HELICOBACTER PYLORI NICKEL METABOLISM ACCESSORY PROTEINS NEEDED FOR THE MATURATION OF BOTH UREASE AND HYDROGENASE

by

NALINI SANJAY MEHTA

(Under the Direction of Dr. ROBERT J. MAIER)

ABSTRACT

Helicobacter pylori is a causative agent of chronic gastritis and peptic ulcer disease. Prolonged infection may lead to gastric cancer. The bacterium has an unique ability to invade and persist in the extremely acidic stomach. *H. pylori* possesses structural and accessory genes for the synthesis and maturation of two nickel containing enzymes, hydrogenase and urease. Insertional mutagenesis of two hydrogenase accessory genes, *hypA* and *hypB*, showed their involvement in the activation of both hydrogenase and urease. The role played by these genes in hydrogenase maturation was not unexpected, however their involvement in urease activation was quite surprising, as the bacterium appeared to have a complete set of urease maturation genes.

To understand the role of HypA and HypB further, both HypA and HypB proteins were purified from *H. pylori*. In some bacteria these accessory proteins have been shown to either bind nickel or hydrolyze GTP. HypA from *H. pylori* bound to two nickel ions per dimer with positive cooperativity, but lacked GTPase activity. A mutant HypA protein (H2A) failed to bind nickel. HypB possessed GTPase activity, but did not bind

nickel. A mutant HypB protein (K59A) showed negligible GTP hydrolyzing activity. Alleles for these two mutant proteins were introduced into *H. pylori.* The resulting mutant strains lacked hydrogenase activity, and had only 2% (*hypA* mutant) and 1.4% (*hypB* mutant) of the urease activity shown by the wild type strain.

Crosslinking studies between HypA and HypB in the presence of dimethyl suberimidate showed formation of a 43-kDa heterodimeric complex, composed of both proteins in a 1:1 molar ratio. A similar complex was seen when a mixture of the two proteins was subjected to gel filtration chromatography. These data suggest that HypA is involved in nickel sequestration and HypB in nickel incorporation via its GTP hydrolyzing activity during the maturation of urease and hydrogenase.

INDEX WORDS: *Helicobacter pylori*, Gastric ulcer, Hydrogenase, Urease, Site-directed mutation, Crosslinking, Dimethyl suberimidate, Heterodimeric, Chromatography

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INTRODUCTION AND LITERATURE REVIEW

Since the time *Helicobacter pylori* was first isolated in 1982 by Marshall and Warren, it has caused a great deal of interest among gastric microbiologists all over the world. This Gram-type negative, spiral-shaped, slow-growing, flagellated, microaerophilic bacterium has the extraordinary ability to survive in the extremely acidic environment of the stomach. Its unique ability to colonize and persist in the gastric mucosa of the human stomach for many years reflects on its physiological capabilities to specifically occupy this unusual niche. According to a CDC report, approximately two-thirds of the world's population is infected with *H. pylori*, which is responsible for causing 90% of duodenal ulcers and up to 80% of gastric ulcers. Early studies of biopsy specimens from the antral mucosa of patients suffering from acute chronic gastritis, duodenal ulcer or gastric ulcer almost always showed the presence of this bacterium (91, 120). Once colonized, the host can be chronically infected for life unless anti-microbial therapy is administered (11, 12). A wide body of evidence supports the fact that *H. pylori* is responsible for causing acute or chronic gastritis and peptic ulcer disease (9, 10). Volunteers who ingested *H. pylori* cells suffered from acute or chronic gastritis (89, 107). Antimicrobial therapy, which eliminated the infection, also eliminated gastritis, but with the recurrence of infection, relapses of gastritis occurred (155). Animals such as gnotobiotic piglets have been shown to develop gastritis after challenging them with *H. pylori* (37). Recent evidence indicates that the gastritis may progress over several decades

to chronic atrophic gastritis, a lesion that has been shown to be a precursor of gastric carcinoma and mucosal associated lymphoid tissue (MALT) lymphoma (39, 118, 142, 162, 170). Although most persons infected with *H. pylori* never suffer symptoms related to the infection, they have a 2- to 6-fold increased risk of developing gastric cancer and MALT lymphoma compared to the uninfected individuals. Treatment of gastric MALToma patients with appropriate antibiotics that eradicated *H. pylori* often lead to the regression of the tumors (6). Gastric cancer is the second most common cause of cancer deaths in the world. Based on the available evidence, the International Agency for Cancer Research declared *H. pylori* a carcinogen for humans in 1994. Thus, *H. pylori,* a previously obscure organism, is now associated with many important gastroduodenal disorders.

Gastritis can be diagnosed using serological tests that can detect the IgG antibodies specific for *H. pylori* or by using the urea breath test (121, 133). Gastritis is usually treated with a combination of proton pump inhibitors and antibiotics for 10 to 14 days (100). Although this infection is common, there appears to be no direct evidence for its mode of transmission. The bacteria may spread from person to person through fecal-oral or oral-oral routes (74, 147). Morphologically *H. pylori* resembles the *Campylobacter* species because of its spiral-shaped structure with bluntly rounded ends (54). The cells show the presence of five to seven unipolar, sheathed flagella (unlike the unsheathed, bipolar flagella of *Campylobacter*), which are not only essential for its motility but also for colonization of the gastric mucosa (38). After prolonged culture, these spiral-shaped bacteria assume a coccoidal morphology. Under electron microscopy these coccoidal forms appear as U-shaped cells with their ends joined by a thin,

membranous structure. Coccoidal forms are metabolically active, although not culturable *in vitro* (13).

To date, genomic sequences of three *Helicobacter* strains have been elucidated. The first reported sequence was for *H. pylori* strain 26695. This strain has a genomic size of 1.67 Mbp, which is one-third the size of *E. coli* genome. It consists of 1,590 coding sequences (151). The second strain, J99, has a slightly smaller genome size of 1.64 Mbp and 1,495 coding sequences (2). There are numerous sequence similarities between these two strains. Sequence analysis has shown that basic amino acids such as arginine and lysine are twice as frequent in *H. pylori* as in *E. coli* and *Haemophilus influenzae*.

The third genome which was sequenced was for *Helicobacter hepaticus*. It has a larger genome size of 1.8 Mbp, and consists of 1,800 coding sequences (presented at the CHRO conf. 2001 by S. Suerbaum, Würzburg Univ; and J. Fox, MIT, Cambridge). *H. hepaticus* is associated with chronic active hepatitis and liver cancer in many strains of immunocompetent mice and can also cause inflammatory bowel disorders in immunocompromised mice (129, 163). It lacks the *cag* (cytotoxin associated antigen) and *vacA* (vacuolating cytotoxin) genes which are essential virulence factors for *H. pylori*, but is bile resistant and commonly found in the intestinal tract of the animal. The genome sequence of *H. hepaticus* also shows other differences as compared to genes from *H. pylori*, and a few similarities to genes from *Campylobacter jejuni* and other bacteria. The availability of these three genome sequences has helped understand the molecular basis of pathogenesis caused by these strains.

H. pylori possesses enzymes for the oxidative and non-oxidative steps of the pentose phosphate pathway and for the Entner-Doudoroff pathway (94, 96). Among the

available carbon sources, *H. pylori* can use only glucose (97). For metabolism of pyruvate it possesses pyruvate oxidoreductase similar to that in hyperthermophiles, and it lacks both the pyruvate dehydrogenase required for aerobic metabolism and the pyruvate formate lyase required for anaerobic metabolism (67). It does not possess genes for synthesis of proteins that can metabolize arginine or histidine, so these two amino acids have to be provided as supplements in the growth media. Although glucose is the preferred carbon source, *H. pylori* can grow in its absence if the medium is supplemented with arginine, aspartate, asparagine, glutamate, glutamine and serine (95). Ammonia formed during urea hydrolysis also serves as a substrate for synthesis of glutamine by glutamine synthetase which is present in *H. pylori* (167).

As in other bacterial pathogens, *H. pylori* possesses genes for ferrous iron regulation (*fur*, *frpB*) (7, 169). The Fur protein is a repressor for iron-regulated genes when iron is in excess. *H. pylori* also has two iron storage proteins, Pfr and NapA (8, 152).

Also present are the enzymes required for synthesis of lipopolysaccharide (151). The polysaccharide component has Lewis^x and Lewis^y antigenic motifs which mimic the blood group antigens and the antigens present on the parietal cells of the human gastric mucosa (3, 4, 136).

In vitro studies have indicated that optimal growth of *H. pylori* occurs at pH between 6.0 and 7.0 and no growth was seen below pH 4.5 or above 8.0 (106). In the presence of urea however, it can survive even at a pH of 3.0 (21). This means that *H. pylori* is an acid-tolerant neutrophile and not an acidophile. To withstand the acidic conditions of the stomach, *H. pylori* has evolved a number of mechanisms, the most

significant one being the production of urease which increases the pH via urea hydrolysis and release of ammonia (90). Other than urease, *H. pylori* also possesses the ability to maintain the transmembrane proton gradient by adjusting the potential difference across the cytoplasmic membrane to compensate for changes in the pH gradient (99, 130). This helps in pH homeostasis. This proton gradient is formed by the reverse functioning of the $F_1 F_0$ ATPase (93). Proteome analysis for three strains of *H. pylori* (strains 26695, J99 and SS1) identified the proteins that are expressed at highest levels. These proteins are GroEL (chaperone), UreB (large subunit of urease), TsaA (Alkyl hydroperoxide reductase), and CagA (cytotoxin associated protein) (72). The intensity of two proteins increased when the pH of the media was increased from pH 5.0 to pH 8.0. These included the VacA protein, responsible for causing vacuolation of gastric epithelium, and a serine protease, HtrA, perhaps responsible for causing destruction of the mucus layer.

To date a number of factors responsible for colonization and virulence have been identified in *H. pylori* including a tuft of five to seven unipolar flagella which helps in penetration and spreading into the viscous mucus layer covering the gastric epithelium (59). That motility is an important factor for colonization was shown by the inability of non-flagellated variants of *H. pylori* to colonize the stomach of gnotobiotic piglets (38). The filament of the *H. pylori* flagella are made up of two flagellins, FlaA and FlaB, the expression of which is controlled by two different sigma factors, σ^{28} and σ^{54}, respectively (143). The protein shaft of the flagellum is protected by a membranous sheath, which is contiguous with the outer membrane and may protect the flagellum from enzymes and acids in the stomach. The bacterium has a corkscrew-type of motion due to its curve shaped body and this presumably helps in movement through the viscous mucin.

Although the majority of *H. pylori* cells are free living in the mucus layer, a proportion appear to adhere to epithelial cells or to the mucin layer by way of adhesins (63, 76). Virulence factors can be classified based upon the pathologic effects, which they cause. Some of them are responsible for the gastric inflammation, some for disruption of the gastric mucosal barrier and still others for changing the gastric physiology. *H. pylori* possesses a vacuolating cytotoxin (VacA) which can produce acidic vacuoles in gastric epithelial cells thus causing tissue damage (124). Inactivation of the *vacA* gene abolishes the cytotoxic activity of cells cultured *in vitro* (53). Strains with VacA producing ability have been isolated more often from patients suffering from peptic ulcer disease (44, 145). One of the main symptoms of gastritis is inflammation of the gastric mucosa which involves the infiltration of polymorphonuclear leukocytes and/ or monocytes. This is caused by a number of factors, such as Interleukin-8 (IL-8), which is induced by *H. pylori* upon infection and is a potent inflammatory mediator (24, 66). CagA, which is encoded by genes on the *cag* pathogenicity island, can induce the production of IL-8 by gastric epithelial cells, thus causing inflammation. Although the *cagA* gene is associated with the *vacA* gene, disruption of *cagA* did not abolish the cytotoxin activity of VacA (18, 25, 154). Another protein, NapA or neutrophil activating protein, activates neutrophiles thus causing them to aggregate (42).

H. pylori can also induce the formation of reactive oxygen species within the tissue which can cause oxidative damage to the host cells, thereby leading to gastric mucosal injury (28). Highly reactive superoxide radicals such as hydrogen peroxide or hydroxyl radicals are also formed as a result of oxidative burst of the infiltrating polymorphonuclear leucocytes. This is a protective mechanism by the host to resist

invasion by the pathogen. These radicals can be highly damaging to the bacterial proteins, lipids and DNA (15, 30, 43, 45, 68, 88). However, *H. pylori* has evolved three enzymatic-based mechanisms to protect itself from the oxidative damage. It synthesizes catalase, superoxide dismutase and alkylhydroperoxide reductase to protect against damage caused by hydrogen peroxide, superoxide radicals and alkyl hydroperoxide respectively (58, 122, 141). Thus these enzymes can also be considered as factors helping in the colonization of *H. pylori*.

One of the most important pathogenesis factors is the nickel containing enzyme urease, which is responsible for both colonization as well as virulence of *H. pylori* (36, 153). Urease is a potent stimulator of mononuclear leukocytes and also induces cytokine production which results in inflammation (55). A number of studies prove that urease is critical for colonization by *H. pylori.* In one such study, urease negative mutants failed to colonize gnotobiotic piglets (35, 36). Urease has also been shown to be responsible for histological damage due of the formation of ammonium hydroxide during urea hydrolysis (138). Another nickel containing enzyme, hydrogenase, has recently been shown to play a role in mouse colonization, since a hydrogenase structural gene mutant (in SS1 strain of *H. pylori*) showed only 24% colonization in contrast to 100% colonization by the parental strain (114).

Nickel metalloenzymes: Hydrogenase and Urease

Proteins containing metallocenters are widely distributed in nature. They participate in a number of essential cellular functions such as enzymatic catalysis, transport processes, regulation, and numerous electron transport reactions. In order for these enzymes to function, they have to undergo metallocenter assembly at their active

site. This process may involve a simple reversible binding of the metal ion, that may not require any accessory proteins, or a more complicated process that may require the participation of up to six or seven accessory proteins. To comprehend the mechanism of metallocenter assembly it is necessary to determine the role of these accessory proteins and the sequence in which they function. Both hydrogenases and ureases have been extensively studied in a number of bacteria. These two enzymes have served as models for study of metallocenter (specifically nickel) assembly.

Hydrogenases. A central metabolic feature of many prokaryotes and eukaryotes is the hydrogenase enzyme which is responsible for catalyzing the interconversion of molecular hydrogen to protons and electrons: $H_2 \leftrightarrow 2H^+ + 2e^-$

Hydrogen oxidation may be coupled to the reduction of different electron acceptors such as O_2, NO_3^-, SO_4^-, CO_2 or fumarate. The low potential electrons that are generated during this process can enter the electron transport chain, thereby generating energy via oxidative phosphorylation. Proton reduction or hydrogen evolution may be essential during fermentations for disposing of excess reducing equivalents. Both processes can contribute to the generation of a transmembrane proton gradient leading to the formation of ATP. Thus hydrogenases may play a significant role in biological energy conservation (158). The significance of hydrogen metabolism in bacteria is evident from the fact that a great metabolic investment goes into synthesizing an active hydrogenase.

