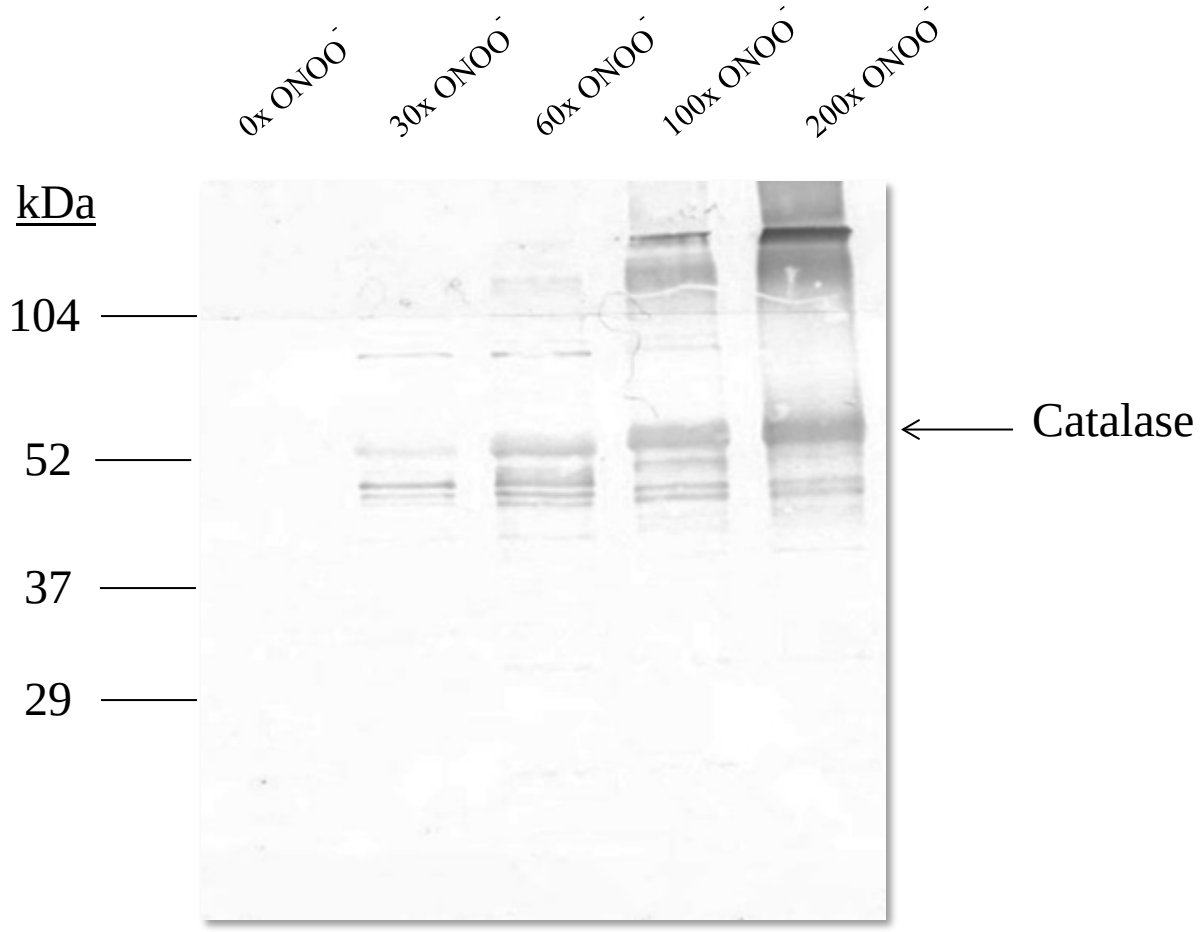

Figure 2.3. Tyrosine nitration of pure catalase. 10 µM of purified *H. pylori* catalase was exposed to increasing concentrations of peroxynitrite for 30 minutes at room temperature. Following dialysis to remove excess peroxynitrite, 5 µg of protein was analyzed by anti-nitrotyrosine immunoblot.

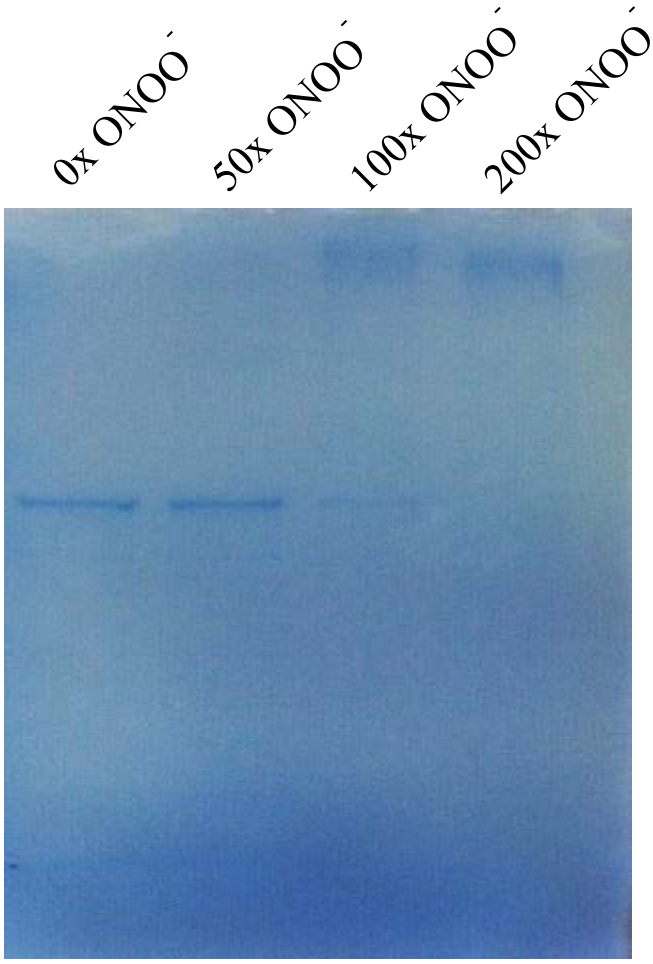

Figure 2.4. SDS-PAGE of peroxynitrite-treated catalase. Pure catalase was exposed to increasing concentrations of peroxynitrite and then analyzed by SDS-PAGE. 5 µg of protein was loaded onto the gel and after running it was stained with Coomassie.

