HELICOBACTER PYLORI NICKEL STORAGE PROTEINS: RECOGNITION AND MODULATION OF DIVERSE METABOLIC TARGETS

by

ZACHARY JAMES SAYLOR

(Under the Direction of Robert J. Maier)

ABSTRACT

Helicobacter pylori nickel metabolism and trafficking are necessarily complex; the bacterium inhabits an environment where nickel concentration is in constant flux while simultaneously requiring nickel for survival. *H. pylori* utilizes nickel transport mechanisms, a nickel-specific regulator, two essential nickel-containing enzymes and their associated accessory proteins, and two nickel storage proteins Hpn and Hpnl. Hpn and Hpnl modulate activity of one of the two nickel-containing enzymes, urease, through their interaction with accessory proteins HypAB. It was hypothesized, but unstudied, that the storage proteins contribute to other aspects of nickel metabolism.

I show, through a crosslinking and affinity pulldown approach, that Hpn and Hpnl interact with up to 215 putative partners. Among these interacting partners are proteins known to be involved in nickel management, including UreAB, UreG, and HypB. Interestingly, many of the most strongly enriched interacting proteins have no known connection to nickel homeostasis. Interactions were explored by several approaches, including phenotypic characterization of mutant strains (Δ*hpn*, Δ*hpnl*, or Δ*hpn*Δ*hpnl*) and interaction verification by tryptophan fluorescence. Hydrogenase activity of the

Δ*hpn*Δ*hpnl* strain was severely impacted by nickel deprivation in contrast to this effect on the wild type (WT). Interactions of two enzymes with both nickel storage proteins were confirmed: leucyl aminopeptidase (PepA) and aliphatic amidase (AmiE) interaction with the storage proteins was validated by observing a shift in tryptophan fluorescence upon incubation with either storage protein. The nickel storage proteins had opposite effects on PepA activity; deletion of *hpn* resulted in a three-fold decrease in activity while deletion of *hpnl* resulted in a seven-fold increase relative to WT. Hpn and Hpnl synergistically suppressed AmiE activity in a nickel-dependent manner – AmiE activity in the mutant strains was higher than in their WT counterpart, and this activity was reduced by supplementation with nickel. Both storage proteins were required for full suppression – the addition of pure Hpn or Hpnl to the extract lacking that protein resulted in suppression back to WT levels. Additionally, I show that recombinant amidase can bind the divalent metals zinc, cadmium, manganese, cobalt, and nickel. I show herein that Hpn and Hpnl may play more diverse roles than previously thought.

INDEX WORDS: *Helicobacter*, nickel, Hpn, Hpn-like, hydrogenase, amidase, peptidase

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B.S., Allegheny College, 2011

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2018

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May 2018

DEDICATION

I dedicate this dissertation to my parents, Jim and Pam, who made innumerable sacrifices to foster my desire to learn and grow. I could not have asked for better role models and more loving parents.

ACKNOWLEDGEMENTS

I would like to acknowledge my advisor, Rob Maier, for his support throughout graduate school. He supported not only my primary project, but also gave me the opportunity to explore secondary projects of my own design. He additionally allowed me the opportunity to foster my love of teaching and to gain experience that will undoubtedly contribute to my professional success in the years to come. I would also like to thank my committee members for their thoughtful contributions and suggestions, and in particular I would like to thank Tim Hoover for giving me the opportunity to teach a microbiology course as instructor of record. Thanks are due to Jennifer Walker for her continual support and mentorship of my teaching. I would like to acknowledge current and past members of the Maier lab for their support, guidance, and advice, and especially Stéphane Benoit for his assistance throughout my graduate career. I appreciate the support of my friends and family throughout this journey. I would especially like to thank my parents for their love and support, and my friend Alex Tinker for his encouragement and positive attitude. Finally, I would like to thank my wife Julie for her unceasing love, support, commiseration, and patience, and our dog, Miles, for always greeting me excitedly when I get home.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

The isolation of *Campylobacter pyloridis*, now known as *Helicobacter pylori*, from the stomach of Dr. Barry Marshall upended long-held beliefs about the root cause of stomach ulcers and provided evidence of bacteria living in an environment once thought to be entirely hostile to bacterial life. After observing curved bacilli in the stomach of patients with gastric and peptic ulcers, Drs. Barry Marshall and Robin Warren initiated a self-infection experiment to fulfill Koch's postulates and identify *H. pylori* as the etiologic agent of chronic gastritis and other gastric related diseases (1-5). Within a decade of its isolation, *H. pylori* was identified by the World Health Organization as a Class I human carcinogen. It is now estimated that *H. pylori* infects half of the worldwide population (6). Along with its ubiquity, *H. pylori* has several interesting characteristics, such as natural transformability, unique nitrogen metabolism, acid-resistance, and reliance on nickel. These factors make it an attractive organism to research from both a humanitarian and academic perspective, and consequently *H. pylori* is a heavily studied organism with more than 40,000 medical and scientific articles published since its isolation.

Helicobacter pylori: a model organism for study

Epidemiology and pathogenesis. The high worldwide infection rate for *H. pylori* belies the variations seen in infection rate between different countries (6). *H. pylori* prevalence is highest in developing countries and lower in industrialized nations (7-9), and these differences have been attributed to higher standards of hygiene and increased use of

antibiotics in industrialized nations. *H. pylori* infection is thought to spread through the oral-oral and/or fecal-oral route (10, 11), and most infections occur during childhood (12). In developing countries and socioeconomically disadvantaged communities of industrialized nations, ingestion of fecal matter through contaminated drinking water sources may contribute to increased rates of infection (13). While there is some evidence of *H. pylori* in environments outside the human host (14-17), the primary methods of transmission appear to be through direct person-to-person contact, and close contact with an infected individual is correlated with increased risk of infection (18-21).

H. pylori colonizes the gastric mucosa of the human stomach. In order to colonize this environment, the bacterium must reach the neutral mucosa (pH ~7) through the acidic environment of the stomach lumen (pH 1-2). To achieve this, the bacterium uses the enzyme urease, which produces ammonia and lowers the pH in the cellular microenvironment while traversing the lumen (22-25). Once the bacterium reaches the gastric mucosa, its spiral shape and flagella aid in burrowing into the viscous mucosa (26). Most *H. pylori* cells will remain in the gastric mucosa, however a small percent will become associated with the epithelial cells (27), where the activity of urease will de-gel the mucous and contribute to stomach acid- and ammonia/VacA-mediated irritation of the epithelial cells and eventual ulceration (28).

Despite the high worldwide prevalence of *H. pylori*, only a small portion of infected individuals experience symptoms of infection (29). Roughly 15% of those infected with *H. pylori* exhibit symptoms, and among symptomatic individuals, 10-20% will develop gastric or duodenal ulcers, and 1-2% will develop *H. pylori* associated cancers (30-32), including mucosal associated lymphoid tissue (MALT) lymphoma or non-Hodgkins

lymphoma (12). One of the bacterial proteins strongly associated with cancer development is CagA (33), which is translated to the host cell in part by energy provided by *H. pylori* hydrogen respiration (34). *H. pylori* infection is generally detected by blood or stool antigen tests, urea breath test, urine ELISA, or a biopsy and histological examination/rapid urea combination test (35, 36). Treatment of *H. pylori* infection is typically a "triple therapy" consisting of two antibiotics (usually amoxicillin, tetracycline, metronidazole, or clarithromycin) and either a colloidal bismuth compound or proton pump inhibitor (37, 38) lasting one to two weeks.

Bacteriology, Genetics, and Metabolism. *H. pylori* is a Gram-negative bacterium that is characterized by its distinct spiral shape. It is classified as a curved bacillus, although a viable but unculturable coccoid form has been observed in the mouse host and in culture (39-41). *H. pylori* is a microaerophilic bacterium and contains a diverse array of oxidative stress response mechanisms (42-52). *H. pylori* has one to seven sheathed, polar flagella that contribute to its survival and colonization in the stomach (26, 53).

H. pylori is part of the ε-proteobacteria class, specifically the *Camphylobacterales* order which it shares with genera *Arcobacter*, *Campylobacter*, *Sulfurospirillum*, *Flexispira*, *Sulfuricurvum*, *Sulfromonas*, *Thiovulum*, and *Wolinella*. Over 500 *H. pylori* genomes have been sequenced to date, including some commonly used lab strains such as 26695 (54) and X47 (55). *H. pylori* genomes are diverse, likely due to their natural competence (56). The *H. pylori* genome is relatively small, on the range of 1500 genes across 1.6 Mb, indicating a species highly specialized for its specific environment and reliant on host resources (57, 58).

Nutritional requirements for *H. pylori* are complex (59), at least partially due to the lack of several key metabolic enzymes (60, 61). Of particular interest in *H. pylori* is its nitrogen metabolism, which relies heavily on metabolism of amino acids and urea (62). The bacterium is auxotrophic for several amino acids, including arginine, histidine, isoleucine, leucine, methionine, phenylalanine, and valine, as well as alanine and serine in some strains (63, 64). Amino acids (65-67) or peptides (68, 69) are transported into *H. pylori* where they can serve as carbon and nitrogen sources (62). Additionally, urease is the major ammonia-producing enzyme for *H. pylori*, and plays a substantial role in nitrogen metabolism for the bacterium (62).

Among the proteins that contribute to *H. pylori* amino acid metabolism, the aminopeptidase PepA is of interest for this study. *H. pylori* leucyl aminopeptidase (PepA) is an allosteric metalloenzyme which catalyzes the removal of N-terminal amino residues from peptides or proteins (70), with high affinity for leucine and arginine (71). While the extent to which PepA plays a role in *H. pylori* metabolism is not fully characterized, PepA is involved in the assimilation of exogenously supplied peptides, presumably by predigesting extracellular peptides and proteins (69), although the exact mechanism of secretion or the relative abundance of PepA inside versus outside the cell are unknown. The smaller peptides are then transported into *H. pylori* by several peptide uptake systems (68). We can predict, based on data from other model organisms, that PepA may additionally be involved in endogenous protein turnover and maturation (72).

PepA can accept a wide range of divalent metal ions for activity, and the enzyme is up-regulated under acidic conditions (73). Zinc-containing PepA was proposed to be the native form, although many other divalent metals, including cobalt, magnesium, manganese, and nickel, appear to be incorporated into the enzyme; incorporation of these

metals increased PepA activity above both apo-enzyme or zinc-loaded enzyme (72). The reliance of *H. pylori* on amino acids for carbon and nitrogen makes defining the role of PepA intriguing, especially considering the possible connections to use of the other major nitrogen source, urease. Additionally, both peptidase and urease are activated by nickel, and would be subject to the complex nickel-regulation and nickel-homeostasis mechanisms of *H. pylori*.

Although urea and amino acids are the major source of nitrogen for *H. pylori*, another source of nitrogen plays an important role. Aliphatic amidase (AmiE) is a *H. pylori* protein that hydrolyzes short-chain aliphatic amides, namely propionamide, acetamide, and acrylamide, into ammonia and their corresponding organic acid (74, 75). This reaction provides the bacterium with nitrogen (ammonia) and carbon (organic acid) from a source independent of the two major nitrogen sources, urea and amino acids.

Interestingly, *H. pylori* amidase activity is tied to urease levels; amidase expression and activity were both increased in a *ureB* deletion mutant (75). In the absence of urease, amidase plays a crucial role in *H. pylori* ammonia generation and nitrogen metabolism, supplying the bacterium with ammonia when the otherwise major source is unavailable (75). Commensurate with its ties to urease, amidase is regulated through many of the same networks as the urease machinery. Like urease (76, 77), amidase is up-regulated by nickel, although this regulation is achieved by different means. The nickel-specific regulator NikR represses the ferric uptake regulator Fur, and Fur in turn directly represses *amiE* (78). In addition to nickel and iron regulation of amidase, the acid-response regulator ArsRS also directly regulates the expression *amiE* (79). Amidase is thus regulated by both nickel and acidity, two important regulators for urease (76-78, 80), supporting a role for this protein

in nickel-mediated ammonia production. Indeed, amidase activity has been directly tied to nickel concentration through the NikR-Fur repressor cascade (78).

Metal homeostasis. A variety of metals are important for *H. pylori* colonization and survival. Metals serve as required cofactors for many enzymes and important cellular functions, but overaccumulation can lead to cell damage through either oxidative stress or mis-incorporation of metals into protein complexes (81, 82). Among the metals that are important for *H. pylori* metabolism are nickel and iron. Iron uptake is accomplished by FeoB (Fe^{2+}) and FrpB1, FrpB2, ExbB, ExbD, TonB, CueE, and FecDE (83-89), which coordinate to transport both ferrous and ferric iron into the cell. Iron storage is accomplished by the putative bacterioferritin NapA (90-92) and the ferritin Pfr (93, 94). The ferric uptake regulator, Fur, is involved in regulation of a variety of iron-related proteins, such as the transport and storage proteins (95-100), as well as several targets unrelated to iron homeostasis, such as *ureA* or *amiE* (78). The relative importance of other metals, including copper, zinc, manganese, cobalt, and cadmium for *H. pylori* is quite limited compared to iron and nickel. Although we do know that many proteins and cellular processes require these metals (101), less is known about how they are imported, regulated, stored, and managed.

Nickel metabolism and homeostasis

Nickel is an important cofactor in many *H. pylori* enzymes, including its essential roles in virulence and metabolic proteins. Despite its fundamental role in *H. pylori* physiology, nickel overaccumulation can pose a serious problem for the bacterium (81). To balance the need for nickel with its possible toxic effects, *H. pylori* has evolved a complex array of proteins for nickel transport, regulation, and storage (102, 103) to ensure

efficient delivery of the metal to its destination in the final nickel sinks urease and hydrogenase.

Nickel transport. *H. pylori* nickel import is accomplished by several proteins and complexes. NixA is an inner-membrane nickel permease with extremely high specificity (K_T=11.3 nM) (104, 105). The membrane topology of NixA has been thoroughly investigated, and several residues important for nickel import, urease activity, and nickel binding were uncovered (106-108). Disruption of NixA by allelic exchange mutagenesis (105) resulted in decreased nickel transport and urease activity, although neither was entirely abolished, suggesting redundant mechanisms for transport. More recently, an additional nickel import apparatus was described. NiuD is an ABC-type inner membrane nickel import protein, and together with NixA, appears to constitute all of the inner membrane nickel import proteins in *H. pylori*; deletion of both NixA and NiuD resulted in complete abolition of nickel import by the bacterium (109). Between these proteins, NixA appears to complete the bulk of nickel transport – nickel import for Δ*nixA* was reduced to 17% of the parent level, while nickel import for Δ*niuD* was reduced to 52% of the parent (109). Along with NiuD, NiuB1 and NiuB2 were also found to contribute to nickel import by delivering nickel to NiuD in the periplasmic space (109). In addition to the inner membrane nickel import proteins, *H. pylori* also contains protein complexes to aid in nickel import through the outer membrane. FrpB4 is capable of binding nickel or nickel-complexes and transporting them through the outer membrane, and is energized by the TonB/ExbB/ExbD machinery to do so (110). In addition to FrpB4, FecA3 has also been proposed as a candidate for nickel uptake across the outer membrane due to the observation that transcription of *fecA3* is controlled by the nickel-specific regulator NikR

(111). Spanning the inner and outer membranes, the CznABC system is responsible for the export of nickel, zinc, and cadmium, and contributes to urease activity by regulating these divalent metals (112). Many of the nickel import and export proteins were discovered primarily because they were regulated by nickel, often mediated by the nickel-specific regulator of *H. pylori*, NikR.

Nickel regulation. Nickel regulation in *H. pylori* is mediated by the pleiotropic autoregulator NikR (77). NikR forms a dimer of dimers wherein each unit binds one nickel ion, and dimer formation is critical to NikR function (113). NikR regulates a variety of targets related to nickel in *H. pylori*, including *ureAB* (urease), *hydABC* (hydrogenase), *nixA*, *hpn*, *hpnl*, *frpB4*, *fecA3*, *exbD*, *tonB*, and *hspA* (77, 114); targets can be activated or repressed based on the nickel loading state of NikR, allowing this protein to function as a nickel sensor for regulation of its target proteins (114). In addition to its nickel-related regulatory targets, NikR also regulates the transcription of many other genes. Among the most interesting of these regulatory targets is *fur*, which encodes the iron uptake regulator. NikR regulation of *fur* has implications for many other downstream Fur targets, including nitrogen metabolism (through urease and amidase) (78, 115). In addition to its metal-related targets, NikR also regulates a wide variety of proteins with no known connection to metals, including targets in stress response, motility and chemotaxis, and outer membrane and hypothetical proteins of unknown function (77). Recently, some of these targets have been assigned putative roles as outer membrane ion transporters, toxin/antitoxin systems, host interaction proteins, and metabolic proteins, indicating that the NikR regulon may be even broader than previously appreciated (115).