Hydrogenases may be divided into hydrogen-evolving or hydrogen-uptake types. Hydrogen evolution is most often cytosolic, whereas hydrogen uptake is usually periplasmic or membrane-localized. Some bacteria may have more than one type of hydrogenase, one performing hydrogen evolution and the other hydrogen utilization,

which indicates the significant role played by hydrogenases in versatile bacteria. Nearly all the hydrogenases are metalloenzymes. There are three phylogenetically distinct classes of hydrogenases, the [Ni-Fe] hydrogenases, the Fe-hydrogenases and the metal-free hydrogenases (123, 158, 159, 173). Some hydrogenases also have selenium coordinated to the Ni^{+2} ion (60). The binuclear metal center consisting of an iron and nickel is ligated to the protein via four cysteine thiolate residues, two of which bridge the two metals. The iron is also liganded to two cyano and one carbonyl groups. Although the mechanism of insertion of the iron is not clear, it is known that it precedes that of nickel insertion (98). The [Ni-Fe] hydrogenase operon shows the presence of many genes, some of which are structural, and others that are primarily accessory genes. The large number of genes present in the operon is indicative of the complexity of the hydrogenase molecular structure and its maturation, and some of this complexity is due to the need for proteins that deal with metal (Fe or Ni) metabolism. The structural genes encode the large and the small subunits, with average masses of 60 and 30 kDa respectively (125), that interact to form a heterodimer. The bimetallic [Ni-Fe] center which is the active site of the enzyme is located within the large subunit. There are three [Fe-S] clusters within the small subunit (2[4Fe-4S] and 1[3Fe-4S]) that conduct the electrons between the hydrogen-activating site in the large subunit and the redox partner of hydrogenase. The third subunit (cytochrome b) acts as an anchor for the binding of the hydrogenase to the membrane. The accessory genes, referred to as the *hyp* genes (*hypA, hypB, hypC, hypD, hypE* and *hypF*), are involved in the maturation of this heterodimeric enzyme (17). These genes affect the activity of hydrogenase pleiotropically because

mutation in any one of them results in a deficiency in more than one type of hydrogenase activity.

Unlike the [Ni-Fe] hydrogenases that require many accessory genes for metallocenter assembly, very few genes may be involved in the maturation of the Fe-hydrogenases (123, 158). Only the structural genes have been well studied. Many of the Fe-hydrogenases are monomeric. The single subunit has the catalytic site consisting of two iron atoms and two [Fe-S] clusters, namely (1[2Fe-2S] and 1[3Fe-4S]) (131). These clusters may be involved in the transport of electrons from the active site to the redox partner. The catalytic site consists of the CO and CN ligands to the Fe atom just like in the case of the [Ni-Fe]-hydrogenases (111). This shows that CO and CN ligands are an absolute requirement for both [Ni-Fe] and Fe-hydrogenases.

Localization of hydrogenases may be intracytoplasmic, membrane-bound or periplasmic. Those hydrogenases that have to be exported into the periplasm or imported into an organelle have a characteristic N-terminal signal peptide in the small subunit. This signal peptide contains a conserved RRxFxK motif that is recognized by the twin-arginine translocase system, by which the fully active and correctly folded dimer can cross the membrane and enter the periplasm (158, 161, 172). The intracytoplasmic hydrogenases however, lack this signal peptide.

Hydrogenase is synthesized in the inactive form when any of the *hyp* accessory genes are mutated, which means that for the enzyme to function, it has to undergo a series of maturation steps involving each of the Hyp proteins. Extensive mutational analyses of hydrogenase accessory genes (*hyp*) from *Escherichia coli*, *Bradyrhizobium japonicum*,

Rhizobium leguminosarum and others, have provided us with a better understanding of the hydrogenase maturation process and the sequence of steps involved.

HypC. The first step in the maturation process is the insertion of iron into the active site of the large subunit precursor. However, because it has not been possible to obtain a Fe-free precursor, this step can only be assumed. The next step involves the formation of a complex between HypC and the precursor of the large subunit, pre-HycE (33). This complex was shown to be formed when a mixture of HypC and pre-HycE were run through a non-denaturing gel. The proof that HypC is involved prior to the nickel insertion steps comes from the observation that *hypC* mutants of *E. coli* accumulated a nickel-free precursor (70). It was further shown that HypC bound to a cysteine residue in the C- terminus of the large subunit and remains bound to it until completion of the nickel insertion step (83). From these results it has been speculated that HypC functions like a chaperone to the pre-HycE, guiding it through the post-translational maturation process.

To further analyze the sites of interaction between HypC and pre-HycE in *E. coli*, site-directed mutations were introduced replacing Cys2 and Pro6 of HypC, and Cys241, Cys244, Cys531 and Cys534 of pre-HycE with alanine. Different combinations of the mutant proteins were run through a non-denaturing gel and anti-HypC antibodies were used to detect any complexes being formed. Hydrogenase activities were also measured. The Pro6 mutant of HypC could form a complex with pre-HycE, however complex formation was abolished in the Cys2 mutant. Among the pre-HycE mutants, complex formation was not seen in Cys241 mutants. These mutants also did not incorporate nickel into hydrogenase and did not show any hydrogenase activity. The Cys244 and Cys531 mutants showed complex formation, but could not incorporate nickel and hence lacked

hydrogenase activity. The Cys534 mutants showed complex formation, nickel insertion and C-terminal processing, but still the enzyme was inactive. From these results, it was concluded that the interaction between the HypC and pre-HycE involved the Cys241 of the precursor and Cys2 of HypC; the Cys244 and Cys531 may bind the Fe and Ni moieties, and the Cys534 may be closing the bridge between the two metals after the processing has taken place (83).

HypF. The next step after formation of the pre-HycE–HypC complex is the insertion of the three diatomic ligands, 2(CN) and 1(CO) to the Fe- atom within pre-HycE (160). The proof that this step precedes the nickel insertion step comes from the observation that when cells are grown in nickel-starved conditions, the pre-HycE can be activated in vitro by nickel supplementation, which is possible only if the Fe $(CN)_2CO$ complex is already present within the precursor (85). To find the source of these ligands, a gene coding for carbamoyl phosphate synthetase was mutated. The mutant accumulated the precursor of the large subunit, which led to the conclusion that carbamoyl phosphate is the source for the CO and CN ligands. Additional proof came from the observation when the carbamoyl phosphate synthetase mutants were grown in the presence of citrulline, a precursor of carbamoyl phosphate, the processing steps took place and hydrogenase activity was observed (119). While *hypF* mutants did not show hydrogenase activity, a sequence motif similar to that seen in proteins catalyzing O-carbamoylations was present suggesting that HypF may be catalyzing the steps leading to the insertion of the CO and CN ligands using carbamoyl phosphate as sources of these ligands.

HypB. HypB is one of the well-studied Hyp proteins. HypB has been proposed to play a major role in nickel sequestration, since mutations in HypB of *E. coli* could be

phenotypically suppressed by addition of high concentrations of nickel (0.6 mM) to the growth medium (164). Additional proof for a Ni-metabolism role came from studies with purified HypB from *R. leguminosarum*, which could bind 3.9 ± 0.1 Ni^{+2} per monomer, and the purified HypB from *B. japonicum* could bind 9.0 ± 0.14 Ni^{+2} per monomer (49, 126). The Ni^{+2} binding ability was attributed to the presence of a histidine-rich N-terminus in HypB of these bacteria. When 23 out of 24 histidine residues were deleted from the N-terminus of HypB from *B. japonicum*, the truncated protein bound 1.19 ± 0.12 Ni^{+2} per monomer, which means that internal residues are also involved in nickel binding (113). A chromosomal *hypB* mutant of *B. japonicum* that lacked the N-terminal histidine residues showed less hydrogenase activity, however the activity could be restored to almost wild type levels by growing the mutant in the presence of 50 μM nickel (112). HypB from *E. coli* and *Ralstonia eutropha* lack the histidine-rich domain, but have efficient nickel uptake systems to fulfill this requirement (40). Many of the HypB proteins also have conserved GTP-binding motifs, which have been shown to hydrolyze GTP. HypB from *E.coli* had low GTPase activity with a K_{cat} of 0.17 min^{-1} and a K_m of 4 μM for GTP (86). HypB from *R. leguminosarum* did not possess GTPase activity, however that from *B. japonicum* had an activity with a K_{cat} of 0.18 min^{-1} and the K_m of 7 μM for GTP (49, 126). A mutant HypB protein in the conserved lysine residue within the GTP-binding motif from *B. japonicum* showed nickel-binding ability similar to the wild-type, but had very low GTPase and no hydrogenase activity. The activity could not be restored even after prolonged incubation with nickel. In contrast, a mutant protein which lacked the 23 N-terminal histidine residues had GTPase activity similar to the wild-type protein, and had low hydrogenase activity (86, 113). From these results it can

be concluded that hydrogenase maturation is completely dependent on the intrinsic GTPase activity of HypB in *B. japonicum*. Similarly mutants in the conserved lysine residue within the GTP-binding motif or the aspartate residue in the G-4 motif of HypB from *E.coli* could not hydrolyze GTP and the hydrogenase activity was also adversely affected (87).

HypA. Although no precise function has yet been identified, HypA has been predicted to interact with metal ions because of its high cysteine content (8.5% for *E. coli*). It has also been predicted that a promoter lying within *hypA* in *E. coli* is responsible for transcription of the *hypB*, *hypC*, *hypD* and *hypE* genes under non-fermentative conditions (80). A mutant in *hypA* lacked only the hydrogenase isoenzyme-3 activity, unlike the mutants in *hypB*, *hypD* or *hypE* which lacked hydrogenase isoenzyme-1, 2 and 3 activities (70). Sequence comparison of HypA from different bacteria showed the presence of a conserved CX_2–C-X_{12-13}-CX_2-C motif near the C-terminus which is characteristic of metal binding domains of non-heme iron proteins (48, 62).

HypD and HypE. Although mutations in *hypD* and *hypE* have been shown to inhibit the activity of all three hydrogenase isoenzymes in *E. coli*, no information is available regarding the precise function of these proteins (70). HypD contains a conserved C-X-X-C motif which is characteristic of metal binding proteins.

Endopeptidase. Pre-HycE is cleaved at a conserved histidine near the C-terminal end only after nickel insertion, because a nickel-free precursor does not undergo proteolysis. Cleavage is brought about by a specific endoprotease (127, 128). The protease, HycI, which is involved in the maturation of hydrogenase isoenzyme-3, has been purified from *E. coli*. Substitution of the nickel in the large subunit with zinc

resulted in an inactive precursor which was no longer amenable to proteolytic processing. This indicates that the recognition site for the endopeptidase is highly specific (82, 146). The C- terminal cleavage triggers a conformational switch in which the free thiol of a C-terminal cysteine (C534) residue closes the bridge between the two metals (Fe and Ni) resulting in the formation of a complete binuclear center.

Working model for hydrogenase metallocenter assembly. Studies with hydrogenase isoenzyme-3 from *E. coli* have predicted the following steps in the maturation of the [Ni-Fe] hydrogenases (Figure 1.1). After Fe insertion, a complex is formed between HypC and pre-HycE, followed by liganding of 1(CO) and 2(CN) to the Fe within the pre-HycE. HypF may play a role in the liganding step. Nickel is then inserted into the partially unfolded large subunit precursor. The HypB accessory protein has been proposed to deliver the nickel, a reaction which requires GTP hydrolysis which is also catalyzed by the same protein (HypB). Throughout the initial maturation steps, the HypC accessory protein remains bound to the N-terminus of the large subunit and is therefore assumed to function as a molecular chaperone. HypC releases the large subunit after nickel insertion, at which point endopeptidase cleavage of the large subunit occurs. The large subunit then forms a heterodimer with the small subunit giving rise to a catalytically active mature enzyme. The enzyme is then translocated through the inner membrane into the periplasmic space or remains in the cytosol. The translocation of the periplasmic hydrogenases is directed by the signal peptide within the small subunit.

Ureases. Urease is a high molecular weight multi-subunit metalloenzyme produced by a number of taxonomically diverse bacterial species, and some plants and fungal species. It was the first enzyme to be crystallized (from jack bean) (144) and also

the first enzyme shown to contain nickel (31). Many years later it was crystallized from *Klebsiella aerogenes* and its three-dimensional structure was elucidated (69). Study of urease is significant because it serves as a model for nickel metallocenter assembly, and also because of its association with health related conditions such as urinary stones, catheter encrustation, acute pyelonephritis, and peptic ulceration (103). Urease catalyzes the hydrolysis of urea to yield ammonia and carbamate. Carbamate again decomposes to yield another molecule of ammonia and carbonic acid:

$$H_2N - CO - NH_2 + H_2O \rightarrow NH_3 + H_2N - C(O)OH$$

$$H_2N - C(O)OH + H_2O \rightarrow NH_3 + H_2CO_3$$

The ammonia formed can elevate the pH of the urine and under such alkaline conditions some salts tend to precipitate leading to the formation of urinary stones.

Nucleotide sequences are available for the urease structural and accessory genes from five bacterial species including *H. pylori* (20, 75), *Proteus mirabilis* (71), *K. aerogenes* (78), *Yersinia enterocolitica* (29) and *Bacillus sp.* strain TB-90 (81). Each of these bacteria have only one chromosomal copy of the urease genes. There are three structural genes, *ureA*, *ureB* and *ureC* in all these bacterial species except for *H. pylori,* and four accessory genes *ureD*, *ureE*, *ureF* and *ureG*. In *H. pylori, ureA* and *ureB* appear to be fused, and is designated as *ureA*. The *ureC* in other bacteria is designated as *ureB* in *H. pylori*. *Bacillus sp.* strain TB-90 has two additional accessory genes, *ureH* and *ureI. H. pylori* also has *ureH* (which is called *ureD* in *K. aerogenes*) and *ureI.* The regulatory gene *ureR* is present only in those gene clusters which are inducible by urea, like in *P. mirabilis* (110). UreR is a positive transcriptional regulator (32, 148). It is absent in *H. pylori*, *Bacillus sp.* strain TB-90, *Y. enterocolitica* and *K. aerogenes*. In *K. aerogenes*, the

urease genes are activated through a two-component signal tranduction system that globally regulates expression of genes in response to nitrogen availability (22, 134). Neither urea nor nitrogen availability affects the urease expression in *Streptococcus salivarius*. However expression of urease in this organism is lowered at neutral pH (19).

All ureases that have been characterized to date have nickel in the active site, and their activity is dependent on the presence of nickel in the growth medium. Urease from *K. aerogenes* is a trimer of trimers with three copies each of UreA, UreB and UreC (molecular weights of 11.1, 11.7 and 60.3-kDa respectively) (108, 150). Atomic absorption shows the presence of two nickel ions separated by 3.6 Å in the active site of each of the large subunits (UreC) (149). The ligands to the nickel ions include a bridging lysine carbamate, a water molecule, four histidine imidazoles with two bound to each nickel ion, and an aspartic acid residue bound to one of the nickel ions (69).