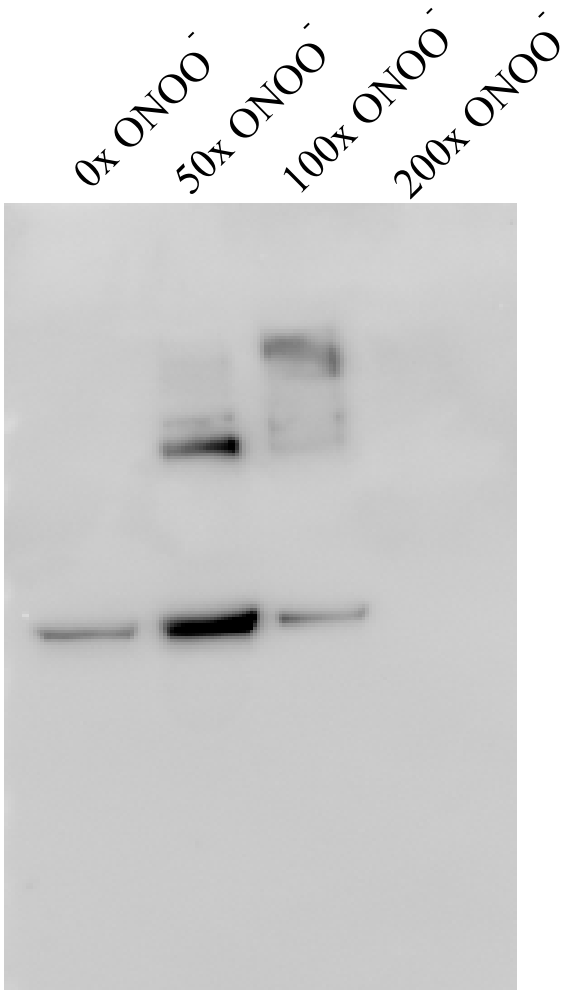

Figure 2.5. Peroxynitrite-treated catalase immunoblot. Pure catalase was exposed to increasing concentrations of peroxynitrite and then analyzed by immunoblot with antibodies directed against catalase.

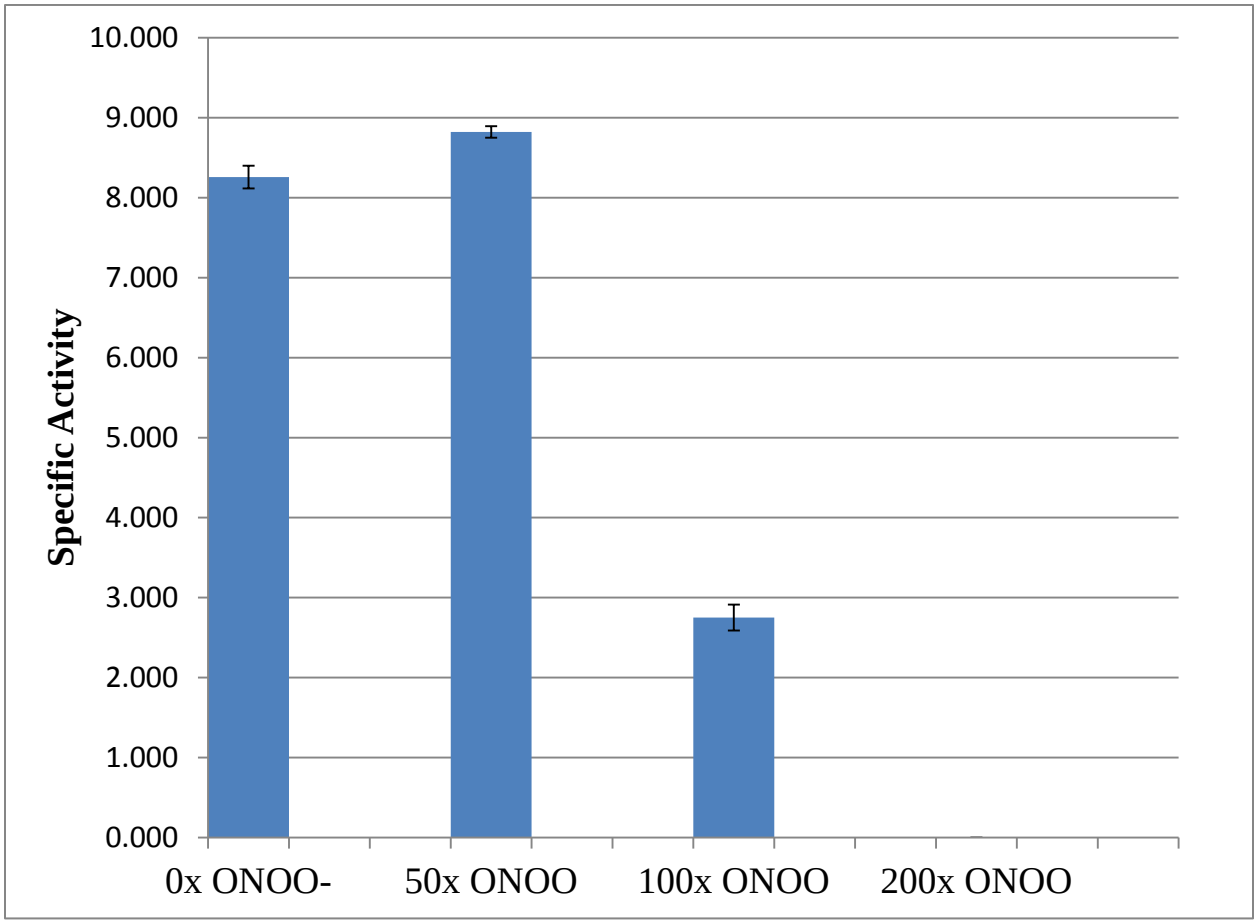

Figure 2.6. Catalase activity following peroxynitrite exposure. Pure catalase was exposed to increasing concentrations of peroxynitrite and its activity was assessed by following the dissipation of H_2O_2 spectrophotometrically.