Urease. The urease enzyme is comprised of the structural subunits UreA and UreB, and together these proteins account for up to 10% of the total cellular protein in *H. pylori* (23). Urease serves as the primary nickel sink for *H. pylori* (116); each dodecameric complex contains 12 nickel ions (117). Urease catalyzes the conversion of urea into ammonia and bicarbonate, which provides a nitrogen source for the bacterium (62), and crucially, raises the pH in the immediate microenvironment of the bacterium, allowing it to buffer against the low pH of the stomach (118, 119). Urease is essential for colonization of the host (25), at least in large part due to its role in pH management. Urease maturation is complex, and requires the coordination of several maturation accessory proteins (102). Urease transcriptional modulation is primarily under the control of NikR (114), although the nickel-regulated protein Mua also controls *ureAB* transcription through an independent, undetermined mechanism (120).

UreI is responsible for transport of urea into the cytoplasm, where the nitrogen-rich substrate is then available for urease to use. The mechanism of UreI transport has been proposed to be a H^+ activated gate, indicating that it may also serve to limit access to urea when the production of ammonia would detrimentally alter pH (121, 122). The four remaining dedicated urease accessory proteins, UreEFGH, have all been shown to be necessary for urease activity (123), and a role for each protein has, to some extent, been observed. UreH and UreF have been shown to interact with each other (124), and this interaction is necessary to form the scaffolding on which UreG can bind with UreF and form a pre-activation complex (125). UreF has additionally been shown to bind nickel, and it is possible that it aids in transferring nickel to the active site of urease for maturation (126). UreG is a GTPase (127) involved in urease maturation. UreG is known to interact

with both UreE (128) and UreF (129), and these interactions contribute to urease activation (129). UreE is a nickel-binding urease accessory protein, and the nickel-binding properties of UreE are essential for urease maturation to occur (130). Recently, several new models for urease maturation have been proposed to account for the newfound interactions between the accessory proteins and the ability of UreG to bind nickel (129).

In addition to the dedicated urease maturation proteins, two hydrogenase accessory proteins also serve a urease maturation role (131). HypA and HypB both play a role in urease maturation through their described roles as a nickel-binding protein and a GTPase, respectively, and these proteins have been shown to interact with other urease accessory proteins. A HypA-UreE interaction was essential for urease maturation (132); it was later discovered that UreE could be recognized by both HypA and UreG, and that the two proteins compete for UreE binding (128). The Hyp-Ure interactions contributing to urease activity are likely contributing to nickel loading of UreE early in the maturation process, since the nickel centers of urease and hydrogenase differ greatly (102). The current model for urease maturation is: HypAB contributes to nickel-loading of UreE, wherein HypA nickel delivery is likely energized by HypB GTP hydrolysis (102). UreFH interact to form a homodimer of heterodimers (two UreFH dimers), which is able to recruit a UreG dimer (129). The UreFGH complex then either interacts with either apo-urease or with UreE – interaction with apo-urease would facilitate UreE nickel delivery to UreG, whereas interaction with UreE would deliver nickel to UreG prior to its interaction with the apo-urease. GTP hydrolysis then facilitates the delivery of nickel from UreG to the apo-urease, generating holo-urease (129).

Hydrogenase. *H. pylori* contains a single nickel-iron, hydrogen-oxidizing type hydrogenase comprised of HydABC (103). Hydrogenase also serves as a nickel sink for *H. pylori*, although in a lesser capacity than urease since it is less abundant (102). Hydrogenase activity impacts gastric colonization; mutants deficient in hydrogenase activity were poorer colonizers than their WT counterparts, and the hydrogen content measured in the host (mouse) stomach suggests that hydrogen may serve as a major energy source for the bacterium (133). Nickel is required for hydrogenase activity, and nickel insertion into hydrogenase is complex in nature. Hydrogen oxidation by the bacterium also promotes carbon dioxide incorporation via up expression of acetyl CoA carboxylase (134).

H. pylori contains eight proteins responsible for hydrogenase maturation: HypABCDEF and HydDE (102, 103, 131, 135). Hydrogenase maturation is less well-characterized than urease maturation, although the roles of some proteins are known. Among the hydrogenase structural subunits, HypA and HypB are nickel binding proteins, and the latter is a GTPase; they are expected to play a role in delivering nickel to the active site of apo-hydrogenase (131). Based on information gleaned from *Escherichia coli* hydrogenase maturation, it is expected that the other Hyp accessory proteins are involved in synthesis [HypEF, (136)] and delivery [HypCD, (137)] of a cyanide ligand to the iron metal center of the hydrogenase (102). HydDE are known to be essential from *H. pylori* work (135), although their exact functions are only inferred as a protease (HydD) or protein involved in assembly (HydE) (103). Two additional proteins are thought to play a role in hydrogenase maturation; the chaperone protein SlyD has been shown to interact with HypB (138, 139), and deletion of HspA, a GroES-like chaperone protein, resulted in drastically reduced hydrogenase activity that could be partially recovered with nickel

supplementation (140). HspA is also considered to be a nickel storage protein in *H. pylori*, since deletion of this gene resulted in lowered intracellular nickel pools (140).

Nickel storage. Nickel storage is crucial to *H. pylori* survival – nickel concentrations in the stomach are low (low nM range), and a constant nickel supply is far from guaranteed (141, 142), so nickel must be retained when it is available. *H. pylori* contains two primary nickel storage proteins, Hpn (***H****elicobacter* ***p****ylori* protein with affinity for **n**ickel (143)) and Hpnl (Hpn-like), which aid in sequestration of nickel when it is in limiting supply (144).

The most striking features of Hpn and Hpnl are their small size (60 and 75 amino acids, respectively) and their primary structure; 47% and 25%, respectively, of the total amino acid residues for each protein are histidines, and Hpnl also contains 42% glutamine residues (143-146). While a role for the histidine residues in nickel binding was apparent, the role for the glutamine residues in Hpnl was less obvious. Additional characterization of Hpnl determined that the glutamine residues were involved in metal complex stability (144, 145, 147, 148). Exemplifying the importance of nickel storage, up to 2% of the total cellular protein may be composed of Hpn (143), with Hpnl likely making up a similar portion of the total cellular protein.

Owing largely to the relatively high percentage of their amino acids being made up of histidine, Hpn and Hpnl have moderate nickel affinity. Between the two, Hpn has higher capacity and lower affinity (Hpn reversibly binds five nickel ions with a K_d of 7.1 μM (145)), while Hpnl has lower capacity and higher affinity (Hpnl reversibly binds two nickel ions with a K_d of 3.8 μM (146)). Whether this difference in capacity and affinity has physiological implications has not been thoroughly explored. NikR up regulates both *hpn*

and *hpnl* in the presence of nickel (114), possibly to allow accumulation of nickel inside the cell without toxic effects, or as a preventative mechanism to combat nickel toxicity; both Hpn and Hpnl have been shown to combat nickel toxicity within the cell (144). In addition to nickel, Hpn and Hpnl have been shown to contribute to detoxification of cobalt and cadmium (144), and Hpn also contributes to bismuth detoxification (149). Recent evidence has suggested more nuanced roles for Hpn and Hpnl, suggesting that Hpn was the major contributor to intracellular nickel storage and detoxification, but that both proteins played a role in urease modulation (150).

As nickel storage proteins, it is unsurprising that Hpn and Hpnl play a role in modulation of urease; urease is the single biggest nickel sink in the cell, and its (nickel mediated) activity is essential for gastric colonization and survival (116, 118, 119). Previous work in the Maier lab has shown that deletion of Hpn or Hpnl increased nickel pools in the urease fraction, indicating a sequestration role for the proteins (144). Similarly, urease activity was increased in these mutants when nickel was not limiting. Addition of a nickel-specific chelator, dimethylglyoxime (DMG), severely reduced the urease activity of the mutant strains, but not the WT, suggesting that urease in the mutants was dependent on exogenous nickel for activation and providing further evidence for a nickel-storage role for Hpn and Hpnl (144). Hpn and Hpnl may supply nickel for urease under acidic conditions; Hpn and Hpnl have been observed to release nickel when exposed to a low pH environment (145, 146).

A more recent study has also supported a role for Hpn and Hpnl as modulators of urease activity and has expanded upon our knowledge of how this modulation occurs. Vinella *et al.* reported that Δ*hpn* and Δ*hpn*Δ*hpnl* mutants lacked the nickel-induced urease

activity phenotype of the WT, and the Δ*hpnl* mutant had exaggerated basal urease activity compared to the WT (150). These results do not agree with previously published data (144), although the parent strain, growth media, mutation type (disruption versus deletion), and nickel content were all different, so the cause of this discrepancy is unclear. In the Vinella report, Hpn and Hpnl interaction were also studied in relation to urease activity. Hpn and Hpnl were both found to interact with each other as well as with the urease and hydrogenase accessory proteins HypA and HypB, and Hpn was also found to interact with urease subunit UreA (150). The authors posit that these interactions facilitate nickel delivery to the maturation machinery for urease, rather than directly delivering nickel to the apo-enzyme. This hypothesis fits well with previous theories that HypA and HypB interaction with urease maturation machinery occurs early in the maturation process, possibly to facilitate nickel delivery to UreE (102). The Hpn and Hpnl modulation of urease also plays a role in host colonization. An *H. pylori* Δ*hpn*Δ*hpnl* mutant was deficient in host (mouse) colonization when nickel was limiting (compared to nickel supplementation), however the WT had similar colonization levels regardless of whether nickel was limiting or supplied (151). More recently, three strains of *H. pylori* (SS1, X47, and B128) were used to determine the role of the storage proteins in mouse colonization, and the Δ*hpn*Δ*hpnl* mutant in all three backgrounds was deficient in colonizing the stomach (150).

Scope of study

Hpn and Hpnl play important roles in urease modulation, nickel homeostasis, and animal colonization, and it is reasonable to suspect that they may play additional roles. A nickel-sensing and regulatory role for Hpn and Hpnl would seem to be a logical extension

of their roles, considering their ability to bind and store nickel. Indeed, Hpn and Hpnl have already been shown to interact with urease maturation proteins to modulate urease activity (150). Nickel is a required cofactor for several essential *H. pylori* enzymes, and recent analyses indicate that bacterial metalloproteomes may be much larger than anticipated (152). Based on these data, it is reasonable to suspect that Hpn and Hpnl may play roles in nickel sensing and regulation for a wider range of target proteins, possibly through direct protein-protein interactions.

Based on this hypothesis, I utilized an affinity pulldown approach to study the protein-protein interactions of Hpn and Hpnl. Previous affinity pulldown approaches to determine *H. pylori* interacting proteins have proven successful and have broadened our understanding of *H. pylori* protein-protein interactions (153, 154). Using this approach, I identified over 200 putative interacting targets for Hpn and Hpnl. Among those targets were many proteins known to be influenced by nickel, although many of the most highly enriched interacting proteins had no known ties to the metal. I additionally investigated three interactions further. An Hpn-HypB interaction prompted an examination of the storage proteins' impact on hydrogenase; hydrogenase is more susceptible to inactivation via nickel deprivation when Hpn and Hpnl are absent. Hpn and Hpnl interactions with two proteins involved in nitrogen metabolism, AmiE and PepA, suggest that these storage proteins may play roles in modulating nitrogen metabolism and in peptide salvage. Overall, my results suggest that Hpn and Hpnl play more diverse roles in *H. pylori* than previously thought.

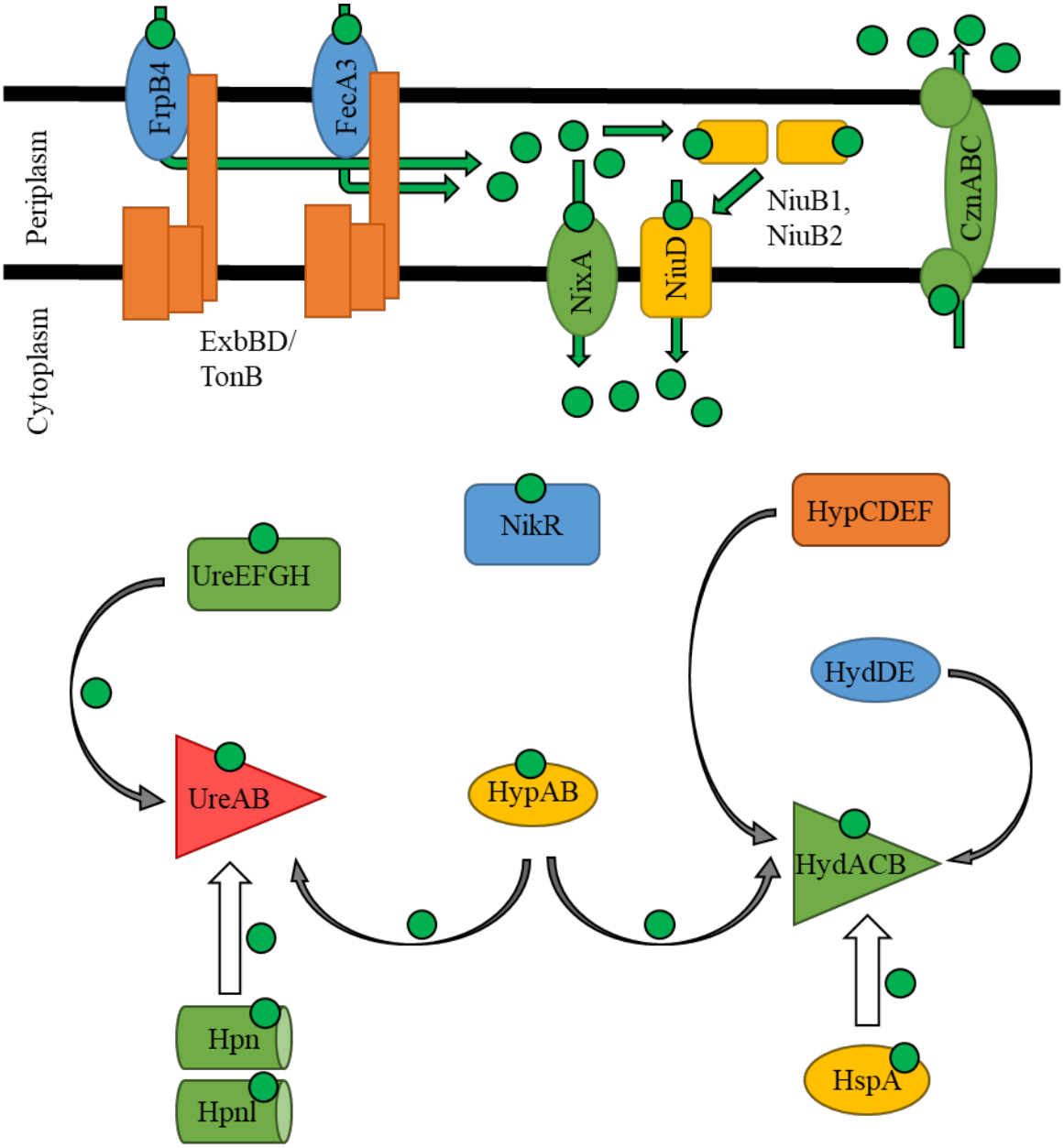

Figure 1.1: Simplified model of H. pylori nickel metabolism. Nickel import (FrpB4, FecA3, NixA, NiuD, NiuB1, NiuB2, and ExbBD/TonB), nickel export (CznABC), nickel regulation (NikR), nickel sinks and associated accessory proteins (UreAB, UreEFGH, HydABC, HydDE, HypAB, HypCDEF, HspA), and nickel storage proteins (Hpn and Hpnl) are represented. Grey arrows indicate maturation proteins for the nickel sinks, and

white arrows indicate interactions with either the sinks or their accessory proteins that are known to contribute to enzyme modulation. The green circle indicates nickel; when overlaid on a protein this indicates that the protein binds nickel (for multi-protein units, at least one protein in that unit binds nickel) and when next to an arrow it indicates interactions or maturation processes that contribute to nickel loading. Nickel import and export are indicated with green arrows.