In vivo activation of urease requires the participation of several accessory genes. Native urease was purified after co-transformation of *E. coli* cells with two plasmids, one which had all the urease structural genes (*ureA, ureB, ureC),* and another which had all the urease accessory genes (*ureD, ureE, ureF* and *ureG)* from *K. aerogenes.* However, an inactive apourease was obtained with only the plasmid carrying the urease structural genes. The apourease had the same subunit stoichiometry and molecular size as the native urease, however it had sixteen-fold less nickel than the native urease. Thus one or more urease accessory genes may be involved in the incorporation of nickel into the active site (108). When *K. aerogenes* was grown in nickel-depleted medium, an inactive apourease was formed that lacked nickel but had the same heteropolymeric structure as the native enzyme (77). This means that nickel is not required for subunit association but is required

for activation of the apourease. The *in vivo* requirement for nickel is very stringent. The reason nickel is preferred over other trace metals is not known.

When *E. coli* cells containing the *K. aerogenes* urease gene cluster were grown in LB medium lacking nickel, a slower migrating complex (UreDFG-apoprotein) was seen in addition to other complexes. This complex has been speculated to be the cellular urease activation complex (105, 115, 116, 117). In the absence of UreE and GTP, a very high concentration of bicarbonate (100 mM) and nickel (100 μM) was required for *in vitro* activation of the complex (140), which is not the case under physiological conditions. Recent studies have shown that the complex could be activated *in vitro* in the presence of low concentrations of nickel (20 μM) and bicarbonate (100 μM), but only if GTP (200 μM) and UreE (20 μM) were also present (139). The authors speculated that the carbon dioxide from bicarbonate reacted with the lysine residues forming lysine carbamate which served as a bridge between the two nickel ions in the active site. The presence of other metals like zinc, cobalt or manganese reduced the activation efficiency of the apoprotein *in vitro*, indicating that the specificity for nickel *in vitro* is low when compared to that *in vivo* (117). All these *in vitro* results highlight the efficiency of the *in vivo* assembly process where not only is the specificity for nickel very high, but it is also bound in a productive way (mobilized to urease), and consequently the Ni-enzyme assembly takes place using physiological concentrations of nickel and bicarbonate.

The significance of the urease accessory genes in metallocenter assembly was shown with mutants that lacked *ureD*, *ureF* or *ureG* in *K. aerogenes*. Mutation in any one of these genes produced an inactive urease (78, 108), indicating that the products of these genes are required for the functional incorporation of the urease metallocenter. There was

no difference in the expression of the urease polypeptides between wild-type and the deletion mutant strains as shown by immunological studies, which means that these accessory genes act at the post-translational level. Urease apoprotein made by these mutants also behaved similarly on SDS-PAGE and gel filtration chromatography. The purified apourease from the mutant strain contained little or no nickel and was also devoid of enzyme activity. A deletion mutation in *ureE* was not totally devoid of urease activity unlike the *ureD, ureF,* and *ureG* mutants (78). Some specific roles played by these accessory proteins have been proposed (see below).

UreD. High levels of urease apoprotein was expressed by *E. coli* cells harboring a plasmid containing the promoter of *ureD* from *K. aerogenes* (altered to introduce an ATG initiation codon and a more efficient ribosome binding site) and the urease structural genes, when grown in the absence of added nickel (see above) (115, 116). The apoprotein was complexed with UreD. Addition of nickel ions (100 μM) and bicarbonate (100 mM) resulted in the activation of the UreD-apoprotein complex *in vitro*. Following activation, UreD dissociated from the complex. Hence UreD may function as a chaperone that stabilizes the apoprotein conformation making it competent for nickel insertion. Although it has been shown that UreD-apoprotein complex can be activated *in vitro* when both nickel and bicarbonate are added, this may not be the case *in vivo*. Deletions in *ureF* or *ureG* resulted in loss of urease activity, which means that in addition to UreD, the cells also need UreF and UreG for activation of urease *in vivo*.

UreG. UreG is the most highly conserved urease accessory protein among ureolytic bacteria. UreG also shares homology with the HypB protein from bacteria such as *E. coli* (86, 171) and *B. japonicum* (48). Four conserved regions are present in UreG,

one of which, the P-loop motif, has been implicated to be a nucleotide-binding motif. This motif is also present in HypB where it has been shown to bind and hydrolyze GTP (49, 87). Hence UreG from *K. aerogenes* may also be capable of binding and hydrolyzing GTP. UreG, however, failed to bind significant levels of ATP or GTP in equilibrium dialysis experiments, nor did it bind to ATP or GTP- linked agarose resins. To assess the importance of the conserved P-loop motif in UreG, site-directed mutants were constructed. Two P- loop mutations K20A and T21A did not affect the formation of the UreD-apoprotein or the UreDF-apoprotein complex, but did affect the formation of the key activation complex, the UreDFG-apoprotein, when cells were grown in the absence of nickel (104). These P-loop variants did not possess any urease activity even when grown in the presence of nickel. Hence an intact P-loop motif may be required *in vivo* for the UreG to carry out nucleotide hydrolysis and help in urease metallocenter assembly.

UreE. An *ureE* deletion mutant showed reduced urease activity. The activity could be restored to wild-type levels by supplementing the medium with high concentrations of nickel. This indicated that the UreE protein may have a role in nickel binding or sequestration. In addition to this, the presence of a histidine-rich tail at the C-terminus made UreE a strong candidate for nickel binding (79). It was shown to reversibly bind six nickel ions per dimer with a K_D of approximately 10 μM in equilibrium dialysis studies. Additional evidence of its ability to bind nickel came from X-ray diffraction studies with UreE crystals which cracked upon addition of nickel. This is consistent with the induction of a conformational change upon binding to nickel. From earlier studies it had been speculated that UreE binds to nickel via its polyhistidine tail and serves as nickel donor during urease activation. However, since the UreE from *H.*

pylori, *Ureaplasma urealyticum* and *Streptococcus salivarius* lack this polyhistidine tail, the role of the C-terminal histidine tail is not yet clear (19, 26, 109). To address the role of the histidine tail, a plasmid was constructed which encoded a truncated UreE (H144*). This protein lacked fifteen residues from the C-terminus, but had a very small effect on the urease activity as compared to the wild-type protein. The truncated UreE (H144*) associated into a homodimer just like the wild-type protein and bound 1.9 ± 0.2 nickel ions per dimer (14). Two internal UreE deletion mutants however, showed significantly lowered urease activity, which indicates that the crucial residues for nickel binding are not localized to the C-terminus, but are distributed in the internal sequence.

Studies with site-directed mutants revealed that the metal-binding site associated with His96 of UreE is critical for the functioning of UreE as a metallochaperone to facilitate urease activation (23). Other studies showed that in the presence of physiological concentrations of bicarbonate (100 μM), nickel (20 μM) and GTP (200 μM), the UreDFG-apourease complex gave very low enzyme activity (approximately 250-500 units/mg). This value was ten-fold less than the wild-type urease activity. At the same concentrations of bicarbonate, nickel, and GTP, a very high activity (approximately 2300 units/mg) was generated from the UreDFG-apourease complex when 20 μM truncated UreE (H144*) was present (139). The ability of H144*UreE to enhance urease activation at physiologically relevant concentrations of nickel and bicarbonate, points to an important role for UreE in metallocenter assembly. Maximal activation was observed at a concentration of UreE (20 μM) equivalent to that of added nickel (20 μM).

UreF. Although deletion mutants have indicated an absolute requirement of UreF for urease activity, the role of this protein is not yet clear. It has been speculated that

UreF prevents nickel from binding to the active site of UreD-apoprotein complex until after the formation of the lysine carbamate metallocenter ligand (105).

Working model for urease metallocenter assembly. In summarizing various findings involving urease genes from *K. aerogenes*, one can speculate on the functions of the accessory proteins and the sequential steps involved for metallocenter assembly (Figure 1.2). UreD may be acting as a molecular chaperone that maintains the apoprotein in a proper conformation so that the complex is competent for nickel insertion. Due to the ability of UreE to bind nickel,UreE seems to be a good candidate for playing a role as a nickel donor to the apoprotein. UreG may function in nucleotide hydrolysis and could bring about GTP-dependent structural changes in the urease apoprotein complex, making it more accessible either to nickel ions, carbon dioxide, or to both substrates. UreF may prevent the binding of nickel to the non-carbamylated apoprotein complex.

Helicobacter pylori: Hydrogenase and Urease

***H. pylori* hydrogenase.** Initial evidence of the presence of hydrogenase in *H. pylori* came from the observation of a significant homology between the hydrogenase structural genes from *Wolinella succinogenes* and a group of genes from *H. pylori* (27). Further studies with whole cells of *H. pylori* showed the presence of an O_2-dependent H_2-uptake activity that was detected amperometrically, so the existence of a respiratory H_2 oxidizing chain was investigated. Membranes isolated from *H. pylori* could couple H_2 oxidation to the reduction of a variety of positive potential electron acceptors like methylene blue, phenazine methosulfate, DCPIP and ferricyanide (84). It was therefore hypothesized that hydrogenase in *H. pylori* is poised at a redox potential which promotes H_2-oxidation, similar to other [Ni-Fe] hydrogenases, thus making it a hydrogen-uptake

type hydrogenase. The coupling of H_2-oxidation to O_2 –reduction also indicates the presence of a complete H_2-oxidizing electron transport chain associated with the membrane of *H. pylori.* The electrons could enter the respiratory chain and thus may contribute to the energy conservation. Immunoblotting of *H. pylori* membrane extracts with antisera prepared against the large and small hydrogenase subunits from *B. japonicum* revealed the presence of two subunits of sizes similar to that in *B. japonicum*. The sizes of the large and the small subunits were approximately 65 and 26 kDa respectively which matched with the subunit size of other [Ni-Fe] hydrogenases (84). Although hydrogenase activity is constitutive in *H. pylori*, it increased five-fold when cells were grown in the presence of 10% hydrogen (114). This increase in activity was shown amperometrically (84). That hydrogenase expression is regulated at the transcriptional level was shown by fusing the promoter of the hydrogenase subunit to the *xylE* reporter gene. A four-fold increase in expression of *xylE* reporter gene in presence of hydrogen indicated that expression of the hydrogenase structural genes is positively regulated in response to hydrogen. The apparent K_m for hydrogen (in whole cells of *H. pylori* grown with H_2) was estimated to be around 1.8 μM. The average hydrogen content of the mucus layer of mouse stomach is approximately 43 μM, indicating that the hydrogenase is saturated and probably functioning at maximum velocity under most conditions inside the animal. The source of hydrogen in the host is probably fermentation reactions carried out by the colonic bacteria. A strain of SS1 (which can colonize the mouse stomach) with an insertional mutation within the hydrogenase large subunit colonized only 24% of mice as compared to 100% colonization by the wild-type strain.

This observation led to the conclusion that hydrogen serves as an energy yielding substrate for *H. pylori* and helps to maintain this pathogen in the stomach (114).

Complete genome sequencing of *H. pylori* strains 26695 and J99 revealed the presence of a single copy of hydrogenase structural (*hydA, hydB, hydC, and hydD)* and accessory (*hypA, hypB, hypC, hypD, hypE, hypF)* genes (2, 151). The function of each of these genes have to be yet elucidated in *H. pylori*. Considerable homology exists between the hydrogenase structural and accessory genes with the corresponding genes from other bacteria. The organization of these genes, however, is remarkably different from one bacterium to another. The *hyp* accessory genes in *E. coli* and *B. japonicum* are present within the same cluster adjacent to the hydrogenase structural genes (although in *E.coli*, they are divergently transcribed) (47, 80). In contrast, the accessory genes of *H. pylori* are not present in a single cluster, nor are they adjacent to the structural genes. The *hypA* gene occurs singly, the *hypB, hypC* and *hypD* form a cluster, and the *hypE and hypF* form yet another cluster. The structural genes, *hydA, hydB, hydC and hydD* are, however, present in a single cluster (2, 151).

Since nickel incorporation into the active site of hydrogenase is essential for the complete maturation and functioning of the enzyme, *H. pylori* has to possess proteins capable of binding and/or mobilizing nickel into the active site. In addition, since HypB from *E. coli* and *B. japonicum*, and UreG from *K. aerogenes* have been speculated to help in the metallocenter assembly via their ability to bind and hydrolyze GTP, it is possible that *H. pylori* has also recruited similar proteins to perform this function (48, 49, 86).

***H. pylori* urease.** Urea, the substrate of urease hydrolysis, is a major nitrogenous waste product of many terrestrial animals. It is carried from the liver via the bloodstream

into the kidneys and excreted in the urine. Serum levels of urea range from 1 to 11 mM. It is also secreted by the exocrine glands. Since there is no active efflux of urea from these secretions, it must diffuse through the cells and tight junctions of the epithelium. Thus, the epithelial surfaces contain urea at presumably similar concentrations as the serum. Although *H. pylori* is known to survive very well in the extremely acidic environment of the gastric mucosa, it is quite sensitive to effects of low pH *in vitro* (90). Once inside the stomach, the lumen is the first barrier that the bacterium faces and because of its extreme acidity (pH values ranging from 1 to 2) the environment encountered is a harsh one. It has therefore been proposed that the organism survives this acidity and enters the mucin layer, pH 7.0 to 8.0, via its ability to hydrolyze urea (Figure 1.3). This reaction is catalyzed by the potent multi-subunit enzyme, urease. It can hydrolyze urea to yield two molecules of ammonia and one of carbonic acid. In solution the carbonic acid and ammonia are in equilibrium with their de-protonated and protonated forms respectively. The net effect of this equilibrium is an increase in the pH of the lumen.

$$H_2CO_3 \leftrightarrow H^+ + HCO_3^-$$

$$2NH_3 + 2H_2O \leftrightarrow 2NH_4^+ + 2OH^-$$

Once the bacteria penetrates the lumen and enters the mucin layer, it adheres to the gastric epithelium by means of adhesions (63). It colonizes, starts multiplying and provokes an inflammatory response using virulence factors such as the reactive oxygen species, vacuolating cytotoxin, CagA, LPS, neutrophil activating protein and urease to cause damage to the mucosa (Figure 1.3) (3, 18, 24, 25, 28, 35, 44, 53, 55, 66, 101, 103, 124, 138, 145, 153).