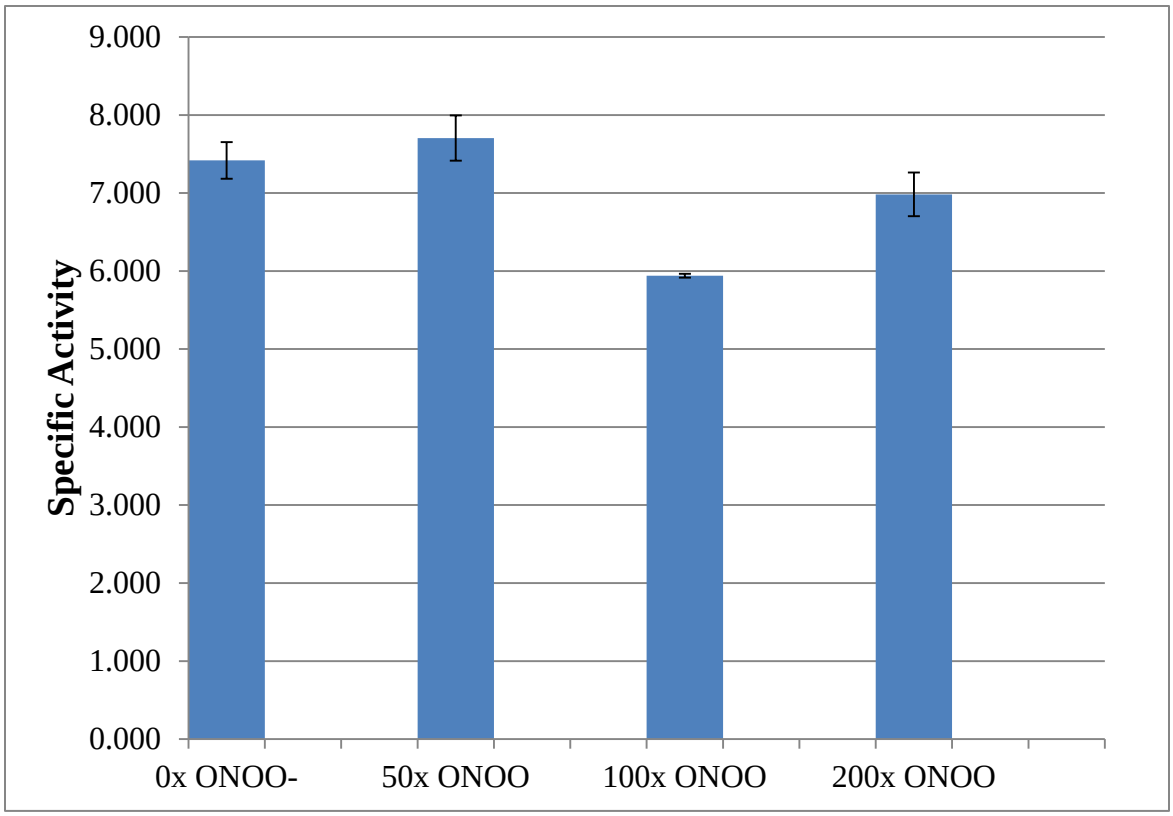

Figure 2.7. Catalase activity following peroxynitrite exposure. Pure catalase was exposed to increasing concentrations of peroxynitrite and its activity was assessed by following the dissipation of H_2O_2 spectrophotometrically.

CHAPTER 3

CHARACTERIZATION OF *HELICOBACTER PYLORI* PROTEIN L-ISOASPARTYL METHYLTRANSFERASE

Introduction

Amino acids in proteins can be modified such that the modification results in significant alterations to protein structure and function. The spontaneous conversion of asparagine and aspartate residues to L-isoaspartate is one such modification. The formation of this residue results from the deamidation and/or isomerization of asparagine or aspartyl residues (6). Isoaspartate is formed through the isomerization of aspartyl and asparagine residue to a succinimide intermediate, which can then spontaneously hydrolyze to a mixture of aspartyl and isoaspartyl residues (6).

Multiple factors can play a role in whether isoaspartate is formed. The primary sequence of a protein can play a major role in whether or not isoaspartate can form. Studies using synthetic peptides have found that the first step in the formation of isoaspartate is the deprotonation of the attacking peptide-bond nitrogen, resulting in the formation of a nuclephilic anion that can attack the side chain carbonyl. The result is the succinimide intermediate, which then hydrolyzes to a mixture of isoaspartyl and aspartyl residues. (3).

When isoaspartate forms in a protein, the charge and structure of the protein are altered. The alteration in structure of the affected protein is due to the introduction of a kink in the polypeptide chain. Multiple examples of human proteins having diminished activity due to isoaspartate formation have been described including human calmodulin (9), human epidermal growth factor (7), and the human antiapoptotic protein Bcl-X_L (4). Since such protein damage occurs, it seems likely that these damaged proteins either have to be degraded or repaired.

In 1965, a methanol-forming enzyme was described through the use of C^{14}-labeled *S*-adenosylmethionine (SAM). This protein was identified as protein L-isoaspartyl methyltransferase (PIMT) (2). In 1984, it was determined that PIMT acts specifically on isoaspartyl residues, which gave rise to the theory of PIMT-induced protein repair (1). The repair mechanism proceeds in three steps:

1) Transfer of a methyl group from SAM to the isoaspartyl residue by PIMT
2) Spontaneous succinimide intermediate formation
3) Spontaneous succinimide hydrolysis to either aspartyl (15-30%) or isoaspartyl (70-85%) residues (10)

In mice, the deletion of PIMT resulted in a number of abnormalities including increased brain size, increased occurrence of epileptic seizures (13), modified insulin receptor signaling factor (5), and premature death (13). In bacteria, the phenotypes associated with deletion of PIMT seem to be less pronounced. Deletion of the gene encoding PIMT in *E. coli* results in phenotypes primarily related to stress response in aging cells. For example, the mutant had increased susceptibility to environmental stresses in stationary phase (12). When young and aging bacterial cells are mixed

together, an interesting phenotype arises. The phenotype known as growth advantage in stationary phase (GASP) occurs when the older cells outcompete the younger cells (14). The GASP phenotype was shown to vary in the *E. coli* mutant strain. Sometimes the mutant would display the GASP phenotype; however, it primarily was unable to display the GASP phenotype (12). Lastly, an *E. coli* PIMT mutant struggles to recover from long-term stationary phase (8).