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CHAPTER 2

HELICOBACTER PYLORI NICKEL STORAGE PROTEINS: RECOGNITION AND MODULATION OF DIVERSE METABOLIC TARGETS[1]

[1]Saylor, Z.J. and R.J. Maier, submitted to *Microbiology* 03/22/2018

Abstract

Nickel metabolism and trafficking in *Helicobacter pylori* is complex, perhaps more so than in any other microorganism. Along with nickel enzymes and their associated nickel-binding maturation machinery, *H. pylori* contains nickel storage proteins, Hpn and Hpnl. Through a combined crosslinking and enrichment approach, we show that Hpn/Hpnl interact with a wide array of partners; up to 215 proteins were captured, including known nickel-enzyme maturation proteins, and other proteins outside known *H. pylori* nickel-associated proteins. Phenotypic characterization of mutant strains (Δ*hpn*, Δ*hpnl*, or Δ*hpn*Δ*hpnl*) was used to explore interactions. Nickel deprivation affected hydrogenase activity of the Δ*hpn*Δ*hpnl* strain much more severely than WT, whereas activities of the single mutants were similar to WT. Tryptophan fluorescence confirmed that both storage proteins interacted with two enzymes known to be influenced by divalent metals. Leucyl aminopeptidase activity was impacted in opposite ways in the mutant strains; Δ*hpn* had a three-fold decrease while Δ*hpnl* had a seven-fold increase, compared to the parent. Similar mutant strain analysis supported Hpn and Hpnl acting synergistically to suppress aliphatic amidase activity in a nickel-dependent manner. Recombinant amidase could bind a variety of divalent metals. Amidase activity was greatest in the mutant strains and it was inhibited by exogenous nickel. Addition of pure storage protein to extracts from the mutants restored suppression of amidase activity only for the mutant strain lacking that protein; both storage proteins are needed for amidase suppression. These results suggest that Hpn and Hpnl play more diverse roles than previously thought.

Introduction

H. pylori is a Gram-negative, microaerophilic, and neutralophilic bacterium colonizing the gastric mucosa of nearly half of the human population worldwide. Chronic infection with *H. pylori* has been linked to increased risk of gastric or duodenal ulceration, chronic gastritis, and gastric cancer (1, 2), and the World Health Organization has classified this infection as a group 1 human carcinogen. Nickel is critical to *H. pylori* colonization due to its role as a required subcomponent of several virulence factors; however, over-accumulation of the metal can contribute to reactive oxygen species generation or interference with normal metal-protein interactions (3). Consequently, *H. pylori* contains extensive nickel homeostasis and management systems (4, 5). *H. pylori* has several nickel import systems (NixA (6), NiuBDE (7), FecA/FrpB (8)), nickel export (CznABC (9)), and nickel-specific regulator (NikR (10)) proteins, along with proteins that are responsible for maturation of the nickel-enzymes urease and hydrogenase (UreEFGH, HypAB, and Mua for urease (11-15) and HypABCDEF (14, 16) for hydrogenase). Among the battery of proteins involved in maturation are two nickel storage proteins, Hpn (***H****elicobacter* ***p****ylori* protein with affinity for **n**ickel (17)) and Hpnl (Hpn-like).

H. pylori proteins Hpn and Hpnl have a substantial number of histidine residues, roughly 47% and 25% of the total amino acid residues, respectively. Hpnl is additionally comprised of roughly 42% glutamine residues; the histidine residues are directly involved in nickel binding, and the glutamine residues are thought to improve the stability of the metal complexes (18-21). Hpn constitutes up to 2% of the total cellular protein (17), and gross observations indicate Hpnl is also made at about these levels (see later text). Hpn and Hpnl bind nickel with moderate affinity; Hpn reversibly binds five nickel ions with a

K_d of 7.1 μM (18) and Hpnl reversibly binds two nickel ions with a K_d of 3.8 μM (22). Both *hpn* and *hpnl* are up-regulated by NikR in the presence of nickel (23), and the proteins contribute to nickel detoxification and storage by sequestering nickel ions within the cell (19). Deletion of Hpn and Hpnl individually or together decrease *H. pylori* tolerance to nickel, cobalt, and cadmium (19), and absence of Hpn has also been correlated to bismuth susceptibility (24).

Deletion of Hpn or Hpnl significantly increases *H. pylori* urease-associated nickel pools (19) and urease activity when nickel availability is not limited (19). Nickel release from Hpn and Hpnl is observed under acidic conditions, suggesting that this protein may supply nickel to the cell when urease activity is needed to maintain pH balance (18, 22). Furthermore, addition of a nickel chelator has little effect on the urease activity of wild type (WT), but significantly decreases the urease activity of Δ*hpn*, Δ*hpnl*, and Δ*hpn*Δ*hpnl* deletion strains (19). Urease activity is more dependent on exogenous nickel in the mutant backgrounds than in the WT, supporting a role for these proteins in nickel storage (19). In addition to their apparent roles in nickel homeostasis and pH balance, Hpn and Hpnl are also important for virulence (25, 26); mutants lacking both storage proteins were poorer colonizers than their wild-type counterparts when the hosts (mice) were subjected to nickel deficient conditions.

H. pylori hydrogenase also serves as a nickel sink for the bacterium (4), and is an enzyme that impacts gastric colonization (27). Like urease, hydrogenase undergoes a complex maturation process to insert the nickel ion required for H_2 oxidation activity. Of the eight accessory proteins involved in hydrogenase maturation (14, 28), two – HypA and HypB – are involved in maturation of both urease and hydrogenase (14). Hpn and Hpnl

have previously been shown to interact with several of the Ni-enzyme maturation proteins; HypA, HypB, and UreA (26).

Given their importance for colonization and virulence, it seems reasonable that Hpn and Hpnl may be involved not only in the storage of nickel, but also in a nickel-sensing or regulatory role for appropriate targets. These roles would require multiple protein-protein interactions. We thus sought to identify additional proteins that interact with the storage proteins via an affinity pulldown approach: such an approach has previously revealed new and unexpected information on *H. pylori* protein-protein interactions (29, 30). Our pulldown results suggest that Hpn and Hpnl interact with a wide array of proteins. A storage protein-AmiE (amidase) interaction suggests a role for Hpn and Hpnl as regulators of ammonia producing amide hydrolysis activity. Our results suggest that Hpn and Hpnl interact with AmiE to suppress its activity, and that both of these proteins are necessary for such suppression to occur. A storage protein-PepA (aminopeptidase) interaction supports these storage proteins involvements in the pathogen's peptide salvage metabolism.

Materials and methods

Bacterial strains and growth conditions. *H. pylori* X47 (31) was used as parental strain in this study. Cells were routinely grown on Brucella agar (BA) with 10% sheep's blood, supplemented with bacitracin (100 μg/mL), vancomycin (10μg/mL) and amphotericin B (10μg/mL), as indicated. BA was supplemented with nickel ($NiCl_2$) or the nickel-specific chelator dimethylglyoxime (DMG, Fluka Analytical) as indicated. For perspective, human blood contains roughly 5 nM nickel (32). *H. pylori* X47 Δ*hpn*, Δ*hpnl*, and Δ*hpn*Δ*hpnl* constructed previously (25) were grown for two days on BA and then passaged twice for two days each on BA with the indicated supplementation (nickel,

DMG), unless otherwise indicated. *Escherichia coli* DH5α (Thermo Fisher Scientific, for genetic manipulation) or BL21RIL (Stratagene, for overexpression) was grown on Luria-Bertani broth or agar, supplemented with 100 μg/mL ampicillin and 30 μg/mL chloramphenicol as needed.

Preparation of cell-free extracts. *H. pylori* cells grown on BA plates were harvested and suspended in the appropriate ice-cold buffers and subjected to sonication on a Heat Systems Ultrasonics W380 sonicator using the microtip attachment at output 3 and continuous cycle for 30 seconds three times, placing on ice between sonications. Sonicated samples were then centrifuged at 10,000 X g for 10 minutes at room temperature, supernatants (cell-free extracts) were removed and stored at 4°C for further use. Phenylmethane sulfonyl fluoride (PMSF, Sigma) was added at a concentration of 1 mM to preserve extracts during storage.

Purification of Hpn and Hpnl. Lysates of *H. pylori* WT, Δ*hpn*, Δ*hpnl*, or Δ*hpn*Δ*hpnl* were suspended in Ni-NTA wash buffer (20 mM sodium phosphate buffer, pH 7.4 with 100 mM imidazole and 500 mM NaCl) and stored on ice. The Ni-NTA column was prepared according to the manufacturer's instructions (Qiagen). Samples for purification were then loaded onto the column and collected as flow-through via gravity filtration. The flow-through was re-run through the column five times and collected. The column was then washed with 10 column volumes of Ni-NTA wash buffer. Ni-NTA elution buffer (20mM sodium phosphate buffer, pH 7.4 with 500 mM imidazole and 500 mM NaCl) was added to the column in three separate one column volume steps, and each step was collected separately. Protein purity in the eluates was determined by visual observation of a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

with a gradient 4-20% gel (NuSep) with coomassie staining, and the relative amount of Hpn and Hpnl produced were also visualized on a gel at this time.

Crosslinking and enrichment for protein-protein interactions. Two crosslinking and enrichment procedures were used in this study. In the first, either purified Hpn or Hpnl (10 μg mL^{-1}) was crosslinked with cell-free extracts (1.5 mg mL^{-1}) from Δ*hpn*Δ*hpnl* by adding 1 mM dimethyl suberimidate (DMS,Thermo Fisher) and incubating at 4°C overnight in phosphate buffered saline (PBS) at pH 7.0. Crosslinking reactions were stopped with the addition of 50 mM Tris-HCl, and stored at 4°C. Crosslinking reactions were diluted 1:1 in Ni-NTA wash buffer and purified by Ni-NTA column chromatography as described above for purification of Hpn and Hpnl. Enriched Hpn and Hpnl interactions were briefly (<10 min) run on a 4-20% gradient SDS-PAGE gel, and the resulting band containing all enriched protein complexes was excised and prepared for LC-MS/MS analysis by suspension in 50 mM ammonium bicarbonate, in 50% methanol. The second system used individual Δ*hpn* and Δ*hpnl* mutant strains, as well as the Δ*hpn*Δ*hpnl* mutant, to generate cell-free extracts lacking in one or both of the storage proteins. The extracts were then treated in the same manner as the first system, including crosslinking, enrichment and analysis.

LC-MS/MS and analysis. Gel bands from crosslinking reactions were sliced into small pieces, and then rinsed with 50 % acetonitrile/20 mM ammonium bicarbonate, pH 7.5, twice. The gel pieces were dehydrated by adding 100 % acetonitrile and dried out on a heat block at 60°C. Trypsin solution (0.01 μg μL^{-1} in 20 mM ammonium bicarbonate) was added until fully absorbed and samples were placed in an incubator at 37°C overnight.

The tryptic peptides were extracted from gel pieces by incubating with 50 % acetonitrile/0.1 % formic acid twice, and extracts were dried down using a SpeedVac.

Mass spectrometry analyses were performed on a Thermo-Fisher LTQ Orbitrap Elite Mass Spectrometer coupled with a Proxeon Easy NanoLC system (Waltham, MA) located at Proteomics and Mass Spectrometry Facility, University of Georgia. The enzymatic peptides were loaded into a reversed-phase column (self-packed column/emitter with 200 Å 5 μM Bruker MagicAQ C18 resin), then directly eluted into the mass spectrometer. Briefly, the two-buffer gradient elution (0.1% formic acid as buffer A and 99.9% acetonitrile with 0.1% formic acid as buffer B) starts with 5% B, holds at 5%B for 2 minutes, then increases to 25% B in 60 minutes, to 40% B in 10 minutes, and to 95% B in 10 minutes.

The data-dependent acquisition (DDA) method was used to acquire MS data. A survey MS scan was acquired first, and then the top 5 ions in the MS scan were selected for following CID and HCD MS/MS analysis. Both MS and MS/MS scans were acquired by Orbitrap at the resolutions of 120,000 and 30,000, respectively. Data were acquired using Xcalibur software (version 2.2, Thermo Fisher Scientific). Proteins identification and modification characterization were performed using Thermo Proteome Discoverer (version 1.4) with Mascot (Matrix Science) and Uniprot database. The spectra of possible modified peptides were inspected further to verify the accuracy of the assignments. The semi-quantitative analyses were achieved using a label-free quantification workflow within Proteome Discoverer, which a Precursor Ion Areas Detector node calculates the average of the top three highest peptide areas in the Extracted Ion Chromatograms (mass precision, 2 ppm).

Normalized fold change of interactions was determined through the following formulas: $\frac{\#PSMs(s)}{Total\ \#PSMs(s)} * Total\ \#PSMs(c) = NPA(s)$ and $\frac{NPA(s)}{NPA(c)} = NFC$, where #PSMs is the number of peptide spectral matches derived from LC-MS/MS for either the sample (s) or the control (c), NPA is the protein abundance for a given protein in either the sample (s) or control (c) normalized to the average abundance of all proteins in both the sample and control, and NFC is the fold change for a given protein, normalized to the abundance of that protein in the control.

Hydrogenase activity. Hydrogen uptake activities were determined amperometrically on whole cells with O_2 as the final electron acceptor as described previously (33).

Expression and purification of recombinant AmiE and PepA. AmiE and PepA were expressed and purified as T7-tagged protein fusions. Briefly, *amiE* and *pepA* genes were PCR amplified using the appropriate primers (Table 2.1) with genomic DNA from WT *H. pylori* as the template. Each PCR product was digested with *Bam*HI and *Hin*dIII, and ligated into similarly digested pET21b vector, yielding pET-T7-pepA and pET-T7-amiE, respectively. *E. coli* BL21RIL cells containing either pET-T7-pepA or pET-T7-amiE were grown in LB for 4 h and induced overnight with 1mM IPTG at either 25°C (for AmiE) or 37°C (for PepA). Cells were broken by sonication (same protocol as above) and proteins were purified from cell-free extracts by using T7-immunoaffinity purification according to manufacturer's instructions (Novagen). Protein purity was determined by SDS-PAGE and coomassie staining. Protein concentration was determined using the BCA kit (Thermo Fisher).

Tryptophan fluorescence. Prior to tryptophan fluorescence spectroscopy, imidazole was gradually removed from Ni-NTA-purified Hpn and Hpnl by stepwise dialysis. After the last dialysis against PBS buffer (pH 7.0), each protein was adjusted to a concentration of 0.15 uM in PBS pH 7.0. Purified AmiE and PepA were used "as purified". Crosslinker (1 mM DMS) was added to the protein samples and incubation was overnight at 4°C. Tryptophan fluorescence was recorded (in triplicate) on a BioTek SynergyMx spectrophotometer with excitation at 295 nm and emission at 315-415 nm. Samples were normalized against their respective (buffer-only) controls and plotted as relative fluorescence against wavelength.

Peptidase activity. Peptidase activity was measured as described previously (34), with some minor modifications. Briefly, bacterial extracts prepared as described above in PBS pH 7.0. Total protein of the extracts was quantified using (BCA) kit, and equal amounts of total protein were incubated with 0.2 mM L-Leucine-7-amido-4-methylcoumarin hydrochloride (Leu-AMC) Sigma). Fluorescence was measured with a BioTek Synergy 2 spectrophotometer with excitation at 360 nm and emission at 460 nm after a 60-minute incubation at 37°C. Peptidase activity was expressed as units of fluorescence per 100 µg total protein.

Amidase activity. Amidase activity was measured as previously described (35), with modifications. Briefly, bacterial extracts were prepared as described above in PBS pH 7.0. Cells were grown with 1 mM DMG to achieve the nickel deprivation condition and the extracts were supplemented with nickel prior to measuring amidase activity unless specified in the relevant figures and results. Total protein concentration of the extracts was measured using the BCA kit, and 50 µL of sample was added to 200 µL of PBS pH 7.0

with 100 mM acrylamide. The reaction mixture was incubated at 37°C for an appropriate amount of time to measure ammonia generation, and then 400 μL of phenol-nitroprusside (36) was added, timing the addition to ensure equal incubation with the amide substrate. Alkaline hypochlorite (36) was added to the reaction mixture (400 μL), and the reaction mixture was incubated at 37°C for 30 minutes. Absorption was read using a BioTek ELx800 microplate reader at 630nm. Ammonia generation was calculated as μmol of ammonia min^{-1} mg total $protein^{-1}$.