Urease constitutes up to 10% of the total soluble cytosolic protein. The fact that it is also present on the cell surface has raised lot of controversies of its exact location (57). The urease activity *per se* does not depend on its localization, since the cytoplasmic membrane is freely permeable to urea. Since urease has a very low K_m for urea (approximately 0.3 ± 0.1 mM), it is efficient even when only submillimolar concentrations of urea are available, thus the pathogen is well suited for the gastric mucosa where the urea concentration is only around 4 mM (34, 65). Under saturating conditions, the specific activity of urease is around 1.7 nmoles urea hydrolyzed per min per mg protein. The structural genes encoding urease and the accessory genes are present in the form of a single cluster on the *H. pylori* chromosome (*ureC, ureD, ureA, ureB, ureI, ureE, ureF, ureG,* and *ureH)* (2, 20, 26, 75, 151). Out of the nine genes, only seven genes are required for synthesis of a functional urease. Mutations within the *ureC* and *ureD* did not affect the urease activity. The *ureA* and *ureB* encode the two structural subunits which make up an inactive apoenzyme in the absence of the accessory proteins (64). The five accessory genes *ureI, ureE, ureF, ureG* and *ureH* are required for making a catalytically active enzyme (26). Except for UreI, the other four accessory proteins are believed to interact with the apoenzyme directly to deliver nickel into its active site. UreI, an integral membrane protein, has been proposed to function as H^+ gated urea channel, which allows the entry of urea into the cell only when the pH of the external medium drops to less than 6.5. This was shown by use of the heterologous *Xenopus oocyte* expression system (16, 165, 166). Once inside the cell, the urea gets hydrolyzed and the pH rises. As the pH reaches neutrality, the UreI pore closes thus stopping further entry of urea and preventing the formation of excessive ammonia (135). The UreI-regulated

production of ammonia in the cytoplasm followed by back diffusion of ammonia into the periplasm is thought to maintain periplasmic pH at harmless levels, even under conditions of high acidity. Thus UreI is a prerequisite for *H. pylori* acid resistance. The ammonia formed by urea hydrolysis may also serve as a source of nitrogen for the synthesis of glutamine. The gene encoding the enzyme glutamine synthetase is present in the *H. pylori* genome (52). The ammonia formed may also have adverse effects on the gastric mucosal tissue because of its cytotoxicity (138).

When crude extracts of *H. pylori* are run on a SDS-polyacrylamide gel followed by staining with Coomasie blue, the large (UreB, 66 kDa) and the small (UreA, 29.5 kDa) subunits of urease were seen as prominent bands (65). Since urease constitutes a major part of the total soluble proteins, it could be easily expressed in *H. pylori* and purified by anion exchange and gel filtration chromatography. Purified urease has an estimated molecular size of 550 kDa with a subunit stoichiometry of six copies each of UreA and UreB (34, 41, 65). The active site of the enzyme is located within the UreB subunit. There are two nickel ions per active site, which means that there are a total of 12 nickel ions per enzyme molecule, since there are six copies of the UreB (56). N- terminal sequence of the small subunit of *H. pylori* urease resembled that of the jack bean urease, although there is only one subunit in jack bean. The *H. pylori* UreA and UreB can be aligned to the UreA, UreB and UreC subunits of *K. aerogenes* and other ureolytic bacteria, and also to the single polypeptide of jack bean urease (Figure 1.4).

A recombinant urease apoenzyme was also purified from *E.coli* transformed with a plasmid which carried the *ureA* and *ureB* genes from *H. pylori*. The K_m of the recombinant urease was around 0.2 to 0.3 mM and the size of the recombinant enzyme

matched that of the native enzyme. The large and the small subunits were also visible on Western blotting with anti-UreA and anti-UreB antibodies. However this enzyme lacked activity, which means that although only the *ureA* and *ureB* genes are required for expression and assembly of the urease apoenzyme, additional genes are required for making a catalytically active enzyme (26, 64, 75, 92). Urease activity was seen temporarily when the shuttle plasmid containing the cloned *H. pylori ureA and ureB* genes was introduced into *Campylobacter jejuni*, which means that the urease accessory genes of *C. jejuni* may be compensating for the role played by the accessory genes of *H. pylori.* Using the shuttle plasmid the *ureC*, *ureD, ureA* and *ureB* genes were sequenced (75). There are two promoter sequences, one preceding UreC and the other preceding UreA and four predicted Shine-Dalgarno sequences, one prior to the start codon of each gene. The accessory genes were also sequenced and they appear to be transcribed from the same promoter and in the same direction as the structural genes (26). Intergenic distance between *ureD* and *ureA* is around 420 base pairs and that between *ureB* and *ureI* is around 200 base pairs.

The urease accessory genes from *H. pylori* have been studied to a lesser extent as compared to those from *K. aerogenes.* A *H. pylori* mutant strain deficient for *ureG* (N_6 strain) was unable to colonize normochlorhydric piglets but could colonize poorly the achlorhydric piglets. Unlike the mutant strain, the wild-type strain could efficiently colonize both the normochlorhydric and the achlorhydric piglets. Although the *ureG* mutant strain was deficient in urease activity, the level of expression of urease was not affected. When the mutant strain was co-inoculated with the wild-type strain, only the wild-type strain colonized the host. From these results one can conclude that urease

activity and not just urease expression is essential for colonization. However since the *ureG* mutant strain could not colonize the achlorhydric piglets very well, it also means that the primary function of urease is not just pH protection, but may be some other as yet unidentified function. Lastly, the inability of the *ureG* mutant strain to colonize the piglets even in presence of the wild-type indicates that the protection is independent of diffusible products of urea metabolism (35, 36, 37).

When *E. coli* cells harboring a plasmid with the entire *H. pylori* urease gene cluster were grown in minimal medium (M_9), supplemented with 0.4% glucose and 10 mM concentration of any single nitrogen source such as L-arginine, L-glutamine, urea, or ammonium chloride, urease activity was seen. However this activity was almost 27-fold less than that shown by wild-type *H. pylori* (26). To identify the genes which might be responsible for regulating urease activity in *E. coli*, the cells were cotransformed with a plasmid which had all the *H. pylori* urease genes along with another plasmid with cloned genes from a ZAP-derived plasmid library. Those genes which increased the activity were called the urease enhancing factors (UEFs) and those which lowered the activity were called the urease decreasing factors (UDFs). Among the common UEFs detected were *helA* and *helB* (both encoding a DNA helicase), HP0511 encoding for a lipoprotein and another gene which encodes an ATP-binding cassette. One significant UEF identified was a gene designated as *nixA* which possessed characteristics of an integral membrane protein. Among the UDFs commonly identified was the *flbA* gene, which is involved in flagella biosynthesis. The significance of the *flbA* gene was also seen in mutation within this gene, which slightly increased the urease activity. From these results one can

conclude that there might be a correlation between flagellar biosynthesis and urease activity (92, 132).

To synthesize catalytically active urease, it is necessary to incorporate two Ni^{+2} ions into each active site of urease (103). Due to the low availability of Ni^{+2} ions in the serum (2-11 nM), and the high requirement of Ni^{+2} (12) ions per urease molecule, the organism has to possess high affinity nickel transporter proteins. In addition to this, urease constitutes almost 10% of the total cytosolic proteins. This must create a heavy metabolic (Ni-sequestering) burden on the pathogen. Sequence homology studies with NixA of *H. pylori*, showed that it shared 25% amino acid sequence identity with HoxN, the high affinity nickel transport protein in *Alcaligenes eutrophus*. HoxN is necessary for high levels of hydrogenase and urease activity when *A. eutrophus* cells are grown in Ni^{+2} limited medium (168). NixA also shared 40% amino acid sequence identity with HupN, an accessory gene in the hydrogenase cluster of *B. japonicum* (46). When a plasmid containing the urease gene cluster was expressed in *E. coli*, the subsequent urease activity was low (< 0.01 μmol ammonia evolved per min per mg protein). However when this plasmid was coexpressed along with another plasmid which had the *nixA* gene from *H. pylori*, the activity went up to 36 μmol ammonia evolved per min per mg protein. Since urease activity went up when the *H. pylori* urease genes were coexpressed along with the *nixA* gene, it was a likely candidate for conferring nickel transport ability (102). It should be noted however that the urease activity seen in *E. coli* containing the *H. pylori* urease genes is 2- to 9-fold less than that seen in *H. pylori.* Further studies showed that NixA has high affinity for Ni^{+2} ions (K_T of 10 nM) and has eight transmembrane domains, with the amino and carboxy termini located within the cytosol (51). Mutation in the highly

conserved aspartate, glutamate or histidine residues located within the transmembrane domain affected the uptake of Ni^{+2} ions and also the urease activity (50).

An insertional mutation within the *nixA* gene on the *H. pylori* chromosome reduced the nickel uptake capacity by almost 30% and the urease activity by almost 42%. Thus NixA seems to be a highly efficient transporter which can scavenge Ni^{+2} ions (5). Since the *nixA* mutation did not completely abolish the nickel uptake or the urease activity, this bacterium must possess alternate Ni^{+2} uptake mechanisms to help provide a source of nickel for activation of urease.

Other proteins which may be likely candidates for nickel binding are the heat shock protein, HspA, because of the presence of a metal binding C-domain and also because it has been shown to bind nickel with a K_D of 1.8 μM. In addition to this, when *hspA* was coexpressed along with the urease gene cluster containing plasmid in *E. coli*, the urease activity increased by four-fold (73). Four genes homologous to the ATP-dependent nickel transport system in *E. coli* were identified in *H. pylori* (*abcABCD*). Allelic exchange mutagenesis in one of the genes, *abcC* decreased the urease activity by 88%. Creation of a double mutant (in *nixA* and *abcC*) almost abolished the urease activity (61).

When a clinical isolate of *H. pylori* or the 26695 strain was grown in BBN medium (*Brucella* broth supplemented with 3% newborn calf serum) with and without nickel supplementation (1 or 100 μM Ni^{+2}), there was an increase in the expression of the UreA and UreB subunits as the nickel concentration increased. This was supported by immunoblotting experiments (156). The increase in urease expression was accompanied by an increase in the urease activity and the number of mRNA transcripts coding for

UreA and UreB. When the *ureA* promoter was fused upstream to a promoterless *lacZ*, and this fragment was introduced into *H. pylori* chromosome by recombination, the β-galactosidase activity increased 3-fold when the medium was supplemented with 100 μM nickel. These results indicate that urease expression in *H. pylori* is mediated at the transcriptional level and depends on the concentration of nickel in the medium. Further studies identified a gene, *nikR* in *H. pylori*, which is homologous to the *nikR* in *E. coli* and was shown to be responsible for nickel responsive induction of urease expression (157). Mutation in *nikR* did not affect urease subunit expression or its activity in unsupplemented media, but the nickel-induced increase in urease expression was not seen in nickel supplemented media. This shows that NikR acts as an inducer of the urease operon in *H. pylori*, unlike in *E. coli*, where NikR acts as a repressor of the nickel-uptake genes.

Another method of regulation of the urease operon which occurs at the transcriptional level has been shown to be due to mRNA decay in response to pH (1). Transcription of urease genes in *H. pylori* occurs from two promoters, one located upstream of *ureA*, and another between *ureB* and *ureI* (137). Studies with mRNA has shown that the urease operon produces three transcripts: the *ureAB*, *ureABIEFGH* and the *ureIEFGH*. The *ureAB* transcript was seen at acidic and also alkaline pH, however the *ureIEFGH* transcript was barely observable at pH above 6.0. The *ureABIEFGH* transcript was predominant at acidic pH. These transcripts were cleaved to produce several different mRNAs in response to environmental pH. Cleavage may be brought about by exoribonucleases or endoribonucleases. Hence it was concluded that regulation of urease appears to take place by fine tuning the post transcriptional expression of the accessory

genes via mRNA decay, and also that the transcriptional organization of the urease operon is designed in such a way so as to allow for maximum expression of the *ureA*, *ureB* and *ureI* genes at all times (1).

Purpose of the study

From previous studies it can be concluded that there are a few striking similarities in the maturation of hydrogenase and urease: both of them require a nickel sequestering protein, a chaperone to hold them in a conformation competent for nickel insertion, and a GTP hydrolyzing protein to catalyze the steps leading to nickel incorporation. The models for hydrogenase and urease maturation in *E. coli* and *K. aerogenes* may share some similarities with the hydrogenase and urease metallocenter assembly in other bacteria, but may not be necessarily equivalent. Although the players for the assembly have been identified, their precise roles and the sequence in which they function needs further investigation. In the case of *H. pylori* the urease accessory genes have been studied to some extent, however no work has been done on the hydrogenase accessory genes. Owing to the pathogenicity of *H. pylori*, it is important to learn more about the function of the hydrogenase accessory proteins, especially in light of the recent observation that a structural hydrogenase gene mutant was less efficient in its ability to colonize the mouse stomach. Since urease is a crucial enzyme for *H. pylori*, it is possible that along with the urease accessory proteins, this pathogen would have recruited one or more of the hydrogenase accessory proteins for the catalytic activation of urease. The study of *H. pylori* accessory proteins will also help in improving our understanding about the role of accessory proteins in the maturation of nickel-containing enzymes.

Bibliography

1. **Akada, J. K., M. Shirai, H. Takeuchi, M. Tsuda, and T. Nakazawa.** 2000. Identification of the urease operon in *Helicobacter pylori* and its control by mRNA decay in response to pH. Mol Microbiol **36:**1071-84.

2. **Alm, R. A., L. S. Ling, D. T. Moir, B. L. King, E. D. Brown, P. C. Doig, D. R. Smith, B. Noonan, B. C. Guild, B. L. deJonge, G. Carmel, P. J. Tummino, A. Caruso, M. Uria-Nickelsen, D. M. Mills, C. Ives, R. Gibson, D. Merberg, S. D. Mills, Q. Jiang, D. E. Taylor, G. F. Vovis, and T. J. Trust.** 1999. Genomic-sequence comparison of two unrelated isolates of the human gastric pathogen *Helicobacter pylori*. Nature **397:**176-80.

3. **Appelmelk, B. J., I. Simoons-Smit, R. Negrini, A. P. Moran, G. O. Aspinall, J. G. Forte, T. De Vries, H. Quan, T. Verboom, J. J. Maaskant, P. Ghiara, E. J. Kuipers, E. Bloemena, T. M. Tadema, R. R. Townsend, K. Tyagarajan, J. M. Crothers, Jr., M. A. Monteiro, A. Savio, and J. De Graaff.** 1996. Potential role of molecular mimicry between *Helicobacter pylori* lipopolysaccharide and host Lewis blood group antigens in autoimmunity. Infect Immun **64:**2031-40.

4. **Aspinall, G. O., M. A. Monteiro, H. Pang, E. J. Walsh, and A. P. Moran.** 1996. Lipopolysaccharide of the *Helicobacter pylori* type strain NCTC 11637 (ATCC 43504): structure of the O antigen chain and core oligosaccharide regions. Biochemistry **35:**2489-97.

5. **Bauerfeind, P., R. M. Garner, and L. T. Mobley.** 1996. Allelic exchange mutagenesis of *nixA* in *Helicobacter pylori* results in reduced nickel transport and urease activity. Infect Immun **64:**2877-80.

6. **Bayerdorffer, E., A. Neubauer, B. Rudolph, C. Thiede, N. Lehn, S. Eidt, and M. Stolte.** 1995. Regression of primary gastric lymphoma of mucosa-associated lymphoid tissue type after cure of *Helicobacter pylori* infection. MALT Lymphoma Study Group. Lancet **345:**1591-4.