To date, PIMT has not been studied in *Helicobacter pylori*. According to a genome-wide transposon study, it was suggested that the *pcm* gene encoding PIMT is essential in *H. pylori* (11). Thus far, all our attempts at construction of a mutant have failed suggesting that *pcm* is an essential gene in *H. pylori*. Due to the seeming essentiality of *pcm*, we have sought alternative methods to investigate the importance of PIMT in *H. pylori*.

Materials and Methods

Bacterial strains and growth conditions

H. pylori strain 26695 was used throughout this study. *H. pylori* was cultured on brucella agar (BA) plates supplemented with 10% defibrinated sheep blood. Incubation occurred at 37°C with 4% O_2, 5%, CO_2, balanced with N_2. Liquid cultures were grown in brain-heart infusion media prepared in sealed bottles, supplemented with 10% O_2 and an anaerobic mixture of CO_2, H_2, and N_2. Where necessary, chloramphenicol or kanamycin was added to the media in concentrations of 25 and 30 µg/mL, respectively.

Inactivation of PIMT *in H. pylori*

To delete *pcm*, an overlapping PCR method was used. Genomic DNA from *H. pylori* strain 26695 was isolated and used as template to amplify a 400 base pair region of HP0363 (*pcm*) as well as part of HP0362, the intergenic region, and the beginning of the *cat* cassette, with primers PIMT_Del_1 and PIMT_Del_2. A second PCR amplified a 350 base pair region consisting of the 3' end of *pcm*, the *pcm*/HP0364 intergenic region, a portion of HP0364, and the end of the *cat* cassette using primers PIMT_Del_3 and PIMT_Del_4. The final amplification step combined the first and second PCR products with the 800 base pair *cat* cassette and primer PIMT_Del_1 and PIMT_Del_4 to generate a 1550 base pair final product. The final product was used to transform *H. pylori* by natural transformation. Screening for clones occurred on BA plates supplemented with chloramphenicol (30 µg/mL).

Preparation of soluble and insoluble fractions

H. pylori strain 26695 was grown for 24-48 hours on BA plates and harvested by scraping into PBS, pH 7.0. Lysed cells by sonication and then centrifuged at 6,000 RPM, 4°C and for 10 minutes. The resulting cell-free extract was fractionated by ultracentrifugation at 45,000 RPM, 4°C, and for 2 hours. The supernatant was designated the soluble fraction and the insoluble pellet was suspended in PBS, pH 7.0 as the insoluble fraction. These samples were then used for anti-PIMT immunoblots.

Immunoblotting

Antibodies against PIMT were raised in rabbits using purified his-tagged PIMT (ProSci). Following SDS-PAGE, samples were electrophoretically transferred to a 0.2 µm

nitrocellulose membrane (Bio-Rad). The membrane was blocked by incubating in PBS, pH 7.0 supplemented with 5% dry milk and 0.1% Tween-20. The blocking step was followed by incubation with 1:1000 dilution of anti-PIMT antibody in blocking buffer for 1-4 hours. The membrane was washed three times with PBS, pH 7.0 plus 0.1% Tween-20, and then incubated with goat anti-rabbit secondary antibody conjugated to alkaline phosphatase (1:1000) in blocking buffer for 1 hour. Again, the membrane was washed three times followed by detection of bound antibodies with nitro blue tetrazolium (0.25 mg ml^{-1}) and 5-bromo-4-chloro-3-indolyl phosphate (0.125 mg ml^{-1}) (Sigma).

Construction of merodiploid strain

The plasmid pEMCm-PIMT was constructed by first digesting pEMCm with NdeI and XhoI, which left a *H. pylori ureA* promoter at the 5' end followed by insertion of a similarly digested *pcm* fragment. The *pcm* fragment was generated by removing it from the pET21b expression vector by digestion with NdeI and XhoI. Following successful transformation into *E. coli* Top10, the plasmid was recovered and naturally transformed into *H. pylori* by adding 3 µL of pEMCm-PIMT to cultures of *H. pylori* strain 26695 and incubating for 10 hours. After 10 hours, the culture was transferred to BA plates supplemented with chloramphenicol. Clones were picked after 48 hours and genomic DNA was isolated. Insertion of *pcm* into HP0405 was confirmed by PCR amplification using the following primers: PIMT_Interior_R and 405 Sty1 .

H. pylori oxidative stress exposure

H. pylori strain 26695 was grown for 36 hours on BA plates and harvested by scraping into PBS, pH 7.0 to OD_{600} of 1.1. 2 mL of the suspension was placed into each of two

petri dishes. One dish was incubated at 37°C, 4% O_2, and 5% CO_2, while the other was incubated at 37°C and atmospheric conditions. The samples were incubated for 4 hours, and hen washed twice with PBS. Final pellets were resuspended in 500 µL PBS and lysed by sonication. Following sonication, the samples were centrifuged at 6,000 RPM, 4°C, and for 10 minutes. The resulting cell-free extracts were then used for anti-PIMT immunoblots.

Results

PIMT appears to be essential in H. pylori

Several attempts at constructing a PIMT deletion mutant resulted in failure. These attempts include multiple strains and conditions including incubation at 30°C in an attempt to recover clones. It does not seem surprising that a mutant could not be recovered due to the findings by Sharma et. al.(11) suggesting that PIMT is essential based on their genome-wide transposon study. Since a mutant could not be recovered, alternative methods were employed to study the importance of PIMT to *H. pylori.*

PIMT cellular localization

In order to determine the cellular localization of PIMT, *H. pylori* was fractionated by ultracentrifugation to give a soluble, cytosolic fraction and an insoluble membrane fraction. These fractions, along with the original extract, were then analyzed by immunoblotting against polyclonal PIMT antibodies (Fig. 3.1). The resulting immunoblot showed that all of the cellular PIMT localizes to the cytosolic fraction and none is localized to the membrane. The higher molecular weight bands in the

immunoblot are non-specific bands, which were present in the pre-immune serum of the rabbit.