AmiE metals analysis. AmiE metals content was determined by equilibrium dialysis against a variety of metals followed by ICP-MS. Briefly, AmiE was dialyzed as described previously (15) against 1 μM calcium, cadmium, cobalt, copper, manganese, magnesium, molybdenum, selenium, zinc, and tungsten, 0.1 μM mercury, and 5 μM nickel along with 50 mM ultrapure NaCl pH 8.25 (all reagents purchased from Sigma). Metal content of the dialysis sample and buffer was observed by ICP-MS and analyzed as described previously (15).

All solutions were prepared with analytical reagent-grade chemicals and ultra-pure (18MΩ) water. Trace-metal grade HNO_3 (67% v/v) was purchased from Fisher Scientific (Thermo Fisher Scientific). Standard stock solutions were purchased from SPEX CertiPrep (New Jersey, USA). Multi-element and individual standard solutions (SPEX CertiPrep) were used to prepare a tuning solution containing: Co, In, Li, Tl, Ce, Ba, and U. Quality control standards and internal standards were purchased from Inorganic Ventures (Virginia, USA).

ICP-MS measurements were performed at Plasma Chemistry Lab, Center for Applied Isotope Studies on a Thermo Scientific X Series II instrument (Thermo Fisher

Scientific, USA) equipped with hexapole Collision Cell Technology. The sample solutions were pumped by peristaltic pump using a Cetac ASX 520 auto-sampler (Cetac, Nebraska, USA). The internal standard, In, was added in-line using a Trident Internal Standard Kit (Glass Expansion, Massachusetts, USA). Sample introduction into the plasma was performed using a MicroMist EzyFit nebulizer (Glass Expansion, Massachusetts, USA), which reduces oxide formation, has a high total dissolved solids tolerance, and has reduced sample uptake rates. The cyclonic spray chamber was maintained at 3 °C to further minimize oxide formation, which would interfere with ^{56}Fe, ^{51}V, ^{52}Cr, and ^{64}Zn analysis. Ion lens voltages, nebulizer flow, and stage positioning were optimized daily using tuning solution to maximize ion signal and stability while minimizing oxide levels (CeO^+/Ce^+) and doubly-charged ions (Ba^{2+}/Ba^+). A multi-elemental analysis was performed in standard mode for all elements excluding ^{51}V, ^{52}Cr, ^{56}Fe, ^{59}Co, ^{60}Ni, ^{75}As, which were analyzed by kinetic energy discrimination (KED) mode with H_2 in a He balance. A fully quantitative approach was employed with external calibration standards. Calibration check standards were analyzed following initial calibration, at the end of the sample run, and, at most, after every 12 samples. Quality control check standards were accepted as passing if the measured concentration of each element was found to be within ±10% of the certified concentration.

Results and Discussion

Hpn and Hpnl interact with a wide range of protein partners

While researchers have elucidated some information on the structure and function of Hpn and Hpnl (4, 17-20, 22, 24-26), currently the breadth of their biological roles are not well defined. We thus sought to expand our knowledge of the Hpn and Hpnl

interactome by identifying transient interactions in a more native state, *e.g.* using *H. pylori* crude extracts. From our results, the interactome for both Hpn and Hpnl appear to be surprisingly broad, albeit these are putative interactions. Both Hpn and Hpnl appear to transiently but intimately recognize proteins involved in many disparate cellular functions (Table S2.1), and the most strongly enriched proteins for transient binding of both Hpn and Hpnl did not appear to have any obvious correlation to nickel. Previously-implicated interactions include Hpn with UreA (urease structural subunit) and both Hpn and Hpnl with HypA and HypB (hydrogenase and urease maturation proteins) (26). Our results support the previously identified Hpn-HypB interaction and extend the previous findings to include additional urease-related proteins; indeed, our results suggest that both Hpn and Hpnl may recognize UreA and UreB (Table 2.2). Furthermore, Hpn and Hpnl appear to interact with the maturation protein UreG (Table 2.2), and Hpn also appears to interact with UreF (Table S2.1). While our results support an interaction between Hpn and HypB, there was no evidence for interaction between Hpnl and HypB or either storage protein with HypA (Table 2.2). Overall, the results largely support the findings that Hpn and Hpnl contribute to urease activity modulation.

Specific discrepancies between our results and those of Vinella *et al.* (26), such as the interaction between the storage proteins and HypA (not found in our study) or the interaction of the storage proteins with UreB (observed in our study, and not described in the previous report) are to be expected; the previous study used a very different approach, namely an *in vivo* bacterial two-hybrid system. One could argue our system is more direct – we probed for interaction in a native *H. pylori* background, and did not rely on external tags or markers. Also, our use of a crosslinking reagent would be expected to capture

transient interactions that would not be apparent with a bacterial two-hybrid system (e.g. due to tags inhibiting interactions). On the other hand, one could also reasonably expect our system to produce some false negative results; for example, interactions that prevent Hpn or Hpnl binding to the affinity column would likely remain undetected. The unexpectedly large number of putative interactions we observe means each putative interaction should be verified with other approaches. Comparing the merits of each of the two storage protein interactome approaches is not the goal here, rather we hoped to identify additional interactions such that storage protein-peptide interactions that impact *H. pylori* physiology are revealed.

Urease maturation in *H. pylori* has been well-studied, and it is clear that urease maturation does not occur (or weakly occurs) in the absence of the maturation chaperone accessory proteins, including for instance UreG, UreE, HypA, and HypB (14, 37, 38). Hpn and Hpnl interaction with accessory proteins likely serves a nickel recruitment role from the nickel storage reservoirs to the nickel mobilizing accessory protein machinery. This model fits well with the model proposed previously (26). Interaction of Hpn with HypB similarly indicates a potential role for Hpn in hydrogenase maturation, and this is likely related to nickel mobilization toward the H_2 utilizing enzyme.

Hpn and Hpnl impact hydrogenase activity

Our data support a role for Hpn in hydrogenase maturation as previously hypothesized (26) – HypB was nearly 3-fold more abundant in the Ni-NTA enrichment when Hpn was present (Table 2.2). An Hpn-HypB interaction would have implications for maturation of hydrogenase. We thus assayed hydrogenase activity in nickel deplete and replete conditions compared to standard conditions (BA) in the WT and mutant strain

backgrounds (Fig. 2.1). Under standard growth conditions, WT and Δ*hpn*Δ*hpnl* hydrogenase activities were similar, and with supplementary nickel added to the growth medium, the activities for both strains increased more than three- to four-fold over the standard conditions.

A previous report from our lab indicated that the storage proteins affect urease activity but have no impact on hydrogenase activity (19), however the mutant strains used were generated differently (insertion previously versus deletion in this study), and the culture conditions used in that study to achieve nickel deprivation prior to performing hydrogenase assays were not rigorous. Herein the nickel-specific chelator dimethylglyoxime (DMG) was added at a concentration of 1 mM (and present throughout growth) to generate a nickel-deprived condition. As expected, both WT and the Δ*hpn*Δ*hpnl* mutant hydrogenase activities were reduced in these conditions. However, the reduction in hydrogenase activity for the WT was mild, while the activity reduction for Δ*hpn*Δ*hpnl* was marked (13-fold reduction compared to the BA condition) and represents a significant deviation from WT activity. These results show that the lack of nickel storage proteins can significantly impact hydrogenase activity. Most likely, the storage proteins influence the overall availability of nickel, so the nickel deprivation condition imparts a severe nickel challenge to the Δ*hpn*Δ*hpnl* strain. We attempted to assign the strong influence of the storage proteins on hydrogenase activity to either Hpn or Hpnl by studying the single mutant strains, and performing the same experiment as in Figure 2.1. However, the Δ*hpn* and Δ*hpnl* strains had activities similar to the wild type (data not shown). It seems the storage proteins may play redundant roles in hydrogenase maturation; alternatively, the phenotypes may not be strong enough to conclude they play individual roles.

Hpn and Hpnl interacting partners are not limited to known nickel sinks

Among the remaining putative interactions (Table 2.2), we chose to explore two interactions that are unrelated to the known nickel sinks urease and hydrogenase. These are the aliphatic amidase (AmiE) and the leucyl aminopeptidase (PepA). Neither of these proteins have previously been shown to interact with Hpn or Hpnl; first the putative interactions needed validation with pure components. We used tryptophan fluorescence to examine the putative interaction of the nickel storage proteins individually with AmiE and PepA, taking advantage of the presence of tryptophan in both AmiE and PepA and the concomitant lack of tryptophan in the storage proteins. As expected, neither Hpn nor Hpnl had measurable fluorescence, while both AmiE and PepA as pure proteins had observable fluorescence profiles (Fig. 2.2). Upon addition of either purified Hpn or purified Hpnl, the fluorescence profile of AmiE and PepA shifted markedly (Fig. 2.2). The observed shift (Fig. 2.2) suggests that the storage proteins have either directly obscured tryptophan(s) within the peptidase and amidase, or that they have caused conformational changes in the target proteins that altered their fluorescence profiles (39). Bovine serum albumin contains three tryptophans, and its fluorescence was not affected by either storage protein (see Figure 2.2 legend).

Hpn and Hpnl control leucyl aminopeptidase activity

H. pylori leucyl aminopeptidase (PepA) is an allosteric enzyme which catalyzes the removal of N-terminal amino residues from peptides or proteins (40). While the extent to which PepA plays a role in *H. pylori* metabolism is unknown, it is predicted to be involved in maturation or turnover of endogenous proteins (41) and it has been shown to be involved in the assimilation of exogenously supplied peptides in *H. pylori* (42). PepA is a

metalloprotein that can accept a range of divalent metal ions for activity and the enzyme is up-regulated under acidic conditions (43). *H. pylori* PepA can utilize many divalent metals in the active site, and nickel-containing PepA was more active than its zinc-containing (native) counterpart (41).

When *H. pylori* strains (WT, Δ*hpn*, Δ*hpnl*, Δ*hpn*Δ*hpnl*) were grown in the presence of 10 μM nickel, a large difference in peptidase activity among the strains was observed. Δ*hpnl* had roughly six-fold higher peptidase activity than the parent, and peptidase activity in Δ*hpn* was nearly three-fold lower than the activity in the WT (Fig. 2.3). These results suggest that Hpn and Hpnl interact with PepA to alter its activity in opposite ways. Given the increased activity of Δ*hpnl* via growth on nickel-containing media (Fig. 2.3) and the effect of nickel on PepA activity compared to its native cofactor (41), along with the putative Hpnl-PepA interaction, our results suggest that Hpnl plays a role in suppressing PepA activity. On the other hand, Hpn seems to play an opposite role, since PepA activity of Δ*hpn* extracts was lower than that of the WT (Fig. 2.3). This suggests a role for Hpn in activation of PepA. It is possible that the interaction of Hpn and Hpnl with PepA is not contributing directly to PepA nickel loading/unloading, but rather is facilitating the proteins recognition of other modulators (*e.g.* within the extract).

Hpn and Hpnl synergistically suppress aliphatic amidase activity

The aliphatic amidase (AmiE) of *H. pylori* hydrolyzes short-chain aliphatic amides into ammonia and their corresponding organic acid, providing the bacterium with carbon and nitrogen (44). AmiE activity in *H. pylori* appears to be confined to three substrates: propionamide, acrylamide, and acetamide (35, 44). Amidase activity in *H. pylori* is also connected to urease levels; deletion of *ureB* resulted in a four-fold increase in amidase

activity and a commensurate increase in *amiE* expression (35). Amidase plays an important role in *H. pylori* nitrogen metabolism and acid resistance, especially in the absence of urease (35). Regulation of amidase occurs through an acid- and metal-responsive repressor cascade – *amiE* is directly repressed by the iron-response regulator Fur, which is in turn repressed by nickel-specific regulator NikR (45), and *amiE* is also directly regulated by the acid-response regulator ArsRS (46). The connection between nickel and amidase has been well established; amidase activity of *H. pylori* extracts was elevated when nickel was supplied in the growth media, and this increase in activity was attributed to NikR-mediated regulation (45). Due to the Hpn-AmiE and Hpnl-AmiE interactions (Table 2.2, Fig. 2.2) and the role of nickel in AmiE expression, studies on the storage protein mutants seemed to be a logical next step.

Consistent with previous studies (45), the specific activity of amidase from WT cells was greater from cells grown with nickel than without (compare Fig. 4a and 4b, bars at extreme left in each panel). For pre-formed amidase (extracts), the activity was inhibited by nickel supplementation (Fig. 2.4). When cells were grown under nickel deprivation, the parent had almost no activity, but all three mutant strains had 20 to 25-fold higher activities than the WT (Fig. 2.4a). This suggests that the storage proteins play an amidase suppression role, but whether this is at the cell expression level or an effect on activity *per se* (*e.g.* due to storage protein binding to AmiE already present in the extract) was not known. The latter possibility was addressed by supplementing extracts with pure storage proteins (Fig. 2.5).

Like in Fig. 2.4(a), the WT had little amidase activity (Fig. 2.5a), and the three mutant strains had activity that was nickel-inhibited (Fig. 2.5b-d). Incubating the Δ*hpn* or

Δ*hpnl* extracts with either pure Hpnl or Hpn respectively (*i.e.* the same protein contained in the extract), resulted in no or a slight reduction in amidase activity compared to the positive control (Figs. 2.5b, 2.5c). Interestingly, supplementation of each mutant with the specific protein lacking in the extract (Δ*hpn* extract with pure Hpn and Δ*hpnl* extract with pure Hpnl) resulted in suppression of the amidase activity; both Δ*hpn* and Δ*hpnl* amidase activities were at least six-fold lower than the positive controls (see Figs. 2.5b, 2.5c). Consistent with the single mutant strain results, supplementation of Δ*hpn*Δ*hpnl* with pure Hpn and Hpnl together resulted in suppression of amidase activity. The suppression was nearly complete (e.g. similar to WT activities, compare Figs. 2.5(a) with 2.5(d), bars at far right). Both WT and Δ*hpn*Δ*hpnl* extracts were also supplemented with Ni-NTA purified proteins from Δ*hpn*Δ*hpnl* strains extract; this ensured that no Ni-NTA contaminants were affecting amidase activity (Fig. 2.5a, d, purification control). Supplementation of either WT or the double mutant with this control did not alter amidase activity of those samples relative to the positive control (Figs 2.5a, 2.5d). Suppression of amidase activity when cells have abundant intracellular (storage protein loaded) nickel would be expected to be of benefit to *H. pylori* as the complementary ammonia producing enzyme (*e.g.* urease) is known to be made in very large amounts when cells are provided with nickel (23, 47).

While there was no previous indication that amidase contains nickel or other metals, it is possible that the *H. pylori* AmiE represents an unusual member of the aliphatic amidases. Metal-binding proteins may comprise a much larger fraction of the total proteome than previously appreciated (48); for example of 343 metal peaks in *Pyrococcus furiosus* chromatography fractions, 158 did not match any predicted metalloprotein. Similar analysis of *E. coli and Sulfolobus solfataricus* yielded numerous unexpected metal-

associating peaks (48). Along the same lines, there is some precedent for *H. pylori* proteins whose homologues in other bacteria are nickel-free, being adapted to incorporate nickel; for instance *H. pylori* protein HspA is a GroES homologue which has a unique C-terminal nickel binding domain involved in nickel sequestration and hydrogenase maturation (49). ICP-MS metal analysis of AmiE indicates that the (recombinant) protein is capable of binding, or associating with, zinc, cadmium, manganese, cobalt, and nickel (molar ratios of 4, 3, 3, 2, and 1 atom per molecule of protein, respectively). Hpn (17) and Hpnl (20, 22) bind all of these metals, with the exception of manganese for Hpnl. Further analysis will be necessary to determine the effect of these metals on amidase activity, especially in the presence of (metal-free and metal-loaded) storage proteins.

Amidase suppression in extracts is only achieved when both Hpn and Hpnl are present and supplementation of one storage protein in excess is not sufficient to suppress activity (Figs. 2.5a-d). Based on these observations, we hypothesize that Hpn and Hpnl play synergistic roles for amidase suppression. As Hpn and Hpnl interact with AmiE (Table 2.2, Fig. 2.2), direct protein-protein interaction may be responsible for this effect. Hpn and Hpnl have been previously shown to interact with each other (26). Knowing this, and that both storage proteins are necessary to suppress amidase activity, it is reasonable to suggest that an Hpn-Hpnl interaction is important to amidase suppression.