7. **Bereswill, S., F. Lichte, T. Vey, F. Fassbinder, and M. Kist.** 1998. Cloning and characterization of the *fur* gene from *Helicobacter pylori*. FEMS Microbiol Lett **159:**193-200.

8. **Bereswill, S., U. Waidner, S. Odenbreit, F. Lichte, F. Fassbinder, G. Bode, and M. Kist.** 1998. Structural, functional and mutational analysis of the *pfr* gene encoding a ferritin from *Helicobacter pylori*. Microbiology **144 (9):**2505-16.

9. **Blaser, M. J.** 1987. Gastric *Campylobacter*-like organisms, gastritis, and peptic ulcer disease. Gastroenterology **93:**371-83.

10. **Blaser, M. J.** 1990. *Helicobacter pylori* and the pathogenesis of gastroduodenal inflammation. J Infect Dis **161:**626-33.

11. **Blaser, M. J.** 1992. Hypotheses on the pathogenesis and natural history of *Helicobacter pylori*-induced inflammation. Gastroenterology **102:**720-7.

12. **Blaser, M. J.** 1997. Not all *Helicobacter pylori* strains are created equal: should all be eliminated? Lancet **349:**1020-2.

13. **Bode, G., F. Mauch, and P. Malfertheiner.** 1993. The coccoid forms of *Helicobacter pylori*. Criteria for their viability. Epidemiol Infect **111:**483-90.

14. **Brayman, T. G., and R. P. Hausinger.** 1996. Purification, characterization, and functional analysis of a truncated *Klebsiella aerogenes* UreE urease accessory protein lacking the histidine-rich carboxyl terminus. J Bacteriol **178:**5410-6.

15. **Burcham, P. C.** 1998. Genotoxic lipid peroxidation products: their DNA damaging properties and role in formation of endogenous DNA adducts. Mutagenesis **13:**287-305.

16. **Bury-Mone, S., S. Skouloubris, A. Labigne, and H. De Reuse.** 2001. The *Helicobacter pylori* UreI protein: role in adaptation to acidity and identification of residues essential for its activity and for acid activation. Mol Microbiol **42:**1021-34.

17. **Casalot, L., and M. Rousset.** 2001. Maturation of the [NiFe] hydrogenases. Trends Microbiol **9:**228-37.

18. **Censini, S., C. Lange, Z. Xiang, J. E. Crabtree, P. Ghiara, M. Borodovsky, R. Rappuoli, and A. Covacci.** 1996. *cag*, a pathogenicity island of *Helicobacter pylori*, encodes type I-specific and disease-associated virulence factors. Proc Natl Acad Sci U S A **93:**14648-53.

19. **Chen, Y. Y., K. A. Clancy, and R. A. Burne.** 1996. *Streptococcus salivarius* urease: genetic and biochemical characterization and expression in a dental plaque *streptococcus*. Infect Immun **64:**585-92.

20. **Clayton, C. L., M. J. Pallen, H. Kleanthous, B. W. Wren, and S. Tabaqchali.** 1990. Nucleotide sequence of two genes from *Helicobacter pylori* encoding for urease subunits. Nucleic Acids Res **18:**362.

21. **Clyne, M., A. Labigne, and B. Drumm.** 1995. *Helicobacter pylori* requires an acidic environment to survive in the presence of urea. Infect Immun **63:**1669-73.

22. **Collins, C. M., D. M. Gutman, and H. Laman.** 1993. Identification of a nitrogen-regulated promoter controlling expression of *Klebsiella pneumoniae* urease genes. Mol Microbiol **8:**187-98.

23. **Colpas, G. J., T. G. Brayman, L. J. Ming, and R. P. Hausinger.** 1999. Identification of metal-binding residues in the *Klebsiella aerogenes* urease nickel metallochaperone, UreE. Biochemistry **38:**4078-88.

24. **Crabtree, J. E., A. Covacci, S. M. Farmery, Z. Xiang, D. S. Tompkins, S. Perry, I. J. Lindley, and R. Rappuoli.** 1995. *Helicobacter pylori* induced interleukin-8 expression in gastric epithelial cells is associated with CagA positive phenotype. J Clin Pathol **48:**41-5.

25. **Crabtree, J. E., N. Figura, J. D. Taylor, M. Bugnoli, D. Armellini, and D. S. Tompkins.** 1992. Expression of 120 kilodalton protein and cytotoxicity in *Helicobacter pylori*. J Clin Pathol **45:**733-4.

26. **Cussac, V., R. L. Ferrero, and A. Labigne.** 1992. Expression of *Helicobacter pylori* urease genes in Escherichia coli grown under nitrogen-limiting conditions. J Bacteriol **174:**2466-73.

27. **Davidson, A. A., Kelly D. J. and Clayton, C. L.** 1995. Molecular cloning and sequencing of a *Helicobacter pylori* hydrogenase. Am. J. Gastro. **89:**1293.

28. **Davies, G. R., N. Banatvala, C. E. Collins, M. T. Sheaff, Y. Abdi, L. Clements, and D. S. Rampton.** 1994. Relationship between infective load of *Helicobacter pylori* and reactive oxygen metabolite production in antral mucosa. Scand J Gastroenterol **29:**419-24.

29. **de Koning-Ward, T. F., A. C. Ward, and R. M. Robins-Browne.** 1994. Characterisation of the urease-encoding gene complex of *Yersinia enterocolitica*. Gene **145:**25-32.

30. **Dix, T. A., K. M. Hess, M. A. Medina, R. W. Sullivan, S. L. Tilly, and T. L. Webb.** 1996. Mechanism of site-selective DNA nicking by the hydrodioxyl (perhydroxyl) radical. Biochemistry **35:**4578-83.

31. **Dixon, N. E., T. C. Gazzola, R. L. blakeley, and B. Zermer.** 1975. Letter: Jack bean urease (EC 3.5.1.5). A metalloenzyme. A simple biological role for nickel? J Am Chem Soc **97:**4131-3.

32. **D'Orazio, S. E., and C. M. Collins.** 1993. The plasmid-encoded urease gene cluster of the family *Enterobacteriaceae* is positively regulated by UreR, a member of the AraC family of transcriptional activators. J Bacteriol **175:**3459-67.

33. **Drapal, N., and A. Bock.** 1998. Interaction of the hydrogenase accessory protein HypC with HycE, the large subunit of *Escherichia coli* hydrogenase 3 during enzyme maturation. Biochemistry **37:**2941-8.

34. **Dunn, B. E., G. P. Campbell, G. I. Perez-Perez, and M. J. Blaser.** 1990. Purification and characterization of urease from *Helicobacter pylori*. J Biol Chem **265:**9464-9.

35. **Eaton, K. A., C. L. Brooks, D. R. Morgan, and S. Krakowka.** 1991. Essential role of urease in pathogenesis of gastritis induced by *Helicobacter pylori* in gnotobiotic piglets. Infect Immun **59:**2470-5.

36. **Eaton, K. A., and S. Krakowka.** 1994. Effect of gastric pH on urease-dependent colonization of gnotobiotic piglets by *Helicobacter pylori*. Infect Immun **62:**3604-7.

37. **Eaton, K. A., D. R. Morgan, and S. Krakowka.** 1989. *Campylobacter pylori* virulence factors in gnotobiotic piglets. Infect Immun **57:**1119-25.

38. **Eaton, K. A., D. R. Morgan, and S. Krakowka.** 1992. Motility as a factor in the colonisation of gnotobiotic piglets by *Helicobacter pylori*. J Med Microbiol **37:**123-7.

39. **Eidt, S., M. Stolte, and R. Fischer.** 1994. *Helicobacter pylori* gastritis and primary gastric non-Hodgkin's lymphomas. J Clin Pathol **47:**436-9.

40. **Eitinger, T., and M. A. Mandrand-Berthelot.** 2000. Nickel transport systems in microorganisms. Arch Microbiol **173:**1-9.

41. **Evans, D. J., Jr., D. G. Evans, S. S. Kirkpatrick, and D. Y. Graham.** 1991. Characterization of the *Helicobacter pylori* urease and purification of its subunits. Microb Pathog **10:**15-26.

42. **Evans, D. J., Jr., D. G. Evans, T. Takemura, H. Nakano, H. C. Lampert, D. Y. Graham, D. N. Granger, and P. R. Kvietys.** 1995. Characterization of a *Helicobacter pylori* neutrophil-activating protein. Infect Immun **63:**2213-20.

43. **Farr, S. B., and T. Kogoma.** 1991. Oxidative stress responses in *Escherichia coli* and *Salmonella typhimurium*. Microbiol Rev **55:**561-85.

44. **Figura, N., P. Guglielmetti, A. Rossolini, A. Barberi, G. Cusi, R. A. Musmanno, M. Russi, and S. Quaranta.** 1989. Cytotoxin production by

Campylobacter pylori strains isolated from patients with peptic ulcers and from patients with chronic gastritis only. J Clin Microbiol **27:**225-6.

45. **Fridovich, I.** 1997. Superoxide anion radical (O2-.), superoxide dismutases, and related matters. J Biol Chem **272:**18515-7.

46. **Fu, C., S. Javedan, F. Moshiri, and R. J. Maier.** 1994. Bacterial genes involved in incorporation of nickel into a hydrogenase enzyme. Proc Natl Acad Sci U S A **91:**5099-103.

47. **Fu, C., and R. J. Maier.** 1993. A genetic region downstream of the hydrogenase structural genes of *Bradyrhizobium japonicum* that is required for hydrogenase processing. J Bacteriol **175:**295-8.

48. **Fu, C., and R. J. Maier.** 1994. Nucleotide sequences of two hydrogenase-related genes (*hypA* and *hypB*) from *Bradyrhizobium japonicum*, one of which (*hypB*) encodes an extremely histidine-rich region and guanine nucleotide-binding domains. Biochim Biophys Acta **1184:**135-8.

49. **Fu, C., J. W. Olson, and R. J. Maier.** 1995. HypB protein of *Bradyrhizobium japonicum* is a metal-binding GTPase capable of binding 18 divalent nickel ions per dimer. Proc Natl Acad Sci U S A **92:**2333-7.

50. **Fulkerson, J. F., Jr., R. M. Garner, and H. L. Mobley.** 1998. Conserved residues and motifs in the NixA protein of *Helicobacter pylori* are critical for the high affinity transport of nickel ions. J Biol Chem **273:**235-41.

51. **Fulkerson, J. F., Jr., and H. L. Mobley.** 2000. Membrane topology of the NixA nickel transporter of *Helicobacter pylori*: two nickel transport-specific motifs within transmembrane helices II and III. J Bacteriol **182:**1722-30.

52. **Garner, R. M., J. Fulkerson, Jr., and H. L. Mobley.** 1998. *Helicobacter pylori* glutamine synthetase lacks features associated with transcriptional and posttranslational regulation. Infect Immun **66:**1839-47.

53. **Ghiara, P., M. Marchetti, M. J. Blaser, M. K. Tummuru, T. L. Cover, E. D. Segal, L. S. Tompkins, and R. Rappuoli.** 1995. Role of the *Helicobacter pylori* virulence factors vacuolating cytotoxin, CagA, and urease in a mouse model of disease. Infect Immun **63:**4154-60.

54. **Goodwin, C. S., R. K. McCulloch, J. A. Armstrong, and S. H. Wee.** 1985. Unusual cellular fatty acids and distinctive ultrastructure in a new spiral bacterium (*Campylobacter pyloridis*) from the human gastric mucosa. J Med Microbiol **19:**257-67.

55. **Harris, P. R., H. L. Mobley, G. I. Perez-Perez, M. J. Blaser, and P. D. Smith.** 1996. *Helicobacter pylori* urease is a potent stimulus of mononuclear phagocyte activation and inflammatory cytokine production. Gastroenterology **111:**419-25.

56. **Hawtin, P. R., H. T. Delves, and D. G. Newell.** 1991. The demonstration of nickel in the urease of *Helicobacter pylori* by atomic absorption spectroscopy. FEMS Microbiol Lett **61:**51-4.

57. **Hawtin, P. R., A. R. Stacey, and D. G. Newell.** 1990. Investigation of the structure and localization of the urease of *Helicobacter pylori* using monoclonal antibodies. J Gen Microbiol **136:**1995-2000.

58. **Hazell, S. L., D. J. Evans, Jr., and D. Y. Graham.** 1991. *Helicobacter pylori* catalase. J Gen Microbiol **137 (Pt 1):**57-61.

59. **Hazell, S. L., A. Lee, L. Brady, and W. Hennessy.** 1986. *Campylobacter pyloridis* and gastritis: association with intercellular spaces and adaptation to an environment of mucus as important factors in colonization of the gastric epithelium. J Infect Dis **153:**658-63.

60. **He, S. H., M. Teixeira, J. LeGall, D. S. Patil, I. Moura, J. J. Moura, D. V. DerVartanian, B. H. Huynh, and H. D. Peck, Jr.** 1989. EPR studies with 77Se-enriched (NiFeSe) hydrogenase of *Desulfovibrio baculatus*. Evidence for a selenium ligand to the active site nickel. J Biol Chem **264:**2678-82.

61. **Hendricks, J. K., and H. L. Mobley.** 1997. *Helicobacter pylori* ABC transporter: effect of allelic exchange mutagenesis on urease activity. J Bacteriol **179:**5892-902.

62. **Hennecke, H.** 1990. Regulation of bacterial gene expression by metal-protein complexes. Mol Microbiol **4:**1621-8.

63. **Hessey, S. J., J. Spencer, J. I. Wyatt, G. Sobala, B. J. Rathbone, A. T. Axon, and M. F. Dixon.** 1990. Bacterial adhesion and disease activity in *Helicobacter* associated chronic gastritis. Gut **31:**134-8.

64. **Hu, L. T., P. A. Foxall, R. Russell, and H. L. Mobley.** 1992. Purification of recombinant *Helicobacter pylori* urease apoenzyme encoded by *ureA* and *ureB*. Infect Immun **60:**2657-66.

65. **Hu, L. T., and H. L. Mobley.** 1990. Purification and N-terminal analysis of urease from *Helicobacter pylori*. Infect Immun **58:**992-8.

66. **Huang, J., P. W. O'Toole, P. Doig, and T. J. Trust.** 1995. Stimulation of interleukin-8 production in epithelial cell lines by *Helicobacter pylori*. Infect Immun **63:**1732-8.

67. **Hughes, N. J., C. L. Clayton, P. A. Chalk, and D. J. Kelly.** 1998. *Helicobacter pylori porCDAB* and *oorDABC* genes encode distinct pyruvate:flavodoxin and 2-oxoglutarate:acceptor oxidoreductases which mediate electron transport to NADP. J Bacteriol **180:**1119-28.

68. **Imlay, J. A., and S. Linn.** 1988. DNA damage and oxygen radical toxicity. Science **240:**1302-9.

69. **Jabri, E., M. B. Carr, R. P. Hausinger, and P. A. Karplus.** 1995. The crystal structure of urease from *Klebsiella aerogenes*. Science **268:**998-1004.