Time-course expression of PIMT

Due to the role of PIMT in protection against stress during late-stage growth in *E. coli* (12), we sought to determine when, during the life cycle, PIMT is expressed in *H. pylori*. Cultures were grown in liquid broth for 25 hours and samples were collected every 5 hours to measure OD_{600} and to analyze by anti-PIMT immunoblot. Due to growth constraints, one bottle was used for each time point. The growth curve of *H. pylori* is shown in Figure 3.2. PIMT expression was analyzed at each time point and was shown to be constant throughout growth, suggesting that it is constitutively expressed throughout the life cycle (Fig. 3.3).

H. pylori PIMT merodiploid

Since a PIMT mutant could not be recovered, we decided to introduce a second copy of *pcm* into the *H. pylori* chromosome. This was accomplished by taking advantage of a recombinant hot spot in the chromosome at HP0405. *Pcm* was introduced into HP0405 and under control of the *ureA* promoter and confirmed by PCR (data not shown). Due to *pcm* being under control of the *ureA* promoter, PIMT expression was analyzed following growth on BA plates supplemented with 50 μM nickel (Fig. 3.4). Based on the immunoblot, it appeared that nickel did not have an effect on PIMT expression in the merodiploid strain. Additionally, the expression of PIMT in the merodiploid and wild-type appear to be identical even under non-inducing conditions. Two interesting

observations of this immunoblot include that PIMT appears to migrate at a higher molecular weight in the merodiploid, relative the wild-type strain and that the high molecular weight non-specific band have disappeared. It is unclear why this may be. It is possible that additional genetic information was recombined with *pcm* resulting in a larger than anticipated protein. The loss of the non-specific band could be due to an incomplete immunoblot or that the merodiploid strain is not what was originally though and could, in fact, be a contaminant. Further experiments showed that the overall protein expression profile of the merodiploid strain was no different than that of the wild-type strain (Fig. 3.5).

PIMT expression under oxidative stress

To identify whether PIMT is up-expressed under oxidative stress, *H. pylori* was grown on BA plates and either kept at 4% O_2 or exposed to atmospheric conditions for four hours, as described in the Methods section. Extracts from both conditions were analyzed by use of anti-PIMT immunoblot (Fig. 6). Based on the results of the immunoblot, I conclude PIMT is not up-expressed under oxidative stress conditions in *H. pylori*.

Discussion

PIMT was studied to identify its importance to *Helicobacter pylori* physiology. In *E. coli*, PIMT appears to play a role in the stress response of aging cells. Here we sought to first generate a PIMT mutant; however, several attempts failed, suggesting the enzyme might play an essential role in *H. pylori*. In order to further investigate PIMT importance, alternative methods were utilized to better understand the role PIMT plays in

H. pylori. We have shown that PIMT is localized to the cytosol (Fig. 3.1) and is constitutively expressed during 25 hours of growth (Fig. 3.3). Furthermore, a second copy of *pcm* was introduced into the *H. pylori* chromosome; however, based on immunoblots PIMT expression did not appear to increase relative to wild-type even under inducing conditions. The loss of the high molecular weight non-specific band in the merodiploid may suggest that the strain is not what was originally thought. In fact, it may have been a contaminant. Lastly, PIMT expression does not appear to be affected when *H. pylori* is exposed to air.

The analysis of PIMT in *H. pylori* sheds some light on how and where it is expressed. Further studies will need to be done to determine its actual role in the cell and whether or not it truly serves an essential role. Firstly, increasing the amount of time in the time-course expression experiment could provide some insight into whether PIMT is up-regulated in late stationary phase. Additional experiments to identify proteins that interact with PIMT in the cell may provide clues as to its role. It is possible through the construction of a conditional mutant essentiality could be rigorously determined. Lastly, it is possible that PIMT has been misannotated; therefore, the pure protein should be assayed for methyltransferase activity using a protein known to contain isoaspartate.

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Table 3.1 - Strains, primers, and plasmids used in PIMT study	
Strains	**Description**
Top 10	*E. coli* cloning strain
26695	*H. pylori* wild-type strain
26695 405-PIMT	*H. pylori* 26695 with *pcm* inserted into HP0405
Primers	
PIMT_Del_1	AAACCCGTAATAAGTGCTCT
PIMT_Del_2	ATCCACTTTTCAATCTATATCAGATTAAAGCATGATTAAACAC
PIMT_Del_3	CCCAGTTTGTCGCACTGATAAACAAGCCCCTTAAAAATCAT
PIMT_Del_4	TGATCAACGCTTTAAGGAAT
PIMT_Interior_R	TTCTTCAAGCTGATCAAT
405 STY1	GTAACGGGAATTCTTA
Plasmids	
pET21b-PIMT	Overexpression vector with *pcm* inserted between NdeI and XhoI sites
pEMCm	Plasmid for insertion of genes into HP0405
pEMCm-PIMT	pEMCm with *pcm* inserted between NdeI and XhoI sites