Nickel homeostasis and its regulatory affects is complicated in whole cells, perhaps involving more components in *H. pylori* than in any other organism (4, 5, 50-52). It is acknowledged that the nickel loading/unloading of Hpn/Hpnl and other factors that control their interactions with and modulations of amidase require additional experimentation. For the suppression of activity *per se*, one possibility is that nickel facilitates the interaction of

Hpn and Hpnl with AmiE, or that only metal-loaded storage proteins are capable of interacting with amidase. There are precedents for bacterial metallochaperones affecting activity and protein-protein interactions of another "downstream" protein upon metal transfer from the chaperone partner (53, 54). It is also possible that nickel directly interacts with amidase to inactivate the protein, and that Hpn and Hpnl facilitate this interaction; nickel is capable of partially suppressing amidase activity, even in the absence of both Hpn and Hpnl.

Concluding remarks

H. pylori nickel trafficking and metabolism is complex, and the bacterium contains specialized nickel storage proteins to facilitate these mechanisms. While Hpn and Hpnl have previously been given roles as nickel storage and detoxification proteins (19), and more recently as mediators of urease activity (26), we show that these proteins play more diverse roles. Our data indicate that Hpn and Hpnl interact with proteins across disparate cellular functions, albeit only two interactions were verified. Still the physiological studies on storage protein mutants support that the storage proteins play roles in the maturation of nickel-enzymes, peptide salvage, and nitrogen acquisition. Hpnl has a stronger affinity for nickel than Hpn (K_d of 3.8 μM and 7.1 μM, respectively (18, 22)). This raises the possibility that Hpnl could contain nickel in a nickel-environment at which Hpn cannot, or Hpnl could perhaps sequester nickel from interacting partners when Hpn cannot. Furthermore, our results suggest a previously undescribed regulatory role for Hpn and Hpnl interactions with two target proteins, PepA and AmiE. We trust that these findings add to our understanding of these nickel storage proteins that are unique to the pathogenic bacterium that colonizes a highly inhospitable environment for bacterial survival.

Acknowledgements

Funding was provided by the University of Georgia Foundation. We acknowledge the contributions by the University of Georgia Proteomics and Mass Spectrometry (PAMS) core facility, which completed the crosslinking mass spectrometry data for this study. We thank Stéphane Benoit for providing the mutant strains used in this study, and for his assistance with both experimental techniques and manuscript editing.

Table 2.1: Primers used in this study. Restriction sites are indicated in bold and *H. pylori* derived sequences are indicated in italics. Primers are listed in the 5' to 3' direction.

PepA	HP0570-1	GATC**GGATCC**G*TTAAAAATCAAATTAGAAAAAACC*
	HP0570-2	CGAC**AAGCTT**GCCTACAGACAAGCCCATTTCA
AmiE	HP0294-1	GATC**GGATCC**G*AGACATGGAGATATTAGTAGC*
	HP0294-2	CGAC**AAGCTT**CCCCTCCTCCTTTTGCCCTTTA

Table 2.2: Selected putative interactions of Hpn and Hpnl target proteins. Cell-free extracts of the double mutant (Δ*hpn*Δ*hpnl)* were incubated with purified Hpn or Hpnl proteins and the crosslinking agent DMS. Crosslinked products were enriched by Ni-NTA column binding and then identified by LC-MS/MS. Normalized fold change (NFC) was calculated by comparing the normalized protein abundance (NPA) of the sample and the NPA of the control. The control sample contained crosslinked and Ni-NTA purified extract of the Δ*hpn*Δ*hpnl* mutant without additional purified Hpn or Hpnl. For the purposes of this manuscript, only the results of the first crosslinking system are shown, although the second system identified many of the same interactions as the first approach (Table S2.1, asterisks).

		Normalized Fold Change	
HP Number	Name	Hpn	Hpnl
HP0068	UreG	2.83	1.59
HP0072	UreB	3.36	7.71
HP0073	UreA	3.56	4.74
HP0294	AmiE	3.93	5.20
HP0570	PepA	9.19	9.19
HP0653	Pfr	9.31	5.18
HP0875	Catalase	3.94	15.31
HP0896	Omp19	4.40	2.00
HP0900	HypB	2.89	N/A
HP0912	Omp20	4.53	2.16
HP0913	Omp21	2.59	1.85
HP1110	PFOR	11.01	4.86

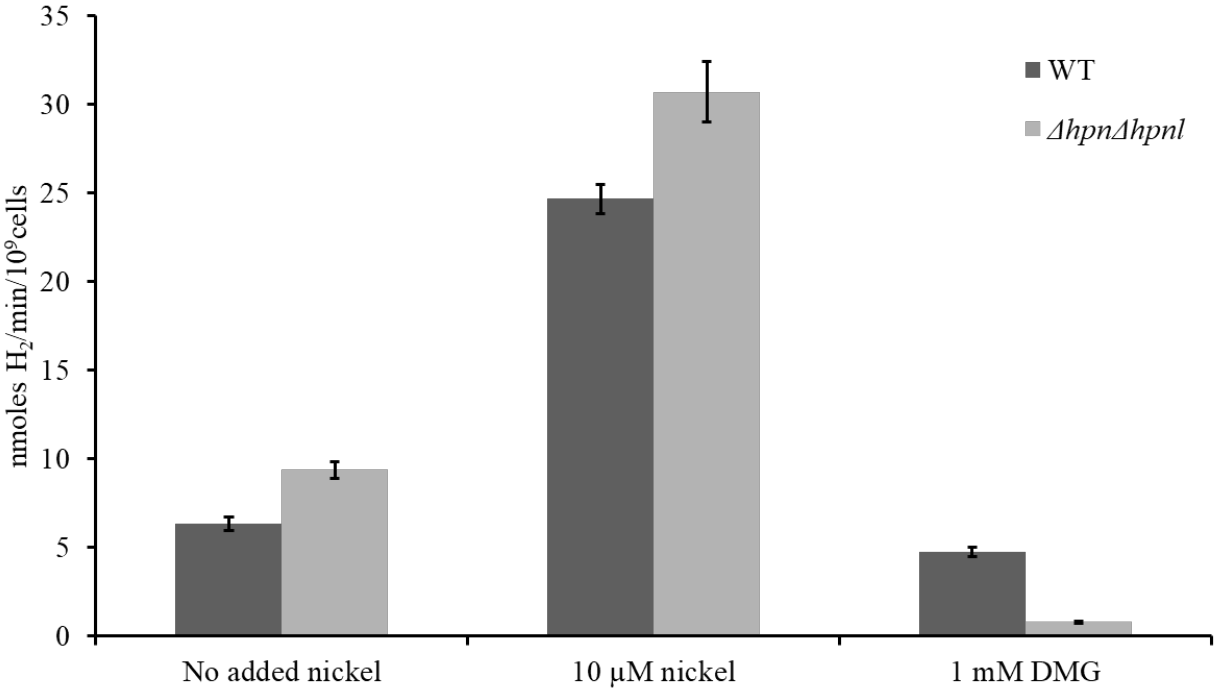

Figure 2.1: Hydrogenase activity in nickel replete and nickel deplete conditions. Results are mean and standard error of one representative experiment, performed with five replicates. The experiment was performed one additional time with similar results.

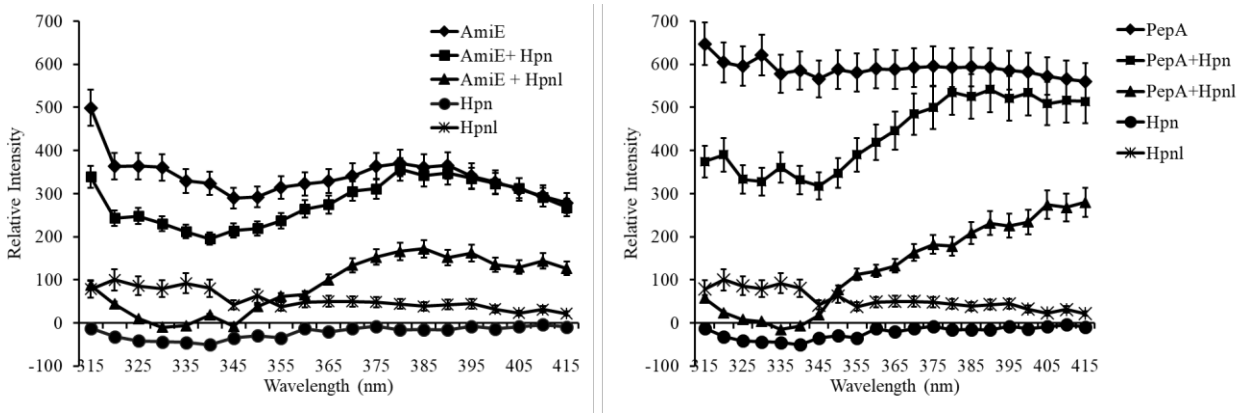

Figure 2.2: Hpn and Hpnl interactions measured by tryptophan fluorescence. T7-tagged AmiE or PepA were purified and crosslinked with purified Hpn or Hpnl. Crosslinked samples were then analyzed by measuring fluorescence of tryptophan with excitation at 295 nm and emission from 315 nm to 415 nm. The crosslinked sample fluorescence was normalized to the fluorescence of a buffer control. Results shown are mean standard error of one representative experiment, done in triplicate. Two additional experiments were performed, and those data provided similar trends to the data shown. Incubation of either Hpn or Hpnl with bovine serum albumin (BSA, Thermo), a non-interacting protein control, had no observable effect on the fluorescence profile of BSA (data not shown), suggesting that addition of the storage proteins does not inherently alter tryptophan fluorescence.

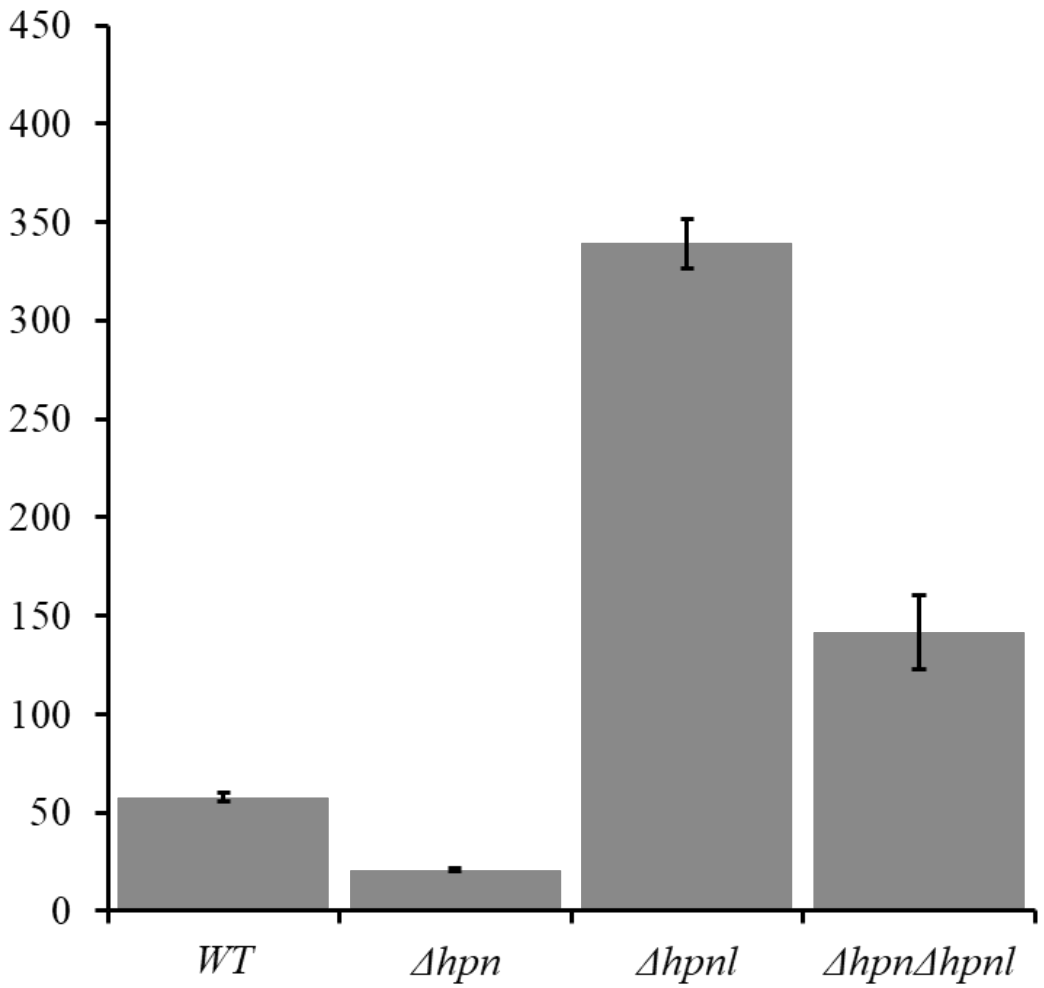

Figure 2.3: Peptidase activity of nickel-supplemented *H. pylori*. Leucine-AMC (Leu-AMC) hydrolysis assays were performed on *H. pylori* extracts. *H. pylori* was supplemented with 10 μM nickel during growth. In both A and B Leu-AMC hydrolysis was measured by increase in fluorescence over 60 minutes. Results are mean and standard error of one representative experiment, in triplicate. Two additional experiments were completed, with similar results.

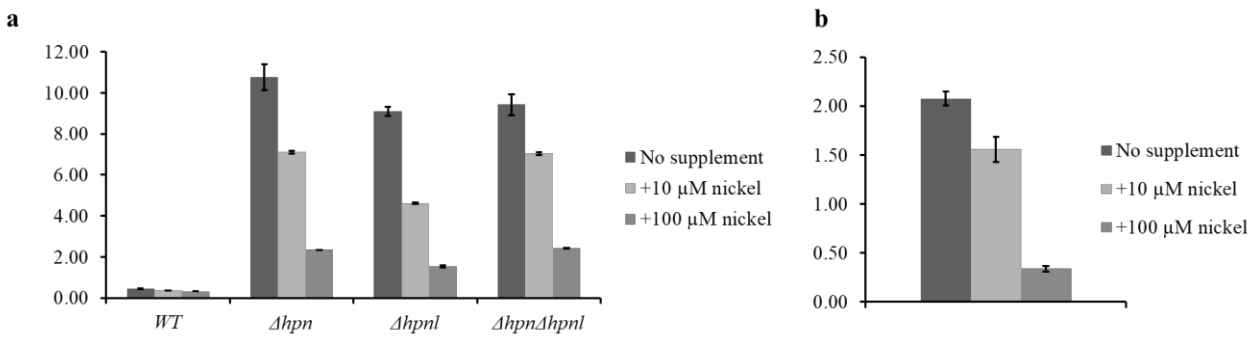

Figure 2.4: Amidase activity of *H. pylori* under various nickel conditions. Amidase assays were performed on **a)** extracts from nickel-deprived *H. pylori* (grown on 1mM DMG) supplemented with additional nickel as indicated and incubated for 2hrs at 37°C prior to measuring activity or **b)** extracts from WT *H. pylori* grown on BA with 10μM nickel and supplemented with additional nickel as indicated and incubated for 2hrs at 37°C prior to measuring activity. Amidase activity was monitored by measuring ammonia generation and is expressed in μM ammonia produced min^{-1} mg total $protein^{-1}$. Results are mean and standard error of one representative experiment in triplicate. Two additional experiments were completed, with similar resulting trends.