70. **Jacobi, A., R. Rossmann, and A. Bock.** 1992. The *hyp* operon gene products are required for the maturation of catalytically active hydrogenase isoenzymes in *Escherichia coli*. Arch Microbiol **158:**444-51.

71. **Jones, B. D., and H. L. Mobley.** 1989. *Proteus mirabilis* urease: nucleotide sequence determination and comparison with jack bean urease. J Bacteriol **171:**6414-22.

72. **Jungblut, P. R., D. Bumann, G. Haas, U. Zimny-Arndt, P. Holland, S. Lamer, F. Siejak, A. Aebischer, and T. F. Meyer.** 2000. Comparative proteome analysis of *Helicobacter pylori*. Mol Microbiol **36:**710-25.

73. **Kansau, I., F. Guillain, J. M. Thiberge, and A. Labigne.** 1996. Nickel binding and immunological properties of the C-terminal domain of the *Helicobacter pylori* GroES homologue (HspA). Mol Microbiol **22:**1013-23.

74. **Klein, P. D., D. Y. Graham, A. Gaillour, A. R. Opekun, and E. O. Smith.** 1991. Water source as risk factor for *Helicobacter pylori* infection in Peruvian children. Gastrointestinal Physiology Working Group. Lancet **337:**1503-6.

75. **Labigne, A., V. Cussac, and P. Courcoux.** 1991. Shuttle cloning and nucleotide sequences of *Helicobacter pylori* genes responsible for urease activity. J Bacteriol **173:**1920-31.

76. **Lee, A., J. Fox, and S. Hazell.** 1993. Pathogenicity of *Helicobacter pylori*: a perspective. Infect Immun **61:**1601-10.

77. **Lee, M. H., S. B. Mulrooney, and R. P. Hausinger.** 1990. Purification, characterization, and in vivo reconstitution of *Klebsiella aerogenes* urease apoenzyme. J Bacteriol **172:**4427-31.

78. **Lee, M. H., S. B. Mulrooney, M. J. Renner, Y. Markowicz, and R. P. Hausinger.** 1992. *Klebsiella aerogenes* urease gene cluster: sequence of *ureD* and demonstration that four accessory genes (*ureD*, *ureE*, *ureF*, and *ureG*) are involved in nickel metallocenter biosynthesis. J Bacteriol **174:**4324-30.

79. **Lee, M. H., H. S. Pankratz, S. Wang, R. A. Scott, M. G. Finnegan, M. K. Johnson, J. A. Ippolito, D. W. Christianson, and R. P. Hausinger.** 1993. Purification and characterization of *Klebsiella aerogenes* UreE protein: a nickel-binding protein that functions in urease metallocenter assembly. Protein Sci **2:**1042-52.

80. **Lutz, S., A. Jacobi, V. Schlensog, R. Bohm, G. Sawers, and A. Bock.** 1991. Molecular characterization of an operon (*hyp*) necessary for the activity of the three hydrogenase isoenzymes in *Escherichia coli*. Mol Microbiol **5:**123-35.

81. **Maeda, M., M. Hidaka, A. Nakamura, H. Masaki, and T. Uozumi.** 1994. Cloning, sequencing, and expression of thermophilic *Bacillus* sp. strain TB-90 urease gene complex in *Escherichia coli*. J Bacteriol **176:**432-42.

82. **Magalon, A., M. Blokesch, E. Zehelein, and A. Bock.** 2001. Fidelity of metal insertion into hydrogenases. FEBS Lett **499:**73-6.

83. **Magalon, A., and A. Bock.** 2000. Analysis of the HypC-hycE complex, a key intermediate in the assembly of the metal center of the *Escherichia coli* hydrogenase 3. J Biol Chem **275:**21114-20.

84. **Maier, R. J., C. Fu, J. Gilbert, F. Moshiri, J. Olson, and A. G. Plaut.** 1996. Hydrogen uptake hydrogenase in *Helicobacter pylori*. FEMS Microbiol Lett **141:**71-6.

85. **Maier, T., and A. Bock.** 1996. Generation of active [NiFe] hydrogenase in vitro from a nickel-free precursor form. Biochemistry **35:**10089-93.

86. **Maier, T., A. Jacobi, M. Sauter, and A. Bock.** 1993. The product of the hypB gene, which is required for nickel incorporation into hydrogenases, is a novel guanine nucleotide-binding protein. J Bacteriol **175:**630-5.

87. **Maier, T., F. Lottspeich, and A. Bock.** 1995. GTP hydrolysis by HypB is essential for nickel insertion into hydrogenases of *Escherichia coli*. Eur J Biochem **230:**133-8.

88. **Marnett, L. J.** 2000. Oxyradicals and DNA damage. Carcinogenesis **21:**361-70.

89. **Marshall, B. J., J. A. Armstrong, D. B. McGechie, and R. J. Glancy.** 1985. Attempt to fulfil Koch's postulates for pyloric *Campylobacter*. Med J Aust **142:**436-9.

90. **Marshall, B. J., L. J. Barrett, C. Prakash, R. W. McCallum, and R. L. Guerrant.** 1990. Urea protects *Helicobacter* (*Campylobacter*) *pylori* from the bactericidal effect of acid. Gastroenterology **99:**697-702.

91. **Marshall, B. J., and J. R. Warren.** 1984. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. Lancet **1:**1311-5.

92. **McGee, D. J., C. A. May, R. M. Garner, J. M. Himpsl, and H. L. Mobley.** 1999. Isolation of *Helicobacter pylori* genes that modulate urease activity. J Bacteriol **181:**2477-84.

93. **McGowan, C. C., T. L. Cover, and M. J. Blaser.** 1997. Analysis of F1F0-ATPase from *Helicobacter pylori*. Infect Immun **65:**2640-7.

94. **Mendz, G. L., and S. L. Hazell.** 1991. Evidence for a pentose phosphate pathway in *Helicobacter pylori*. FEMS Microbiol, Lett. **84:**331-336.

95. **Mendz, G. L., and S. L. Hazell.** 1995. Aminoacid utilization by *Helicobacter pylori*. Int J Biochem Cell Biol **27:**1085-93.

96. **Mendz, G. L., S. L. Hazell, and B. P. Burns.** 1994. The Entner-Doudoroff pathway in *Helicobacter pylori*. Arch Biochem Biophys **312:**349-56.

97. **Mendz, G. L., S. L. Hazell, and B. P. Burns.** 1993. Glucose utilization and lactate production by *Helicobacter pylori*. J Gen Microbiol **139 (Pt 12):**3023-8.

98. **Menon, A. L., and R. L. Robson.** 1994. In vivo and in vitro nickel-dependent processing of the [NiFe] hydrogenase in *Azotobacter vinelandii*. J Bacteriol **176:**291-5.

99. **Meyer-Rosberg, K., D. R. Scott, D. Rex, K. Melchers, and G. Sachs.** 1996. The effect of environmental pH on the proton motive force of *Helicobacter pylori*. Gastroenterology **111:**886-900.

100. **Misiewiez, J. J.** 1996. One week low dose triole therapy for eradication of *Helicobacter pylori* - A large multicenter randomnized trial. Gastroenterology**:**A 198.

101. **Mobley, H. L., M. J. Cortesia, L. E. Rosenthal, and B. D. Jones.** 1988. Characterization of urease from *Campylobacter pylori*. J Clin Microbiol **26:**831-6.

102. **Mobley, H. L., R. M. Garner, and P. Bauerfeind.** 1995. *Helicobacter pylori* nickel-transport gene *nixA*: synthesis of catalytically active urease in *Escherichia coli* independent of growth conditions. Mol Microbiol **16:**97-109.

103. **Mobley, H. L., and R. P. Hausinger.** 1989. Microbial ureases: significance, regulation, and molecular characterization. Microbiol Rev **53:**85-108.

104. **Moncrief, M. B., and R. P. Hausinger.** 1997. Characterization of UreG, identification of a UreD-UreF-UreG complex, and evidence suggesting that a nucleotide-binding site in UreG is required for in vivo metallocenter assembly of *Klebsiella aerogenes* urease. J Bacteriol **179:**4081-6.

105. **Moncrief, M. B., and R. P. Hausinger.** 1996. Purification and activation properties of UreD-UreF-urease apoprotein complexes. J Bacteriol **178:**5417-21.

106. **Morgan, D. R., R. Freedman, C. E. Depew, and W. G. Kraft.** 1987. Growth of *Campylobacter pylori* in liquid media. J Clin Microbiol **25:**2123-5.

107. **Morris, A., and G. Nicholson.** 1987. Ingestion of *Campylobacter pyloridis* causes gastritis and raised fasting gastric pH. Am J Gastroenterol **82:**192-9.

108. **Mulrooney, S. B., and R. P. Hausinger.** 1990. Sequence of the *Klebsiella aerogenes* urease genes and evidence for accessory proteins facilitating nickel incorporation. J Bacteriol **172:**5837-43.

109. **Neyrolles, O., S. Ferris, N. Behbahani, L. Montagnier, and A. Blanchard.** 1996. Organization of *Ureaplasma urealyticum* urease gene cluster and expression in a suppressor strain of Escherichia coli. J Bacteriol **178:**647-55.

110. **Nicholson, E. B., E. A. Concaugh, P. A. Foxall, M. D. Island, and H. L. Mobley.** 1993. *Proteus mirabilis* urease: transcriptional regulation by UreR. J Bacteriol **175:**465-73.

111. **Nicolet, Y., C. Piras, P. Legrand, C. E. Hatchikian, and J. C. Fontecilla-Camps.** 1999. *Desulfovibrio desulfuricans* iron hydrogenase: the structure shows unusual coordination to an active site Fe binuclear center. Structure Fold Des **7:**13-23.

112. **Olson, J. W., C. Fu, and R. J. Maier.** 1997. The HypB protein from *Bradyrhizobium japonicum* can store nickel and is required for the nickel-dependent transcriptional regulation of hydrogenase. Mol Microbiol **24:**119-28.

113. **Olson, J. W., and R. J. Maier.** 2000. Dual roles of *Bradyrhizobium japonicum* nickelin protein in nickel storage and GTP-dependent Ni mobilization. J Bacteriol **182:**1702-5.

114. **Olson, J. W., and Maier, R. J.** 2002. Molecular hydrogen as energy source for *Helicobacter pylori*. Science. In Press.

115. **Park, I. S., M. B. Carr, and R. P. Hausinger.** 1994. In vitro activation of urease apoprotein and role of UreD as a chaperone required for nickel metallocenter assembly. Proc Natl Acad Sci U S A **91:**3233-7.

116. **Park, I. S., and R. P. Hausinger.** 1995. Evidence for the presence of urease apoprotein complexes containing UreD, UreF, and UreG in cells that are competent for in vivo enzyme activation. J Bacteriol **177:**1947-51.

117. **Park, I. S., and R. P. Hausinger.** 1996. Metal ion interaction with urease and UreD-urease apoproteins. Biochemistry **35:**5345-52.

118. **Parsonnet, J., S. Hansen, L. Rodriguez, A. B. Gelb, R. A. Warnke, E. Jellum, N. Orentreich, J. H. Vogelman, and G. D. Friedman.** 1994. *Helicobacter pylori* infection and gastric lymphoma. N Engl J Med **330:**1267-71.

119. **Paschos, A., R. S. Glass, and A. Bock.** 2001. Carbamoylphosphate requirement for synthesis of the active center of [NiFe]-hydrogenases. FEBS Lett **488:**9-12.

120. **Patchett, S., S. Beattie, E. Leen, C. Keane, and C. O'Morain.** 1992. *Helicobacter pylori* and duodenal ulcer recurrence. Am J Gastroenterol **87:**24-7.

121. **Perez-Perez, G. I., B. M. Dworkin, J. E. Chodos, and M. J. Blaser.** 1988. *Campylobacter pylori* antibodies in humans. Ann Intern Med **109:**11-7.

122. **Pesci, E. C., and C. L. Pickett.** 1994. Genetic organization and enzymatic activity of a superoxide dismutase from the microaerophilic human pathogen, *Helicobacter pylori*. Gene **143:**111-6.

123. **Peters, J. W., W. N. Lanzilotta, B. J. Lemon, and L. C. Seefeldt.** 1998. X-ray crystal structure of the Fe-only hydrogenase (CpI) from *Clostridium pasteurianum* to 1.8 angstrom resolution. Science **282:**1853-8.

124. **Phadnis, S. H., D. Ilver, L. Janzon, S. Normark, and T. U. Westblom.** 1994. Pathological significance and molecular characterization of the vacuolating toxin gene of *Helicobacter pylori*. Infect Immun **62:**1557-65.

125. **Przybyla, A. E., J. Robbins, N. Menon, and H. D. Peck, Jr.** 1992. Structure-function relationships among the nickel-containing hydrogenases. FEMS Microbiol Rev **8:**109-35.

126. **Rey, L., J. Imperial, J. M. Palacios, and T. Ruiz-Argueso.** 1994. Purification of *Rhizobium leguminosarum* HypB, a nickel-binding protein required for hydrogenase synthesis. J Bacteriol **176:**6066-73.

127. **Rossmann, R., T. Maier, F. Lottspeich, and A. Bock.** 1995. Characterisation of a protease from *Escherichia coli* involved in hydrogenase maturation. Eur J Biochem **227:**545-50.

128. **Rossmann, R., M. Sauter, F. Lottspeich, and A. Bock.** 1994. Maturation of the large subunit (HYCE) of *Escherichia coli* hydrogenase 3 requires nickel incorporation followed by C-terminal processing at Arg537. Eur J Biochem **220:**377-84.

129. **Russell, R. J., D. C. Haines, M. R. Anver, J. K. Battles, P. L. Gorelick, L. L. Blumenauer, M. A. Gonda, and J. M. Ward.** 1995. Use of antibiotics to prevent hepatitis and typhlitis in male scid mice spontaneously infected with *Helicobacter hepaticus*. Lab Anim Sci **45:**373-8.

130. **Sachs, G., K. Meyer-Rosberg, D. R. Scott, and K. Melchers.** 1996. Acid, protons and *Helicobacter pylori*. Yale J Biol Med **69:**301-16.

131. **Santangelo, J. D., P. Durre, and D. R. Woods.** 1995. Characterization and expression of the hydrogenase-encoding gene from *Clostridium acetobutylicum* P262. Microbiology **141:**171-80.

132. **Schmitz, A., C. Josenhans, and S. Suerbaum.** 1997. Cloning and characterization of the *Helicobacter pylori flbA* gene, which codes for a membrane protein involved in coordinated expression of flagellar genes. J Bacteriol **179:**987-97.

133. **Schuman, R., B. Rigas, A. Prada, and G. Minoli.** 1995. Diagnosis of *Helicobacter pylori* infection by the Lara system - towards a simplified breath test. Gastroenterlogy **108:**A 215.