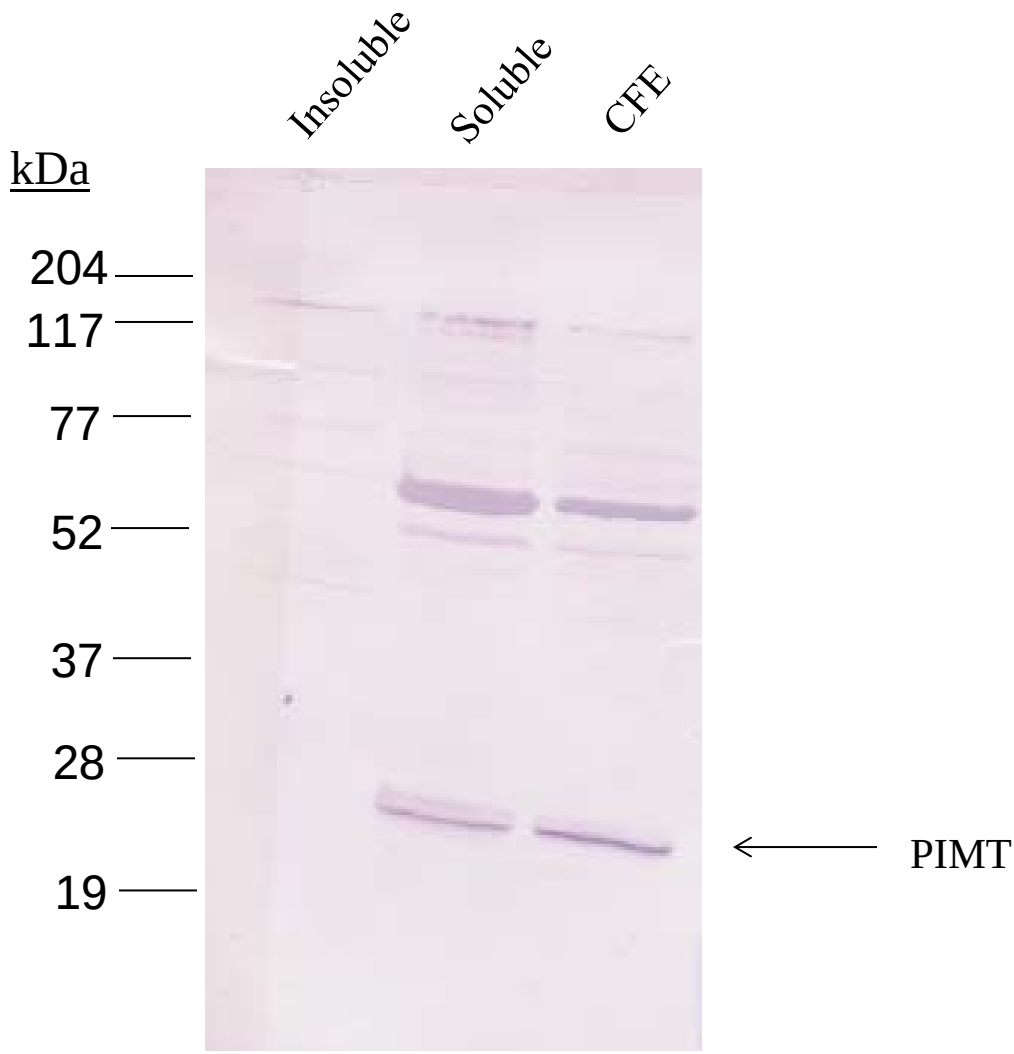

Figure 3.1. Cellular localization of PIMT. An *H. pylori* cell-free extract was fractionated by ultracentrifugation and then 5 µg of the resulting fractions were analyzed by anti-PIMT immunoblot. The high molecular weight bands are non-specific and have existed since the pre-bleed during antibody preparation.

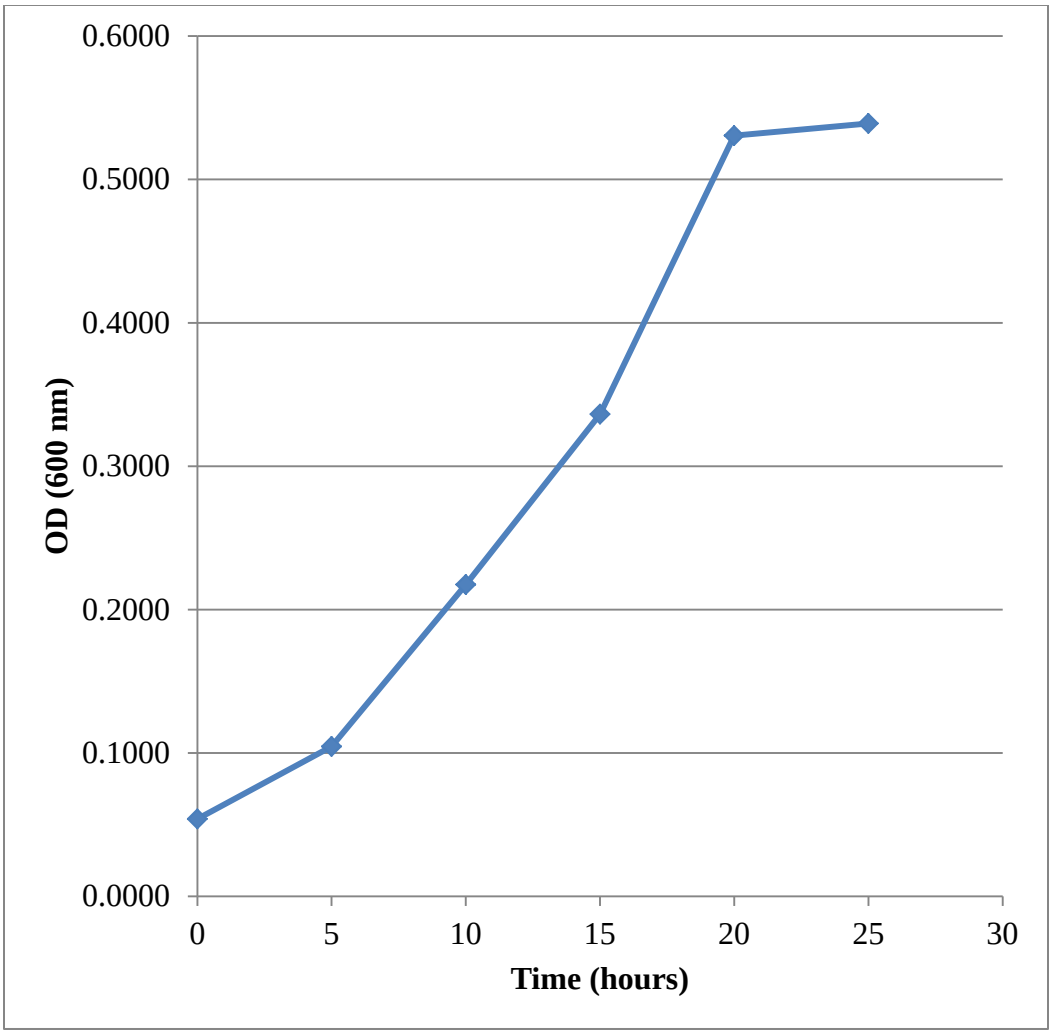

Figure 3.2. *H. pylori* strain 26695 growth curve. *H. pylori* was grown in BHI + 0.04% β-cyclodextran for 25 hours and the A_{600} was read every 5 hours.