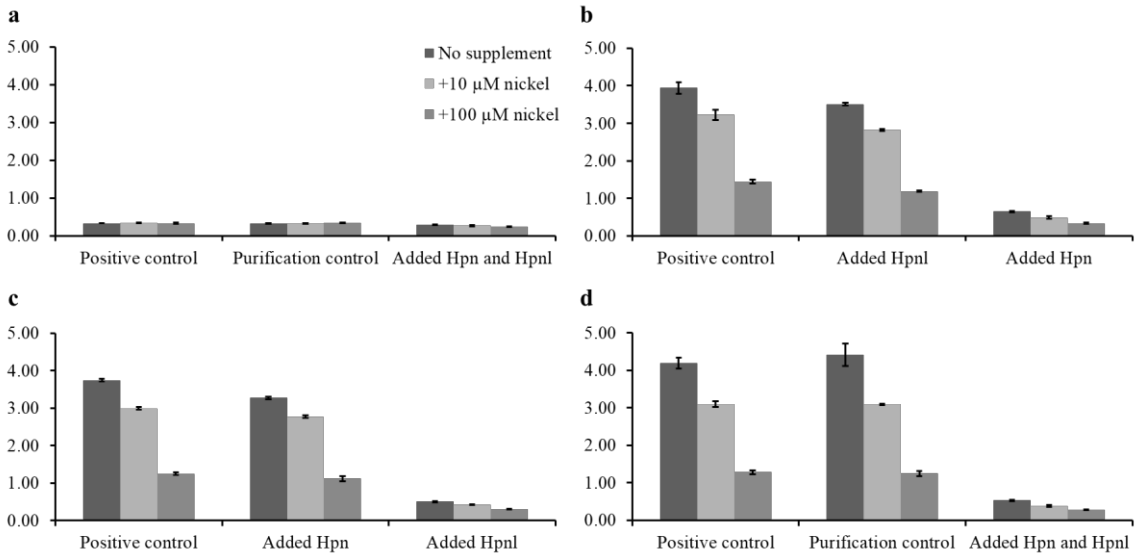

Figure 2.5: Supplementation with exogenous storage protein yields suppressed (like WT) amidase activity of the corresponding mutant. Amidase assays were performed on the extracts from nickel-deprived *H. pylori* (grown on 1 mM DMG), but with extracts supplemented with additional nickel and purified proteins as indicated, and incubated for 2hrs at 37°C prior to measuring activity. Amidase activity was monitored by measuring ammonia generation, and is expressed in µM ammonia produced min^{-1} mg total $protein^{-1}$. Results are mean and standard error of one representative experiment in triplicate. Two additional experiments were completed, with similar results. The positive control in all panels is the indicated lysate incubated with the supplement (no supplement, 10 µM nickel, or 100 µM nickel). As expected, the positive control for each panel mimics the data seen for that strain seen in Fig. 4(c). WT **(a)** and ΔhpnΔhpnl **(d)** *H. pylori* extracts were incubated with purified Hpn+Hpnl. For these two panels, an additional purification control, consisting of Ni-NTA purified extract from Δ*hpn*Δ*hpnl* was assayed to ensure

that no Ni-NTA contaminants contributed to observed phenotypes. Δ*hpn* **(b)** and Δ*hpnl* **(c)** *H. pylori* extracts were incubated with purified Hpn or purified Hpnl.

Supplemental Table 2.1: Putative interactions of Hpn and Hpnl target proteins.

Target proteins with a NFC>1 for either Hpn or Hpnl are shown. Target proteins with NFC≤1 for both Hpn and Hpnl have been omitted from this data. Proteins indicated with * were found using both pulldown enrichment methods.

HP Number	Name	Normalized Fold Change Hpn	Hpnl
HP0010*	chaperone and heat shock protein (groEL)	1.35	1.68
HP0012	DNA primase (dnaG)	2.19	N/A
HP0017	virB4 homolog (virB4)	2.48	N/A
HP0026	citrate synthase (gltA)	10.97	17.82
HP0027	isocitrate dehydrogenase (icd)	12.40	9.72
HP0028	conserved hypothetical secreted protein	2.48	N/A
HP0031	hypothetical protein	2.48	N/A
HP0044	GDP-D-mannose dehydratase (rfbD)	2.19	N/A
HP0056*	delta-1-pyrroline-5-carboxylate dehydrogenase	8.68	4.45
HP0059	hypothetical protein	N/A	4.50
HP0068	urease accessory protein (ureG)	2.83	1.59
HP0069	urease accessory protein (ureF)	1.24	N/A
HP0072*	urease beta subunit (urea amidohydrolase) (ureB)	3.36	7.71
HP0073*	urease, alpha subunit (ureA)	3.56	4.74
HP0082	methyl-accepting chemotaxis transducer (tlpC)	2.19	N/A
HP0083	ribosomal protein S9 (rps9)	2.48	2.23
HP0084	ribosomal protein L13 (rpl13)	4.96	N/A
HP0087	lipoprotein, putative	1.65	0.74
HP0097*	hypothetical protein	2.45	1.24
HP0098	threonine synthase (thrC)	2.19	N/A
HP0109	chaperone and heat shock protein 70 (dnaK)	1.32	0.89
HP0114	hypothetical protein	1.10	N/A
HP0115	flagellin B (flaB)	0.25	1.23
HP0121	phosphoenolpyruvate synthase (ppsA)	0.62	3.38
HP0127*	outer membrane protein (omp4)	4.59	2.63
HP0129*	hypothetical protein	N/A	4.86
HP0130	hypothetical protein	1.53	0.56
HP0134	3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (dhs1)	2.19	N/A
HP0136	bacterioferritin comigratory protein (bcp)	1.10	N/A
HP0137	hypothetical protein	2.19	N/A

HP0144	cytochrome c oxidase, heme b and copper-binding subunit, membrane-bound (fixN)	1.86	N/A
HP0147	cytochrome c oxidase, diheme subunit, membrane-bound (fixP)	1.10	N/A
HP0153	recombinase (recA)	3.29	N/A
HP0166	response regulator (ompR)	0.78	2.57
HP0169	collagenase (prtC)	1.10	N/A
HP0175	cell binding factor 2	0.21	1.39
HP0180	apolipoprotein N-acyltransferase (cute)	11.01	6.13
HP0182	lysyl-tRNA synthetase (lysS)	2.19	N/A
HP0184	hypothetical protein	1.24	N/A
HP0191	fumarate reductase, iron-sulfur subunit (frdB)	4.34	4.86
HP0193	fumarate reductase, cytochrome b subunit (frdC)	1.86	N/A
HP0201	fatty acid/phospholipid synthesis protein (plsX)	2.48	N/A
HP0207	ATP-binding protein (mpr)	2.19	N/A
HP0210	chaperone and heat shock protein C62.5 (htpG)	1.65	0.74
HP0221	nifU-like protein	1.24	2.23
HP0227	outer membrane protein (omp5)	2.48	2.25
HP0229	outer membrane protein (omp6)	3.72	N/A
HP0231	hypothetical protein	3.72	2.23
HP0234	conserved hypothetical integral membrane protein	2.48	N/A
HP0243	neutrophil activating protein (napA) (bacterioferriti)	2.48	6.13
HP0252	outer membrane protein (omp7)	3.66	3.24
HP0254	outer membrane protein (omp8)	1.52	2.16
HP0267	chlorohydrolase	6.13	N/A
HP0272	hypothetical protein	11.01	N/A
HP0294*	aliphatic amidase (amiE)	3.93	5.20
HP0295	flagellin B homolog (fla)	2.48	N/A
HP0296	ribosomal protein L21 (rpl21)	0.99	2.70
HP0305	hypothetical protein	3.72	N/A
HP0308	hypothetical protein	2.19	N/A
HP0310	conserved hypothetical protein	1.86	N/A
HP0324	outer membrane protein (omp10)	1.10	N/A
HP0331	cell division inhibitor (minD)	2.19	N/A
HP0338	hypothetical protein	N/A	9.00
HP0346	hypothetical protein	11.01	N/A
HP0362	conserved hypothetical integral membrane protein	2.48	N/A
HP0370	biotin carboxylase (accC)	2.19	N/A
HP0382	zinc-metallo protease (YJR117W)	2.19	N/A
HP0390	adhesin-thiol peroxidase (tagD)	1.02	0.32
HP0391	purine-binding chemotaxis protein (cheW)	4.39	N/A
HP0393	chemotaxis protein (cheV)	1.10	N/A
HP0397	phosphoglycerate dehydrogenase (serA)	2.48	2.23
HP0400	penicillin tolerance protein (lytB)	2.19	N/A
HP0415	conserved hypothetical integral membrane protein	2.48	N/A

HP0421	type 1 capsular polysaccharide biosynthesis protein J (capJ)	1.10	N/A
HP0447	conserved hypothetical protein	6.13	N/A
HP0467	conserved hypothetical integral membrane protein	2.48	N/A
HP0468	conserved hypothetical protein	2.19	N/A
HP0470	oligoendopeptidase F (pepF)	2.48	N/A
HP0472*	outer membrane protein (omp11)	2.39	1.96
HP0486	hypothetical protein	1.74	1.08
HP0501	DNA gyrase, sub B (gyrB)	4.39	N/A
HP0516	heat shock protein (hslU) ORF1	1.24	N/A
HP0558	beta ketoacyl-acyl carrier protein synthase II (fabF)	2.48	N/A
HP0561	3-ketoacyl-acyl carrier protein reductase (fabG)	2.48	2.23
HP0562	ribosomal protein S21 (rps21)	0.73	1.48
HP0569	GTP-binding protein (gtp1)	1.10	N/A
HP0570*	aminopeptidase a/i (pepA)	9.19	9.19
HP0572	adenine phosphoribosyltransferase (apt)	2.48	N/A
HP0574*	galactosidase acetyltransferase (lacA)	6.13	3.06
HP0576	signal peptidase I (lepB)	2.19	N/A
HP0589	ferredoxin oxidoreductase, alpha subunit	4.96	N/A
HP0590	ferredoxin oxidoreductase, beta subunit	2.19	2.23
HP0591	ferredoxin oxidoreductase, gamma subunit	2.48	N/A
HP0596	lipoprotein, putative	2.63	0.56
HP0599	hemolysin secretion protein precursor (hylB)	1.25	0.32
HP0605	hypothetical protein	8.23	4.50
HP0606	membrane fusion protein (mtrC)	N/A	1.11
HP0617	aspartyl-tRNA synthetase (aspS)	6.20	4.45
HP0618	adenylate kinase (adk)	1.24	N/A
HP0626	tetrahydrodipicolinate N-succinyltransferase (dapD)	4.96	N/A
HP0630	modulator of drug activity (mda66)	1.24	N/A
HP0653*	nonheme iron-containing ferritin (pfr)	9.31	5.18
HP0655	protective surface antigen D15	3.29	N/A
HP0671*	outer membrane protein (omp14)	5.00	2.65
HP0679	lipopolysaccharide biosynthesis protein (wbpB)	2.48	N/A
HP0686	iron(III) dicitrate transport protein (fecA)	2.30	1.24
HP0696	N-methylhydantoinase	2.19	N/A
HP0701	DNA gyrase, sub A (gyrA)	6.13	4.45
HP0706*	outer membrane protein (omp15)	2.04	1.25
HP0710*	conserved hypothetical protein	3.93	3.24
HP0719	hypothetical protein	2.19	N/A
HP0720	hypothetical protein	N/A	4.45
HP0721*	hypothetical protein	0.37	3.24
HP0746	lipoprotein, putative	N/A	4.45
HP0760	conserved hypothetical protein	2.19	N/A
HP0779*	aconitase B (acnB)	22.01	4.50
HP0786	preprotein translocase subunit (secA)	6.13	1.11
HP0791	cadmium-transporting ATPase, P-type (cadA)	1.65	0.56

HP0797	flagellar sheath adhesin hpaA	3.19	0.98
HP0808	holo-acp synthase (acpS)	6.13	4.86
HP0815	flagellar motor rotation protein (motA)	2.19	N/A
HP0836	lipoprotein, putative	1.10	2.23
HP0837	hypothetical protein	N/A	1.50
HP0838	lipoprotein, putative	2.48	N/A
HP0863	lipoprotein, putative	1.24	N/A
HP0875*	catalase [1.11.1.6]	3.94	15.31
HP0896*	outer membrane protein (omp19)	4.40	2.00
HP0900	hydrogenase expression/formation protein (hypB)	2.89	N/A
HP0912*	outer membrane protein (omp20)	4.53	2.16
HP0913*	outer membrane protein (omp21)	2.59	1.85
HP0954	oxygen-insensitive NAD(P)H nitroreductase	9.92	17.82
HP0977	conserved hypothetical secreted protein	1.10	N/A
HP0983	conserved hypothetical integral membrane protein	2.48	N/A
HP1019	serine protease (htrA)	0.88	1.63
HP1027	ferric uptake regulation protein (fur)	3.29	N/A
HP1037	conserved hypothetical protein	1.37	N/A
HP1040	ribosomal protein S15 (rps15)	2.19	N/A
HP1045	acetyl-CoA synthetase (acoE)	2.19	1.11
HP1048	translation initiation factor IF-2 (infB)	2.19	N/A
HP1103	glucokinase (glk)	N/A	4.45
HP1104	cinnamyl-alcohol dehydrogenase ELI3-2 (cad)	12.07	4.45
HP1108	pyruvate ferredoxin oxidoreductase, gamma subunit	3.29	1.80
HP1110	pyruvate ferredoxin oxidoreductase, alpha subunit	11.01	4.86
HP1111	pyruvate ferredoxin oxidoreductase, beta subunit	1.65	2.23
HP1125*	peptidoglycan associated lipoprotein precursor (omp18)	2.45	1.94
HP1131	ATP synthase F1, subunit epsilon (atpC)	N/A	12.25
HP1137	ATP synthase F0, subunit b' (atpF')	2.48	2.23
HP1151	ribosomal protein S16 (rpS16)	N/A	1.11
HP1154	hypothetical protein	1.24	N/A
HP1155	transferase, peptidoglycan synthesis (murG)	N/A	2.23
HP1156	outer membrane protein (omp25)	4.94	4.86
HP1157	outer membrane protein (omp26)	2.19	4.45
HP1168	carbon starvation protein (cstA)	2.19	N/A
HP1172	glutamine ABC transporter, periplasmic glutamine-binding protein (glnH)	2.19	1.67
HP1173*	hypothetical protein	1.75	0.95
HP1177	outer membrane protein (omp27)	1.10	1.11
HP1190	histidyl-tRNA synthetase (hisS)	2.19	N/A
HP1195	translation elongation factor EF-G (fusA)	17.56	13.36
HP1196	ribosomal protein S7 (rps7)	0.94	1.39
HP1197	ribosomal protein S12 (rps12)	0.33	1.29
HP1198	DNA-directed RNA polymerase, beta and beta' subunit (rpoBC)	14.29	5.20

HP1200	ribosomal protein L10 (rpl10)	2.19	N/A
HP1205*	translation elongation factor EF-Tu (tufB)	3.09	0.77
HP1237	carbamoyl-phosphate synthetase (pyrAa)	1.10	N/A
HP1241	alanyl-tRNA synthetase (alaS)	1.24	N/A
HP1246	ribosomal protein S6 (rps6)	2.48	N/A
HP1257	orotate phosphoribosyltransferase (pyrE)	2.19	N/A
HP1266	NADH-ubiquinone oxidoreductase, NQO3 subunit (NQO3)	1.46	N/A
HP1272	NADH-ubiquinone oxidoreductase, NQO13 subunit (NQO13)	2.19	4.45
HP1286	conserved hypothetical secreted protein	4.39	N/A
HP1293	DNA-directed RNA polymerase, alpha subunit (rpoA)	1.86	N/A
HP1295	ribosomal protein S11 (rps11)	0.83	1.62
HP1302*	ribosomal protein S5 (rps5)	1.10	N/A
HP1304	ribosomal protein L6 (rpl6)	N/A	2.43
HP1305	ribosomal protein S8 (rps8)	1.37	1.11
HP1307	ribosomal protein L5 (rpl5)	1.02	N/A
HP1309	ribosomal protein L14 (rpl14)	1.10	1.27
HP1310	ribosomal protein S17 (rps17)	2.19	1.48
HP1312	ribosomal protein L16 (rpl16)	1.10	N/A
HP1314	ribosomal protein L22 (rpl22)	1.24	N/A
HP1316	ribosomal protein L2 (rpl2)	0.59	1.48
HP1318	ribosomal protein L4 (rpl4)	1.55	1.11
HP1320	ribosomal protein S10 (rps10)	1.65	N/A
HP1341	siderophore-mediated iron transport protein (tonB)	6.13	N/A
HP1345	phosphoglycerate kinase	1.59	1.93
HP1354	putative adenine specific DNA methyltransferase	N/A	6.13
HP1364	signal-transducing protein, histidine kinase	6.13	N/A
HP1378	competence lipoprotein (comL)	1.10	N/A
HP1379	ATP-dependent protease (lon)	2.19	N/A
HP1380	prephenate dehydrogenase (tyrA)	11.01	N/A
HP1385	fructose-1,6-bisphosphatase	2.19	N/A
HP1395*	outer membrane protein (omp30)	6.13	N/A
HP1397	hypothetical protein	2.19	N/A
HP1398	alanine dehydrogenase (ald)	N/A	2.23
HP1422	isoleucyl-tRNA synthetase (ileS)	2.19	N/A
HP1444	small protein (smpB)	1.24	N/A
HP1445	biopolymer transport protein (exbB)	2.19	N/A
HP1450	60 kDa inner-membrane protein	1.10	N/A
HP1454*	hypothetical protein	4.96	N/A
HP1456*	membrane-associated lipoprotein (lpp20)	3.68	1.62
HP1458	thioredoxin	1.24	N/A
HP1463	hypothetical protein	2.19	N/A
HP1496	general stress protein (ctc)	3.29	2.23
HP1507	conserved hypothetical ATP-binding protein	1.10	N/A