134. **Schwacha, A., and R. A. Bender.** 1993. The product of the *Klebsiella aerogenes* nac (nitrogen assimilation control) gene is sufficient for activation of the *hut* operons and repression of the *gdh* operon. J Bacteriol **175:**2116-24.

135. **Scott, D. R., E. A. Marcus, D. L. Weeks, A. Lee, K. Melchers, and G. Sachs.** 2000. Expression of the *Helicobacter pylori ureI* gene is required for acidic pH activation of cytoplasmic urease. Infect Immun **68:**470-7.

136. **Sherburne, R., and D. E. Taylor.** 1995. *Helicobacter pylori* expresses a complex surface carbohydrate, Lewis X. Infect Immun **63:**4564-8.

137. **Shirai, M., R. Fujinaga, J. K. Akada, and T. Nakazawa.** 1999. Activation of *Helicobacter pylori ureA* promoter by a hybrid *Escherichia coli-H. pylori rpoD* gene in *E. coli.* Gene **239:**351-9.

138. **Smoot, D. T., H. L. Mobley, G. R. Chippendale, J. F. Lewison, and J. H. Resau.** 1990. *Helicobacter pylori* urease activity is toxic to human gastric epithelial cells. Infect Immun **58:**1992-4.

139. **Soriano, A., G. J. Colpas, and R. P. Hausinger.** 2000. UreE stimulation of GTP-dependent urease activation in the UreD-UreF-UreG-urease apoprotein complex. Biochemistry **39:**12435-40.

140. **Soriano, A., and R. P. Hausinger.** 1999. GTP-dependent activation of urease apoprotein in complex with the UreD, UreF, and UreG accessory proteins. Proc Natl Acad Sci U S A **96:**11140-4.

141. **Spiegelhalder, C., B. Gerstenecker, A. Kersten, E. Schiltz, and M. Kist.** 1993. Purification of *Helicobacter pylori* superoxide dismutase and cloning and sequencing of the gene. Infect Immun **61:**5315-25.

142. **Strickland, R. G., and I. R. Mackay.** 1973. A reappraisal of the nature and significance of chronic atrophic gastritis. Am J Dig Dis **18:**426-40.

143. **Suerbaum, S.** 1995. The complex flagella of gastric *Helicobacter* species. Trends Microbiol **3:**168-70; discussion 170-1.

144. **Sumner, J. B.** 1926. The isolation and crystallization of the enzyme urease. J. Biol. Chem. **69:**435-441.

145. **Tee, W., J. R. Lambert, and B. Dwyer.** 1995. Cytotoxin production by *Helicobacter pylori* from patients with upper gastrointestinal tract diseases. J Clin Microbiol **33:**1203-5.

146. **Theodoratou, E., A. Paschos, A. Magalon, E. Fritsche, R. Huber, and A. Bock.** 2000. Nickel serves as a substrate recognition motif for the endopeptidase involved in hydrogenase maturation. Eur J Biochem **267:**1995-9.

147. **Thomas, J. E., G. R. Gibson, M. K. Darboe, A. Dale, and L. T. Weaver.** 1992. Isolation of *Helicobacter pylori* from human faeces. Lancet **340:**1194-5.

148. **Thomas, V. J., and C. M. Collins.** 1999. Identification of UreR binding sites in the *Enterobacteriaceae* plasmid-encoded and *Proteus mirabilis* urease gene operons. Mol Microbiol **31:**1417-28.

149. **Todd, M. J., and R. P. Hausinger.** 1989. Competitive inhibitors of *Klebsiella aerogenes* urease. Mechanisms of interaction with the nickel active site. J Biol Chem **264:**15835-42.

150. **Todd, M. J., and R. P. Hausinger.** 1987. Purification and characterization of the nickel-containing multicomponenet urease from *Klebsiella aerogenes*. J Biol Chem **262:** 5963-7.

151. **Tomb, J. F., O. White, A. R. Kerlavage, R. A. Clayton, G. G. Sutton, R. D. Fleischmann, K. A. Ketchum, H. P. Klenk, S. Gill, B. A. Dougherty, K. Nelson, J. Quackenbush, L. Zhou, E. F. Kirkness, S. Peterson, B. Loftus, D. Richardson, R. Dodson, H. G. Khalak, A. Glodek, K. McKenney, L. M. Fitzegerald, N. Lee, M. D. Adams, J. C. Venter, and et al.** 1997. The complete genome sequence of the gastric pathogen *Helicobacter pylori*. Nature **388:**539-47.

152. **Tonello, F., W. G. Dundon, B. Satin, M. Molinari, G. Tognon, G. Grandi, G. Del Giudice, R. Rappuoli, and C. Montecucco.** 1999. The *Helicobacter pylori*

neutrophil-activating protein is an iron-binding protein with dodecameric structure. Mol Microbiol **34:**238-46.

153. **Tsuda, M., M. Karita, M. G. Morshed, K. Okita, and T. Nakazawa.** 1994. A urease-negative mutant of *Helicobacter pylori* constructed by allelic exchange mutagenesis lacks the ability to colonize the nude mouse stomach. Infect Immun **62:**3586-9.

154. **Tummuru, M. K., T. L. Cover, and M. J. Blaser.** 1994. Mutation of the cytotoxin-associated *cagA* gene does not affect the vacuolating cytotoxin activity of *Helicobacter pylori*. Infect Immun **62:**2609-13.

155. **Valle, J., K. Seppala, P. Sipponen, and T. Kosunen.** 1991. Disappearance of gastritis after eradication of *Helicobacter pylori*. A morphometric study. Scand J Gastroenterol **26:**1057-65.

156. **van Vliet, A. H., E. J. Kuipers, B. Waidner, B. J. Davies, N. de Vries, C. W. Penn, C. M. Vandenbroucke-Grauls, M. Kist, S. Bereswill, and J. G. Kusters.** 2001. Nickel-responsive induction of urease expression in *Helicobacter pylori* is mediated at the transcriptional level. Infect Immun **69:**4891-7.

157. **van Vliet, A. H., S. W. Poppelaars, B. J. Davies, J. Stoof, S. Bereswill, M. Kist, C. W. Penn, E. J. Kuipers, and J. G. Kusters.** 2002. NikR mediates nickel-responsive transcriptional induction of urease expression in *Helicobacter pylori*. Infect Immun **70:**2846-52.

158. **Vignais, P. M., B. Billoud, and J. Meyer.** 2001. Classification and phylogeny of hydrogenases. FEMS Microbiology Revs. **25:** 455-501.

159. **Volbeda, A., M. H. Charon, C. Piras, E. C. Hatchikian, M. Frey, and J. C. Fontecilla-Camps.** 1995. Crystal structure of the nickel-iron hydrogenase from *Desulfovibrio gigas*. Nature **373:**580-7.

160. **Volbeda, A., J. C. Fontecilla-Camps, and M. Frey.** 1996. Novel metal sites in protein structures. Curr Opin Struct Biol **6:**804-12.

161. **Voordouw, G.** 1992. Evolution of Hydrogenase Genes. Adv. Inorg. Chem. **38:**397-422.

162. **Walker, I. R., R. G. Strickland, B. Ungar, and I. R. Mackay.** 1971. Simple atrophic gastritis and gastric carcinoma. Gut **12:**906-11.

163. **Ward, J. M., M. R. Anver, D. C. Haines, J. M. Melhorn, P. Gorelick, L. Yan, and J. G. Fox.** 1996. Inflammatory large bowel disease in immunodeficient mice naturally infected with *Helicobacter hepaticus*. Lab Anim Sci **46:**15-20.

164. **Waugh, R., and D. H. Boxer.** 1986. Pleiotropic hydrogenase mutants of *Escherichia coli* K12: growth in the presence of nickel can restore hydrogenase activity. Biochimie **68:**157-66.

165. **Weeks, D. L., S. Eskandari, D. R. Scott, and G. Sachs.** 2000. A H+-gated urea channel: the link between *Helicobacter pylori* urease and gastric colonization. Science **287:**482-5.

166. **Weeks, D. L., and G. Sachs.** 2001. Sites of pH regulation of the urea channel of *Helicobacter pylori*. Mol Microbiol **40:**1249-59.

167. **Williams, C. L., T. Preston, M. Hossack, C. Slater, and K. E. McColl.** 1996. *Helicobacter pylori* utilises urea for amino acid synthesis. FEMS Immunol Med Microbiol **13:**87-94.

168. **Wolfram, L., T. Eitinger, and B. Friedrich.** 1991. Construction and properties of a triprotein containing the high-affinity nickel transporter of *Alcaligenes eutrophus*. FEBS Lett **283:**109-12.

169. **Worst, D. J., B. R. Otto, and J. de Graaff.** 1995. Iron-repressible outer membrane proteins of *Helicobacter pylori* involved in heme uptake. Infect Immun **63:**4161-5.

170. **Wotherspoon, A. C., C. Ortiz-Hidalgo, M. R. Falzon, and P. G. Isaacson.** 1991. *Helicobacter pylori*-associated gastritis and primary B-cell gastric lymphoma. Lancet **338:**1175-6.

171. **Wu, L. F.** 1992. Putative nickel-binding sites of microbial proteins. Res Microbiol **143:**347-51.

172. **Wu, L. F., and M. A. Mandrand.** 1993. Microbial hydrogenases: primary structure, classification, signatures and phylogeny. FEMS Microbiol Rev **10:**243-69.

173. **Zirngibl, C., R. Hedderich and R. K. Thauer.** 1990. N^5 , N^{16} Methylene tetrahydromethanopterin dehydrogenase from *Methanobacterium thermoautotrophicum* has hydrogenase activity. FEBS Lett **261:**112-16.

Figure 1.1. Maturation of hydrogenase (model based on studies with *E. coli* hydrogenase isoenzyme 3): The maturation of the precursor of the large subunit (PreLSU) starts with the formation of a complex with HypC followed by liganding of carbonyl (CO) and cyano (CN) groups to Fe within PreLSU. HypF may help in the insertion of these ligands. HypB may be responsible for delivery of one Ni^{+2} ion into the active site of PreLSU in a GTP-hydrolysis dependent manner. HypC remains bound to the PreLSU till the nickel insertion step, hence may act as a chaperone. Once Ni^{+2} is inserted, the PreLSU undergoes C-terminal processing to give the processed large subunit (LSU). This is followed by dimerization with the small subunit (SSU) to give rise to the mature hydrogenase.

Figure 1.1

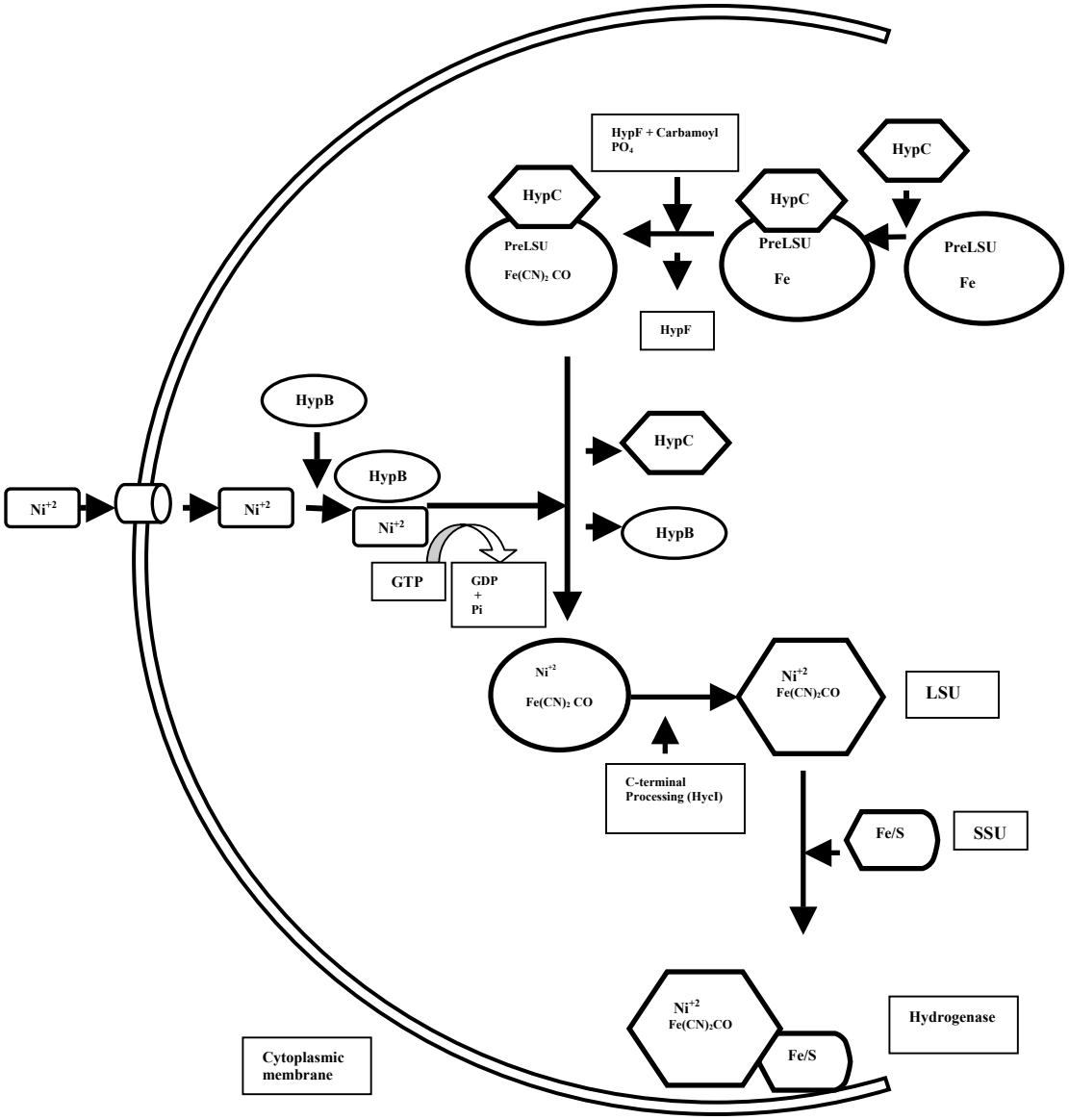

HypF + Carbamoyl PO4
HypC
HypC
HypC
PreLSU
Fe(CN)2 CO
PreLSU
Fe
PreLSU
Fe
HypF
HypB
HypB
HypC
HypB
Ni+2
Ni+2
Ni+2
GTP
GDP + Pi
Ni+2 Fe(CN)2 CO
Ni+2 Fe(CN)2CO
LSU
C-terminal Processing (HycI)
Fe/S
SSU
Ni+2 Fe(CN)2CO
Fe/S
Hydrogenase
Cytoplasmic membrane

Figure 1.2. Maturation of urease (model based on studies with *Klebsiella aerogenes* urease): The apourease $(UreABC)_3$ first forms a complex with urease accessory proteins, UreD, UreF and UreG (UreDFG). A fourth accessory protein, UreE, may function as a metallochaperone because of its ability to bind nickel. Incorporation of nickel and bicarbonate/CO_2 may be coupled to GTP hydrolysis. UreG may be catalyzing the GTP hydrolysis step. Bicarbonate/CO_2 is a source of the lysine carbamate bridge between the two Ni^{+2} ions in the active site of the large subunit (UreC). UreD may be acting as a chaperone to ensure proper nickel insertion. UreF may be preventing the binding of Ni^{+2} ions to the active site till the formation of the carbamylated lysine.