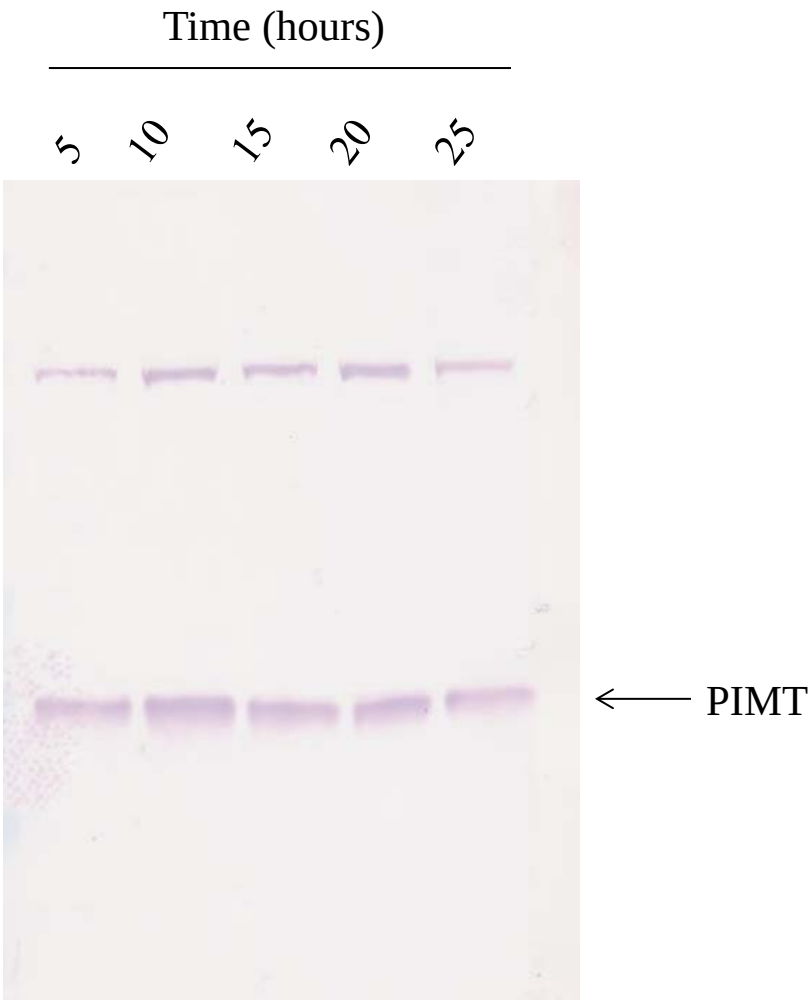

Figure 3.3. PIMT expression over time. *H. pylori* strain 26695 was grown for 25 hours and samples taken every 5 hours. 5 µg of cell-free extract from each time point was analyzed by anti-PIMT immunoblot.

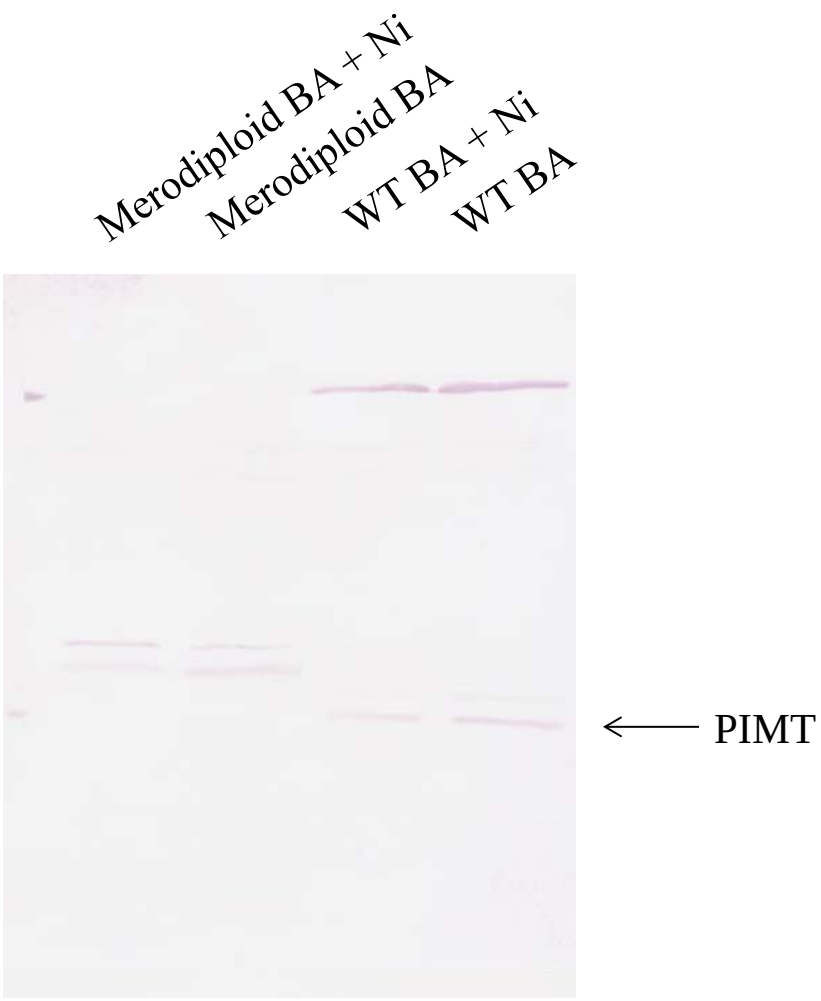

Figure 3.4. PIMT expression in the presence of nickel. *H. pylori* strain 26695 wild-type and PIMT merodiploid were grown on either BA plates or BA plates supplemented with 50 µM nickel. Cell-free extracts were prepared and 5 µg of protein was analyzed by anti-PIMT immunoblot.