HP1509	conserved hypothetical integral membrane protein	N/A	2.23
HP1517	type IIS restriction enzyme R and M protein (ECO57IR)	1.24	N/A
HP1521	type III restriction enzyme R protein (res)	N/A	4.86
HP1538	ubiquinol cytochrome c oxidoreductase, cytochrome c1 subunit (fbcH)	1.65	2.43
HP1547	leucyl-tRNA synthetase (leuS)	1.10	N/A
HP1550	protein-export membrane protein (secD)	2.48	N/A
HP1551	conserved hypothetical secreted protein	8.78	N/A
HP1555	translation elongation factor EF-Ts (tsf)	1.10	N/A
HP1563*	alkyl hydroperoxide reductase (tsaA)	3.32	5.10
HP1564	outer membrane protein	2.48	N/A
HP1582*	pyridoxal phosphate biosynthetic protein J (pdxJ)	6.20	4.50
HP1588*	conserved hypothetical protein	3.03	1.69

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CHAPTER 3

CONCLUSIONS AND FUTURE DIRECTIONS

Helicobacter pylori is the etiological agent of chronic gastritis, gastric ulceration, and gastric cancer, and has been classified by the World Health Organization as a class I carcinogen (1, 2). *H. pylori* colonize the stomach of 50% of the world population, at least in part due to its extraordinary ability to colonize a niche environment with little bacterial competition (3). A major contributor to *H. pylori* survival in the host is the pathogen's ability to neutralize the acidic pH of the stomach, a process made possible by the urease enzyme (4). Urease catalyzes the conversion of urea into ammonia and bicarbonate, which in turn provides the bacterium with a source of nitrogen and buffers the pH in the immediate vicinity of the cell (5-7). Further, the activity of urease is essential for colonization and virulence (4). The need for urease activity poses another problem for *H. pylori* – urease requires nickel in the active site (6, 8, 9), and nickel concentrations in the stomach are thought to be relatively low and in constant flux (10, 11).

Since *H. pylori* maintains a large amount of urease, roughly 10% of its total cellular protein (12), one can speculate that acquisition and maintenance of nickel pools would be important for survival. Indeed, deletion of either nickel import proteins (NiuBDE) or nickel storage proteins (Hpn and Hpnl) resulted in *H. pylori* that were deficient in host (mouse) colonization (13-15). Additionally, nickel storage proteins Hpn and Hpnl have been shown to influence urease activity (15), and more recently to directly interact with urease maturation proteins to modulate urease activity (14). Hpn and Hpnl are well known

for their roles in nickel storage and detoxification (15), and additional roles for the two storage proteins have been hypothesized (16, 17). Based on the broader importance of nickel in *H. pylori* (*e.g.* for hydrogenase (18)), I sought to characterize additional protein-protein interactions for the nickel storage proteins that may affect *H. pylori* cellular functions or survival.

I used a protein-protein interaction based approach to identify possible additional roles for Hpn and Hpnl (Chapter 2). The storage proteins naturally bind to several molecules of nickel with moderate affinity (19-22), so I used a combination of crosslinking (to capture transient or unstable interactions) and enrichment (affinity purification of crosslinked samples on a nickel column) to identify transiently interacting partners of the storage proteins. Using this crosslinking and enrichment approach, I determined that Hpn and Hpnl interacted putatively with over 200 *H. pylori* proteins (Table S2.1). As expected, many of the interacting partners were involved in nickel metabolism. I found that Hpn and Hpnl interacted with urease maturation (UreG for both storage proteins and UreF and HypB for Hpn) and structural proteins (UreA and UreB for both storage proteins), as well as with nickel import machinery (TonB and ExbB, Hpn only). My results confirmed and expanded upon previous findings wherein Hpn interacted with UreA and both storage proteins interacted with HypB (14).

Interestingly, Hpn and Hpnl interactions were not limited to proteins known to be influenced by nickel, including targets in diverse areas of cellular functions such as replication, translation, motility, stress response, iron metabolism, carbohydrate metabolism, and numerous hypothetical and outer membrane proteins of unknown function. Only two of the interactions found were verified, however the breadth of proteins

that interacted, even transiently, with the storage proteins was unexpected, and may indicate that Hpn and Hpnl play roles well beyond their established functions of nickel storage and urease modulation.

Additional verification would be required to explore these interactions further, and the interactions of most initial interest would be those with some connection to nickel or metals homeostasis, such as Fur, Pfr, NapA (related to iron), TonB and ExbB (facilitate metals transport), FixN and FixP (metal-containing cytochrome c oxidase). Additionally, there were some trends in the interacting proteins that may warrant further investigation: many outer membrane proteins of unknown function were present in the pulldown of both Hpn and Hpnl, and these may represent potential metal-binding or transporting proteins, or may interact with the storage proteins for some unknown role. Hpn has previously been found in the periplasm in similar abundance to the cytoplasm, indicating potential for the storage proteins to interact with periplasmic or outer membrane proteins (19). Hpn and Hpnl also putatively interacted with several proteins involved in motility and chemotaxis, including FlaB, MotA, CheV, and CheW. These interactions may contribute to previously observed *H. pylori* chemotactic response to metals (23), although the proteins I have found to putatively interact with the storage proteins were not investigated in that study.

Following the identification of the putative storage protein interactions, I investigated one of the storage protein-nickel enzyme interactions further by phenotypic characterization of mutant strains. The Hpn-HypB putative interaction had already been postulated to influence urease activity (14), and the potential influence of Hpn and Hpnl on hydrogenase activity had been previously explored, although not stringently (14, 15). In chapter 2, I show that a Δ*hpn*Δ*hpnl* double mutant influences hydrogenase activity,

especially in the nickel deplete conditions. Hydrogenase activity was increased more than three- to four-fold when cells were grown on supplemental nickel, as expected (Fig. 2.1). The hydrogenase activity of the double mutant grown on 1 mM DMG was significantly lower than its WT counterpart and nearly 13-fold lower than the double mutant activity under standard growth conditions (Fig. 2.1). Nickel deprivation lowered the hydrogenase activity of all strains tested, but none were as strongly inhibited as the double mutant. Based on these data, I concluded that the lack of nickel storage proteins severely impacted hydrogenase activity. I was unable to assign the strong effect of nickel deprivation on hydrogenase activity to either or the storage mutants individually – both single mutants had a similar phenotype to the WT (see Appendix A). This likely indicates that the storage proteins play redundant roles in modulating hydrogenase activity, or that the observed reduction in hydrogenase activity is a result of altered nickel pools in the double mutant.

The Hpn-HypB interaction was only verified by the crosslinking and pulldown approach, so conclusions from this putative interaction are not rigorous. These proteins are not good candidates on which to use tryptophan fluorescence to verify the interaction; neither HypB nor Hpn contains tryptophan residues. The interaction could be verified by purifying the proteins and visually examining crosslinked products of those purified proteins by SDS-PAGE. Additional characterization of the interaction may provide insight into the mechanism by which the storage proteins affect hydrogenase activity. HpnI and HypB have been previously shown to interact (14), so it is possible that this interaction was missed by my crosslinking and enrichment method. Indeed, interactions that impede Hpn or HpnI binding to the nickel column would be expected to remain undetected, so it is possible that this occurred for an HpnI-HypB interaction. This may be the most likely

scenario, since only the double mutant showed significant differences in hydrogenase activity relative to the WT.

Among all the putative interactions discovered (Table 2.2 and S2.1), two were chosen for further characterization. These two had potential ties to nickel-metabolism and interacted with both storage proteins; AmiE is regulated by a repression cascade involving Fur and NikR (24) and is involved in ammonia production in the absence of urease (25), and PepA contains divalent metals in the active site (including nickel, which increased its activity) (26). The putative interactions of both proteins with the two storage proteins was verified using tryptophan fluorescence. Both AmiE and PepA contain tryptophan residues, and neither Hpn nor Hpnl contain any, making this detection method ideal to observe the interactions. When either AmiE or PepA were incubated with the storage proteins, their tryptophan fluorescence profiles shifted (Fig. 2.2), indicating that an interaction between the storage protein and their interacting partner was obscuring tryptophan residues or causing a conformational change (27). After the interactions were verified, phenotypic characterization of storage protein mutant strains was conducted to further characterize the roles for these interactions.

The Hpn and Hpnl interactions with PepA were characterized by growing *H. pylori* with supplemental nickel and measuring peptidase activity. Under these conditions, each strain had unique peptidase activity phenotype (Fig. 2.3). Peptidase activity was altered in opposite ways in the individual storage protein mutants; Δ*hpn* peptidase activity was nearly three-fold lower than its parent, while Δ*hpnl* had six-fold higher activity (Fig. 2.3). The Hpnl-PepA interaction, along with the phenotype of the Δ*hpnl* mutant, suggest that Hpnl contributes to peptidase suppression, possibly mediated by nickel. Hpn seems to play the

opposite role – the PepA activity of Δ*hpn* mutant was reduced compared to the WT, suggesting that Hpn may play an activation role for PepA (Fig. 2.3). The opposite roles for Hpn and Hpnl may suggest an antagonistic action wherein Hpn contributes to nickel loading (and therefore activation) of PepA while Hpnl contributes to nickel unloading (and inactivation) of PepA. This is supported by the known effect of nickel on PepA activity (26). Peptidase is activated by a variety of divalent metals, and both Hpn and Hpnl are known to bind metals besides nickel (19-22, 28-30), indicating that nickel may not be the only metal to influence peptidase activity via Hpn and Hpnl. It would be of great interest to determine whether other divalent metals known to influence PepA activity had any storage protein mediated effects. It may also be interesting to determine whether there is an effect of metal-loaded versus non metal-loaded storage protein on PepA activity, which may indicate that the storage proteins are capable of directly supplying metal to the peptidase.

Amidase has previously been shown to be influenced by nickel, and this influence has been attributed to the repressor cascade of Fur and NikR (24). Due to the known regulatory effects of nickel on amidase activity, I decided to take a unique approach to exploring the effects of Hpn and Hpnl on the activity of the enzyme *per se*. Pre-formed amidase was assayed for activity by measuring ammonia production in cell-free extracts using nickel deprivation conditions. While I demonstrated [consistent with previous studies, see (24)] that amidase activity of the WT was increased when cells were grown on nickel, I also demonstrate that pre-formed amidase is inhibited by supplementation with nickel (Fig. 2.4).

Unsurprisingly, when *H. pylori* was grown in conditions of nickel deprivation the WT extracts had almost no amidase activity, and that (already low) activity was unaffected by nickel supplementation (Fig. 2.4). Interestingly, the amidase activity of all mutant strains was 20 to 25-fold higher than their WT counterpart, and nickel supplementation suppressed amidase activity in those strains (Fig. 2.4). It was still plausible that the amidase activity phenotype seen here was due to differential amidase expression and not an effect of the storage proteins on the amidase enzyme, and that possibility was addressed through a pure protein supplementation approach. By incubating extracts of storage protein mutants with pure storage proteins, I was able to tease apart the effects of the storage proteins separately from the regulatory effects, since within a given extract the concentration of AmiE would be the same for any supplementation condition. Incubating extracts with additional pure storage protein for the protein already present in the extract (*e.g.* incubating Δ*hpn* extract with pure Hpnl added) resulted in no discernable reduction in amidase activity relative to the positive (no added pure protein) control. When the missing storage protein was added to the relevant extracts (*e.g.* Δ*hpn* extract with pure Hpn added), amidase activity was reduced, nearly six-fold, to near WT levels (Fig. 2.5). In keeping with the single mutant results, incubation of the Δ*hpn*Δ*hpnl* extract with pure Hpn and Hpnl resulted in nearly complete suppression of amidase activity (Fig. 2.5). Taken together, these results suggest that amidase activity is suppressed by nickel, and that nickel-mediated amidase activity suppression requires the presence of both storage proteins for full effect.

Based on these observations, I concluded that Hpn and Hpnl act synergistically to suppress amidase activity, since amidase is only fully suppressed in the presence of both

storage proteins (Figs. 2.4 and 2.5). I also observed interaction between each storage protein and AmiE, indicating that protein-protein interaction between the storage proteins and amidase may contribute to this suppression effect. Hpn and Hpnl have previously been shown to interact with each other (14), so it is plausible that suppression is mediated by a complex of both storage proteins with the amidase. Observing the three-protein complex would greatly add to our knowledge of this suppression phenotype, and it may be possible to observe this by crosslinking and subsequent SDS-PAGE analysis. However, Hpn and Hpnl are both known to form multimers of various sizes (19-22, 31), and these multimers may be captured by crosslinking and impede visual observation of AmiE-storage protein complexes.

Amidase has not previously been annotated as a nickel- or metal-containing enzyme. My results suggest that nickel was inhibiting or suppressing amidase activity, and it is plausible that the *H. pylori* amidase is an unusual member of the aliphatic amidases in this regard. Recent studies have indicated that metal-binding proteins are often under-annotated in bacterial proteomes (32). Additionally, some *H. pylori* proteins that are nickel free in most bacteria have specifically adapted to incorporate the metal (33). To address the possibility that *H. pylori* amidase contains metals, I performed equilibrium dialysis against a variety of divalent metals and measured their relative abundance by ICP-MS. This analysis indicated that recombinant AmiE was capable of binding several divalent metals, including zinc, cadmium, manganese, cobalt, and nickel (listed in decreasing number of atoms per molecule of protein). All of these metals (with the exception of manganese for Hpnl) have been shown to bind the storage proteins (19, 21, 22). Additional metals analysis is necessary to determine the affinity of AmiE for these metals, and it would

be of great interest to measure the effect that each of these metals has on amidase activity, especially in the presence of the storage proteins.

Nickel metabolism is of vital importance for *H. pylori*; it contributes to urease and hydrogenase activities, both of which are important for colonization and virulence (4, 34). In order to facilitate efficient maturation of nickel-containing enzymes, *H. pylori* has adapted a wide range of nickel trafficking, regulation, and storage proteins. The nickel storage proteins Hpn and Hpnl contribute to nickel storage, nickel detoxification, and urease modulation (14, 15). While their roles for maturation of urease have been investigated, little was known about other roles that they may play in *H. pylori*. I have described herein the interaction of the storage proteins with possibly over 200 partners, and provided evidence for their importance in metabolic functions ranging from energy conservation (hydrogenase activity) to nitrogen metabolism (ammonia generation via amidase) and peptide import and turnover (peptidase activity). It is clear from these results that *H. pylori* Hpn and Hpnl play important roles that extend beyond urease activity, and they likely contribute to many disparate cellular functions.

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APPENDIX A

UNPUBLISHED DATA FOR NICKEL STORAGE PROTEIN INTERACTIONS

Addressing possible biases in the crosslinking procedure. The method used to crosslink, enrich, and identify proteins interacting with Hpn and Hpnl (discussed in Chapter 2) produced an unexpectedly high number of putative interacting partners; 215 partners using the NFC>1 cutoff and over 100 partners with the more stringent cutoff of NFC>2 (Table S2.1). Due to the high number of putative interactions (roughly 15% of the genome is represented among the putative interacting proteins) and the potential for false positives, I investigated several possible causes for non-specific interactions.

The crosslinking reagent that was used for the affinity pulldown experiments was dimethyl suberimidate, an imidoester crosslinker that irreversibly links amine residues. DMS proved capable of generating crosslinked products with Hpn and Hpnl, however, it may have also introduced a bias for proteins containing exposed amines in the form of lysine. It is possible to envision a scenario wherein lysine-rich proteins are bound with higher frequency to the crosslinker, and therefore have more potential to non-specifically interact with the storage proteins to be pulled down by affinity purification. I compared the lysine content of all putative interacting proteins with an NFC>5, and found that there was no correlation between lysine content and relative abundance (Fig. A.1). In addition, the average lysine content of *H. pylori* is 9.2%, and the top 5 most abundant putative interacting proteins all had lysine content below average. Based on these data, it appears

that the crosslinking and affinity pulldown approach was not biased towards lysine-rich proteins.

Another possible bias was inherent to the storage proteins and their unique makeup. Both Hpn and Hpnl are extremely rich in basic histidine residues, and these may be predisposed to interact with acidic amino acids. To examine whether the acidic amino acids were introducing bias into the crosslinking and affinity pulldown results, the aspartic acid and glutamic acid distribution of the two verified interacting proteins (AmiE and PepA, see Chapter 2), six of the most abundant putative interacting proteins, and a non-interacting control were compared. There does not appear to be any obvious clustering of acidic residues on the putative or confirmed interacting proteins, and none of the examined structures suggest a pattern for interaction that involves acidic amino acids (Fig. A.2). Based on this observation, it does not appear that the distribution of acidic amino acids on the surface of the proteins influences interaction with Hpn or Hpnl.

Hydrogenase activity of single mutants. In an attempt to assign the strong phenotype (reduced hydrogenase activity under nickel deprivation conditions) observed in the Δ*hpn*Δ*hpnl* mutant (see Chapter 2) to one of the two storage proteins individually, each single mutant was assayed for hydrogenase activity under nickel supplemented and nickel deprived (grown on 10 μM nickel or grown on 1 mM DMG, respectively) conditions. Both Δ*hpn* and Δ*hpnl* single mutants exhibited hydrogenase activity similar to the WT under all conditions tested (Fig. A.3). This suggests that either the storage proteins are playing redundant roles in modulating hydrogenase activity (*e.g.* when one storage protein is absent the other can supplement and maintain activity), or that the hydrogenase activity phenotype found in the double mutant was caused by altered nickel pools. The hydrogenase activity

of the single mutants was only analyzed one time, since their activity was so similar to that of the WT.

Further evidence for possible metal binding by amidase. Prior to this study, amidase was not purported to bind any metals. In chapter 2, I show that amidase is capable of binding or associating with several divalent metals. Using the Phyre2 and Jsmol software, I developed a model for AmiE protein structure (based on similar, known protein structures from other organisms), and used this to provide evidence for possible metal binding locations or residues near the putative active site of the enzyme. *H. pylori* AmiE contains 11 cysteines and 7 histidines (out of 339 total amino acid residues), and several of these metal-binding residues are clustered near the putative active site of the enzyme (Fig. A.4). These clustered histidine and cysteine residues may represent a metal binding center for this protein, and their location near the putative active site may indicate a role for metals in modulation of activity. This information may provide a framework for future researchers wishing to investigate the metal binding properties of AmiE and further elucidate the physiological implications of metal binding for this protein.

Determining nickel transfer between storage proteins and interacting targets. Observing direct or indirect nickel transfer between the storage proteins and their targets would provide additional verification for the interactions, as well as providing insight into the nature of the interaction (*e.g.* whether Hpn and Hpnl donate or strip nickel from AmiE would characterize the interaction between those proteins further). The setup for this experiment was to purify the Hpn, Hpnl, AmiE, and PepA and dialyze them against EDTA to remove all metals and create apo-proteins. Some of the apo-protein would be reserved, while the remainder would be dialyzed against a nickel-containing buffer to generate holo-

proteins. The apo-proteins and holo-proteins would then be combined in pairs (*e.g.* Hpn and PepA) within a single dialysis bag and dialyzed against mass spectrometry buffer, and the resulting samples sent for electrospray ionization mass spectrometry (ESI-MS) to determine nickel content of each protein in the sample. This was attempted with all possible pairs of storage protein-target (apo-Hpnl with holo-AmiE, holo-Hpnl with apo-AmiE, etc.), as well as a control containing all four apo-proteins (so that the metal content of each protein prior to incubation with its partner could be established). Unfortunately, I was unable to get reliable MS data from this experiment; MS was unable to detect any metals or proteins in the control samples. Following so many dialysis steps, the concentration of protein was too low to be detected in the controls. It is possible that the experiment could be repeated with pre-concentrated proteins to ensure that enough sample made it through to the ESI-MS analysis, although the dialysis procedure often increased the volume of the sample, and concentration frequently led to a loss of sample amount.

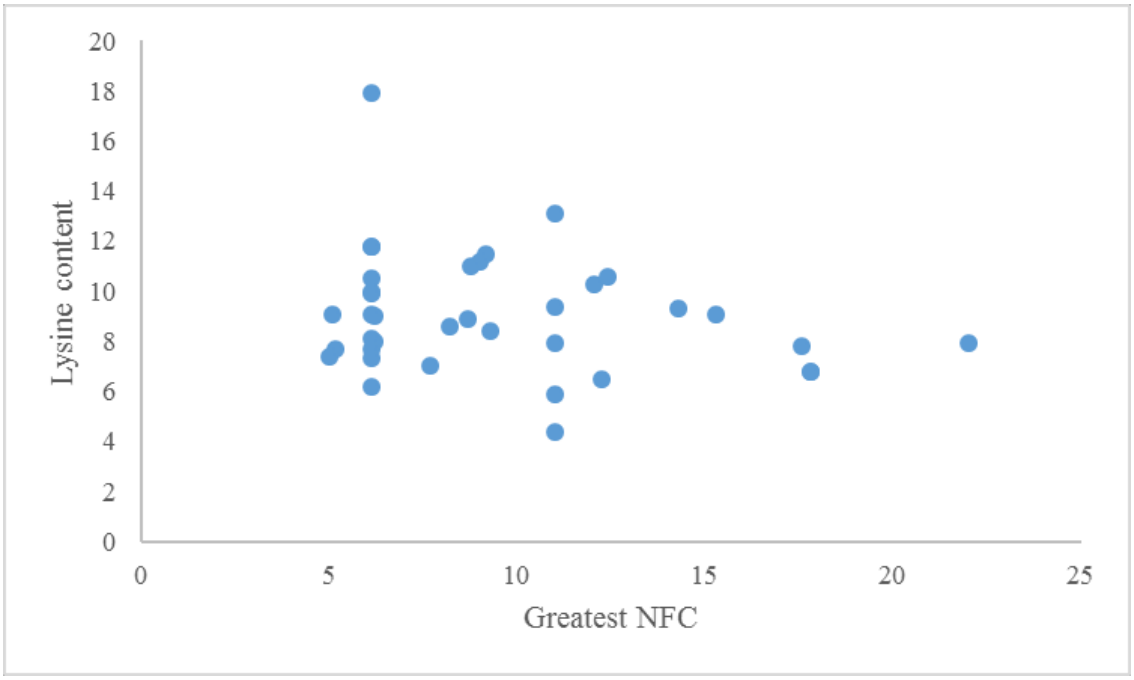

Figure A.1: Lysine content does not predict pulldown abundance. The lysine content of all putative interacting proteins with NFC>5 were analyzed and compared to the relative abundance of those proteins. For reference, the average lysine content of *H. pylori* is 9.2%.

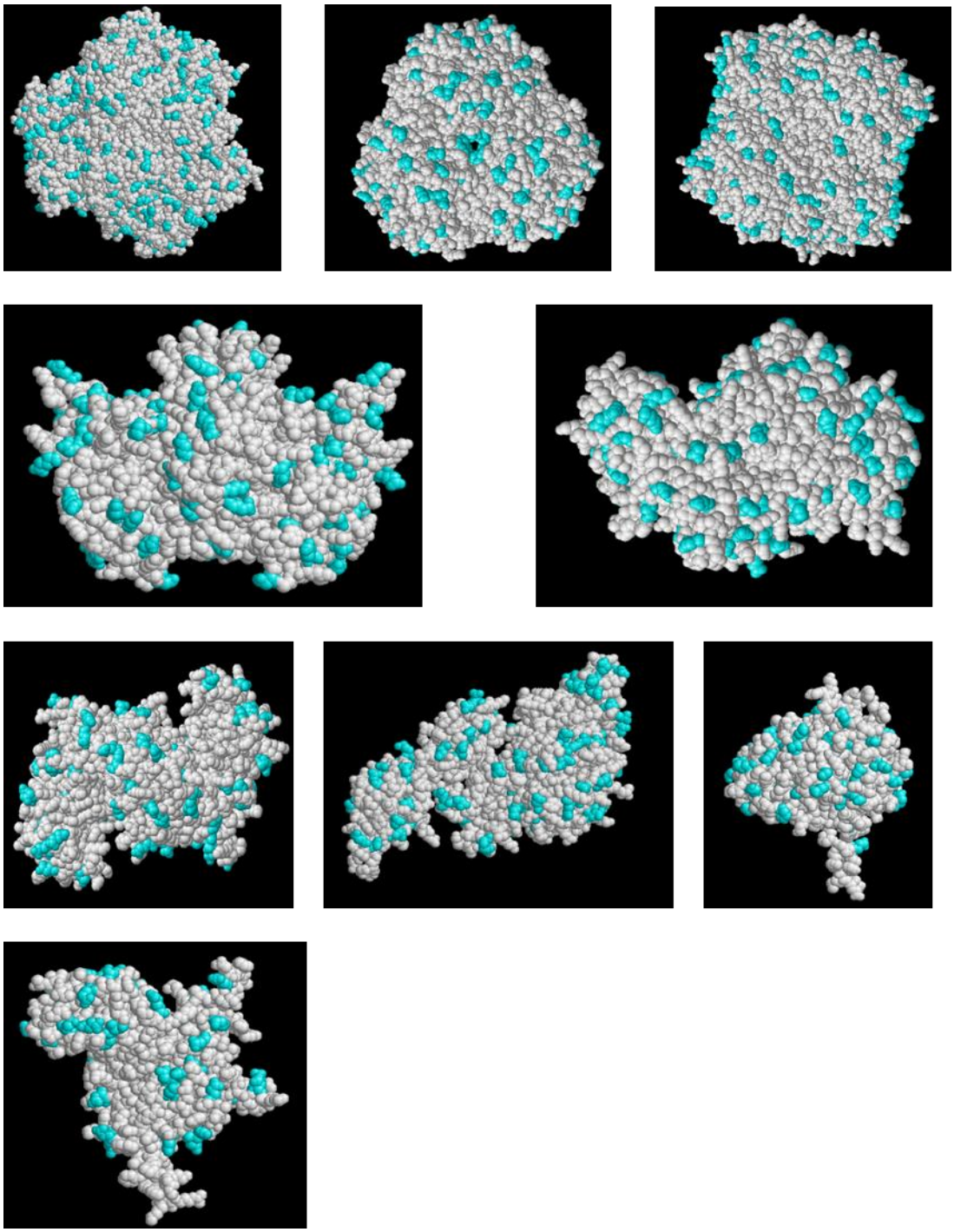

Figure A.2: Distribution of acidic residues on putative interacting protein surfaces. Glutamic acid and aspartic acid are shown highlighted in cyan on the space-fill model of verified or putative interacting proteins, as well as for a non-interacting control.

The image captured was chosen as the best representative for distribution of those residues by manual inspection of the 3D model generated by SWISS-MODEL and manipulated in RasMol. The proteins represented are (from top-left to bottom-right) PepA, AmiE, Catalase, Icd, AcnB, GltA, FusA, oxygen-insensitive NAD(P)H nitroreductase, and ClpX. PepA and AmiE are verified to interact with Hpn and Hpnl (see Chapter 2), and ClpX is a non-interacting control. The remaining proteins are putative interacting partners for the storage proteins.

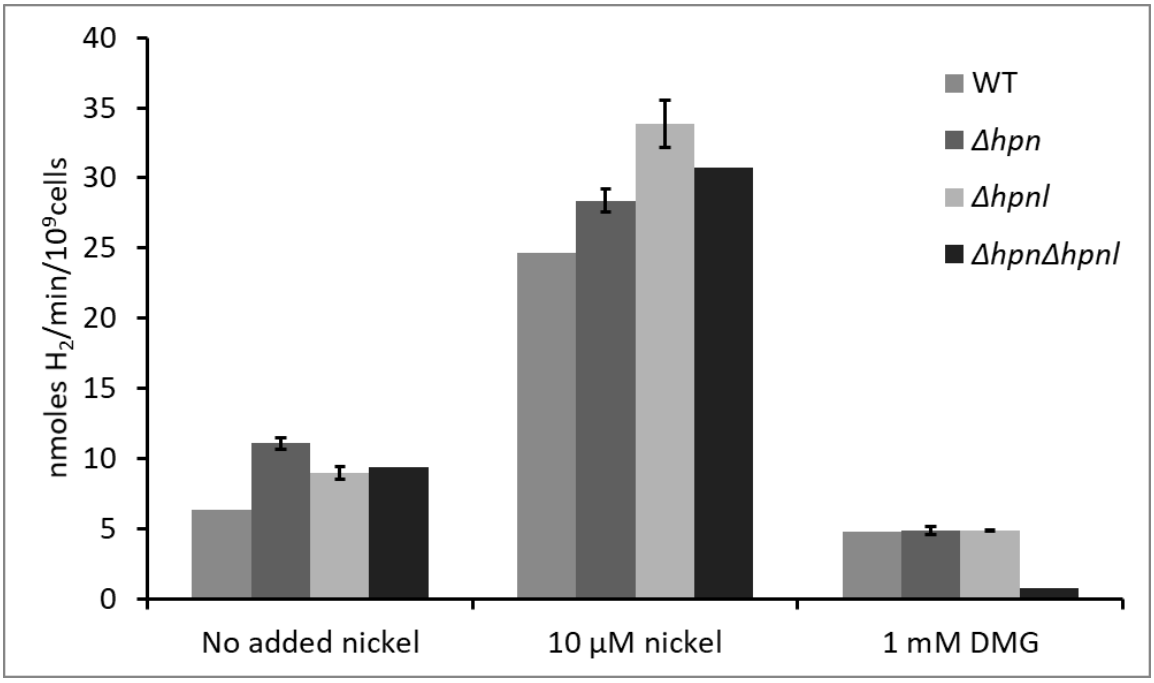

Figure A.3: Hydrogenase activity of nickel storage mutants in nickel deplete and replete conditions. Results are mean and standard error of one representative experiment, performed with five replicates.

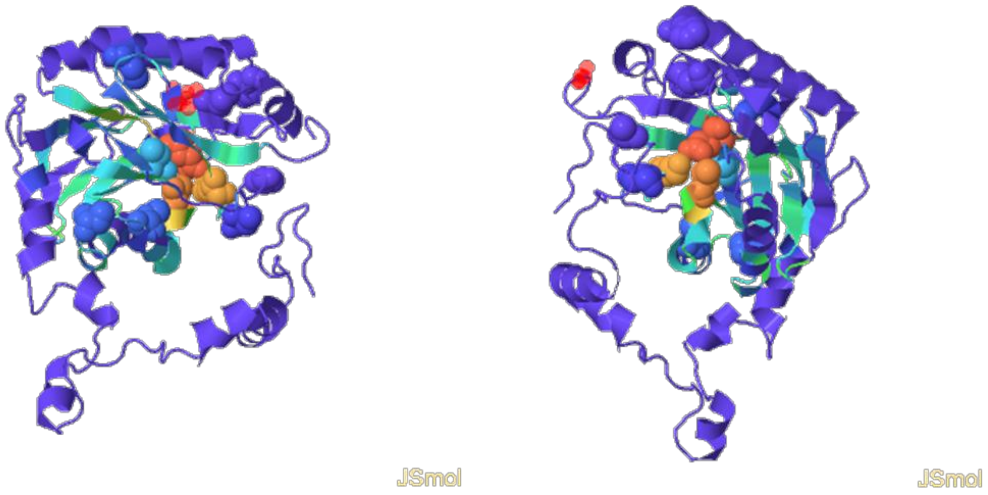

Figure A.4: AmiE protein model. The amidase (AmiE) protein model, with cysteine and histidine residues represented with space-fill and the putative active site in orange and red. Two different angles showing metal-binding residues near the active site are displayed.