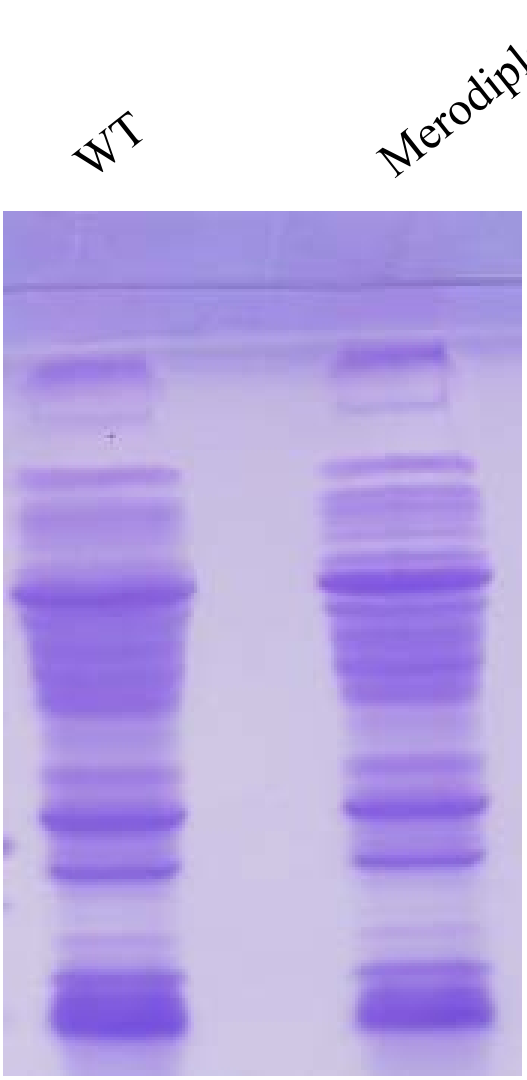

Figure 3.5. Protein expression profile of wild-type and merodiploid. *H.pylori* strain 26695 wild-type and PIMT merodiploid were grown on BA plates at 4% O2 for 48 hours, harvested, and cell-free extract was prepared. The extract was then separated by SDS-PAGE and the gel was stained with Coomassie.

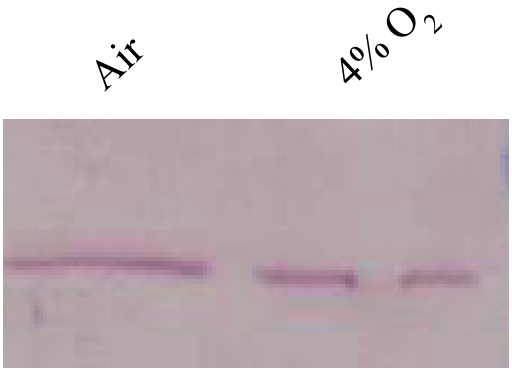

Figure 3.6. PIMT expression under oxidative stress conditions. *H. pylori* strain 26695 was grown for 36 hours on BA plates at 4% O2 and then exposed to atmospheric oxygen conditions at 37°C for 4 hours. Cell-free extracts were prepared and 5 µg of protein was analyzed by anti-PIMT immunoblot.

CHAPTER 4

CONCLUSIONS

Peroxynitrite and nitrotyrosine

Peroxynitrite is a potent oxidizing and nitrating agent produced by macrophages during infection (1). In fact, peroxynitrite formation is increased during *Helicobacter pylori* infection (5). The reactivity of peroxynitrite can result in the formation of 3-nitrotyrosine, which has been shown to be deleterious to the affected protein. For these reasons, we sought to identify *H. pylori* proteins that are targets for tyrosine nitration in the presence of peroxynitrite.

We have shown that *H. pylori* proteins are susceptible to tyrosine nitration and through a proteomics-based approach; we were able to identify several potential targets. One target, catalase, was the only consistent protein identified throughout all three identification experiments. Initial experiments that involved exposing pure catalase to peroxynitrite resulted in increased levels of tyrosine nitration and a propensity for oligomerization. However, decreased catalase activity as a result of peroxynitrite exposure was not consistent between experiments. Several attempts at reproducing the loss of activity met with failure. The true extent of peroxynitrite damage on the *H. pylori* proteome is not clear; however, it appears to be an important mechanism utilized by the host to combat infection with multiple *H. pylori* proteins affected by tyrosine nitration.

PIMT

Isoaspartate formation can render some proteins nonfunctional through alterations in their structure (2-4). In order to combat isoaspartate damage, PIMT facilitates its reversion back to aspartate via multiple rounds of methyl transfer from S-adenosylmethionine to the affected protein. *H. pylori* PIMT has yet to be studied and its role in *H. pylori* physiology is not well understood. According to a previous study, PIMT appears to be essential for survival (6) and our attempts at mutant generation seems to support this notion.

Due to inability to obtain a mutant strain, we sought to investigate the role of PIMT through alternative methods. Through immunoblot analysis we have shown that PIMT is localized to the cytosol. Additionally, it is constitutively expressed throughout 25 hours of growth and is not up-expressed under oxidative stress conditions. A merodiploid strain, constructed through the addition of a second copy of *pcm* into the *H. pylori* chromosome, does not appear to express additional PIMT despite the addition of nickel to drive expression from the introduced *ureA* promoter.

It is interesting that we were unable to recover a mutant deficient in *pcm*. This is a direct contradiction to the mutational studies performed in *E. coli*. If PIMT is truly essential to the survival of *H. pylori* that would seem to imply a much more important role for the enzyme than what was found in *E. coli*.

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