Figure 1.2

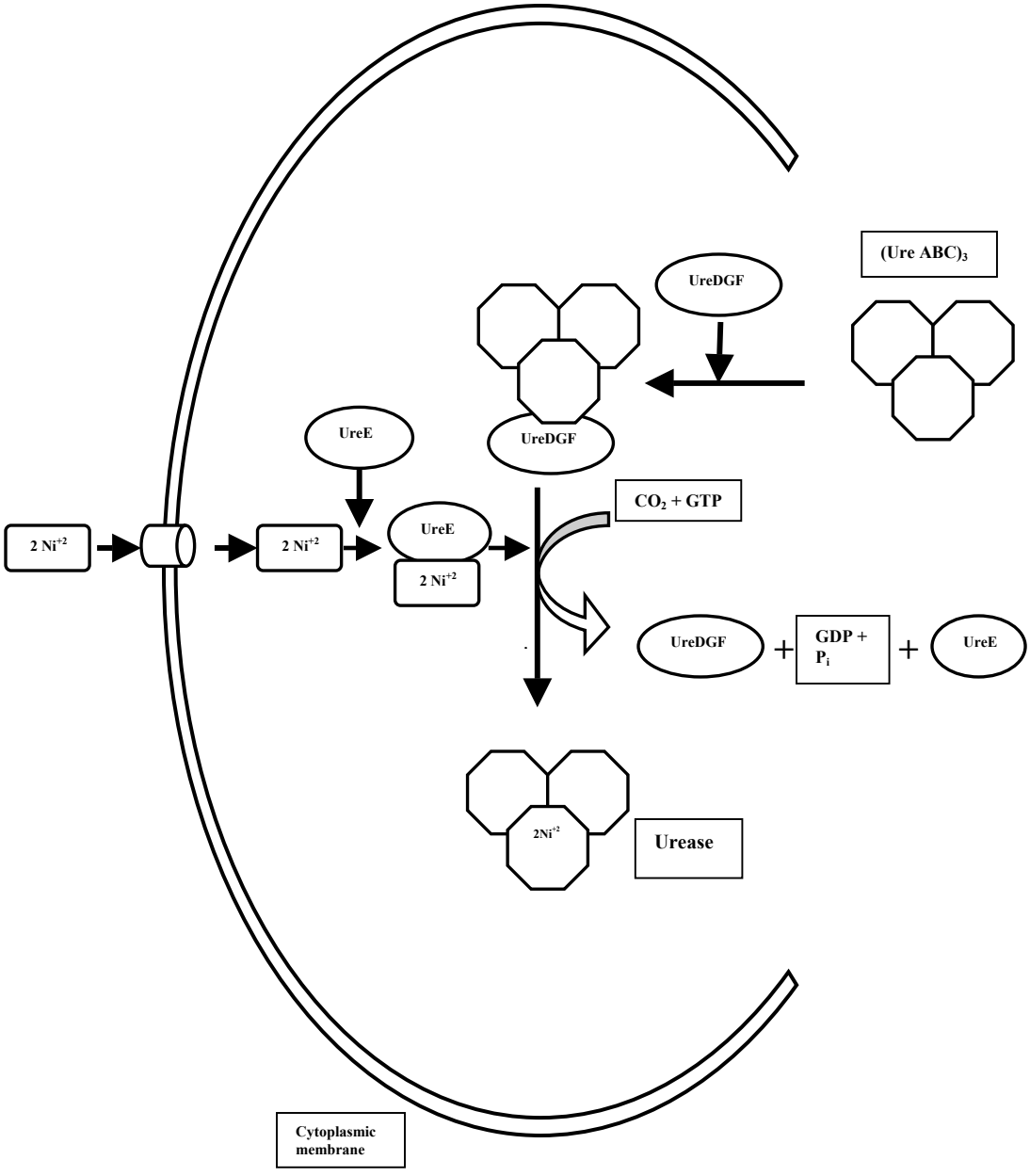

(Ure ABC)3
UreDGF
UreDGF
UreE
2 Ni+2
2 Ni+2
UreE
2 Ni+2
CO2 + GTP
UreDGF
+
GDP + Pi
+
UreE
2Ni+2
Urease
Cytoplasmic membrane

Figure 1.3. Steps leading to colonization and gastric mucosal damage by *H. pylori*: The presence of flagella, a spiral-shaped structure, and the ability to hydrolyze urea are the primary colonization factors for this bacterium, which help in its penetration into the almost neutral mucin layer after crossing the highly acidic lumen. Once inside the mucin layer it may adhere to the gastric epithelium and start multiplying. *H. pylori* induces an inflammatory response via VacA (vacuolating cytotoxin), NapA (neutrophil activating protein), CagA (cytotoxin associated antigen), LPS (lipopolysaccharide) and urease (Reproduced with permission from editorial coordinator, ASM Press from book titled "Bacterial pathogenensis, a molecular approach").

Figure 1.3

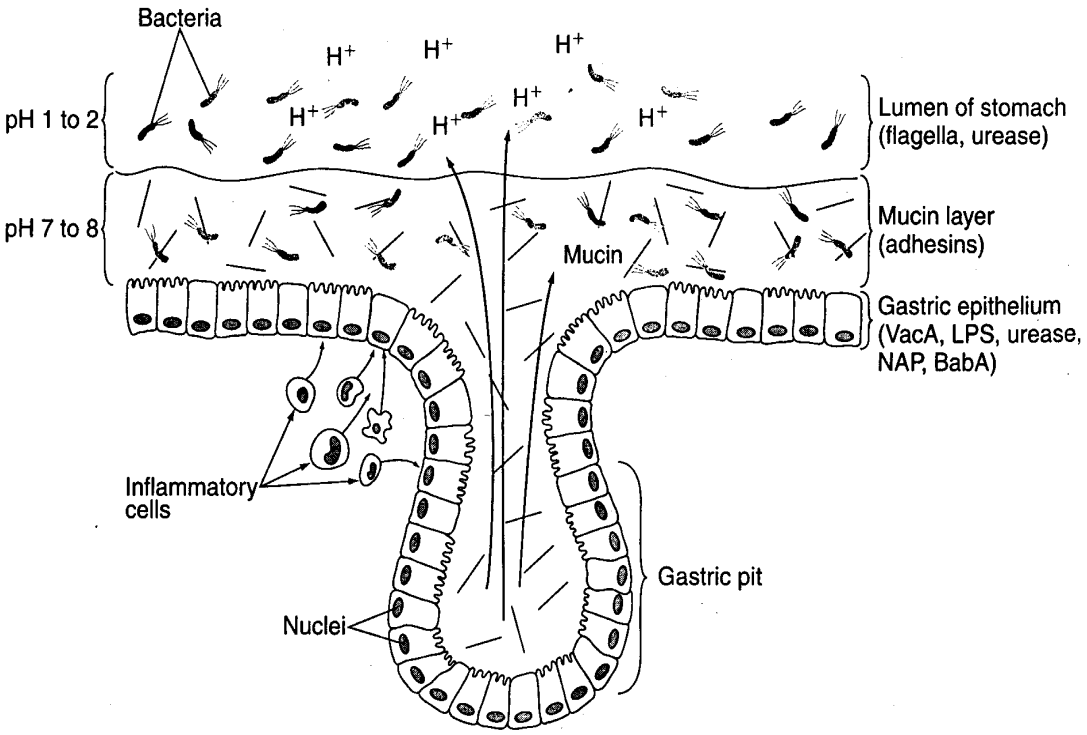
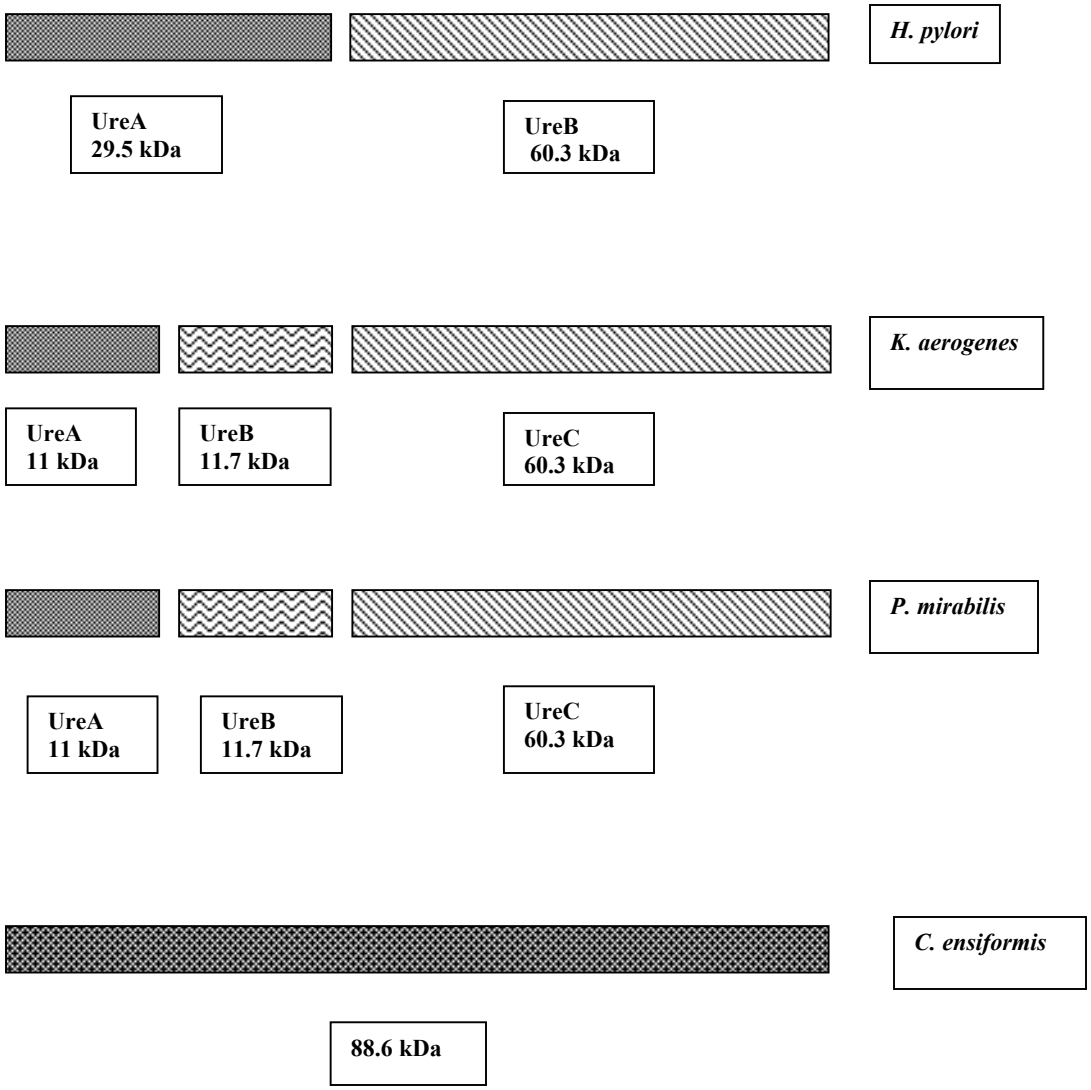

Bacteria
H+
H+
H+
H+
H+
H+
H+
pH 1 to 2
pH 7 to 8
Lumen of stomach
(flagella, urease)
Mucin layer
(adhesins)
Mucin
Gastric epithelium
(VacA, LPS, urease,
NAP, BabA)
Inflammatory
cells
Gastric pit
Nuclei

Figure 1.4. Relative sizes of urease subunits from *H. pylori*, *K. aerogenes*, *Proteus mirabilis* and jack bean (*Canavalia ensiformis*): *H. pylori* UreA subunit can be aligned to the UreA and UreB subunits of *K. aerogenes* and *P. mirabilis*. Both UreA and UreB of *H. pylori* can be aligned to the single subunit of jack bean urease. Molecular weight of each subunit is indicated in kDa.

Figure 1.4


H. pylori
UreA
29.5 kDa
UreB
60.3 kDa
K. aerogenes
UreA
11 kDa
UreB
11.7 kDa
UreC
60.3 kDa
P. mirabilis
UreA
11 kDa
UreB
11.7 kDa
UreC
60.3 kDa
C. ensiformis
88.6 kDa

Chapter 2

HYPA AND HYPB ARE NEEDED FOR FULL ACTIVITY OF BOTH HYDROGENASE AND UREASE IN *HELICOBACTER PYLORI* [1]

[1] This work was part of a manuscript published in Mol. Microbiol. 2001. 39(1): 176-182. Reprinted here with the permission of the publisher (02/18/02).

Abstract

The nickel-containing enzymes hydrogenase and urease require accessory proteins in order to properly incorporate nickel into their active sites. The *Helicobacter pylori* genome encodes the full complement of both urease and hydrogenase accessory proteins. Two of these, the hydrogenase accessory proteins HypA (encoded by *hypA*) and HypB (encoded by *hypB*), are required for the full activity of both the hydrogenase and urease enzymes in *H. pylori*. Under normal growth conditions, hydrogenase activity is abolished in strains in which either *hypA* (HypA:kan) or *hypB* (HypB:kan) have been interrupted by a kanamycin resistance cassette. Urease activity in these strains is 200- (HypA:kan) and 40- (HypB:kan) fold lower than that of the wild-type strain 43504. Nickel supplementation in the growth medium restored urease activity of these mutants to almost wild-type levels. Hydrogenase activity was restored to a lesser extent, as has been observed for *hyp* mutants in other H_2-oxidizing bacteria. Urease activity was not affected by insertional mutagenesis in the *hypD*, *hypE* or *hypF* genes, although these mutants were all deficient in hydrogenase activity. This indicates that the deficiency in urease activity in the *hypA* and *hypB* mutant strains was not due to a lack of hydrogenase activity.

Introduction

Maturation of metal-containing enzymes requires the participation of numerous accessory proteins, presumably due to active site assembly of these metalloenzymes. The specific steps performed by the accessory proteins are not well-defined in the synthesis of nickel-containing enzymes such as urease, hydrogenase and carbon monoxide dehydrogenase (4, 12). Nevertheless, progress in identifying the roles of some of these proteins in nickel storage, in energy dependent mobilization of Ni^{+2} into the active site, or

in forming a complex with other accessory proteins or with the apoprotein to enable Ni^{+2} insertion, is proceeding rapidly (8, 18, 21).

The peptic ulcer-associated pathogen *H. pylori* contains two distinct nickel-containing enzymes that differ greatly in their molecular properties and in their Ni-containing active sites. These are a membrane-bound H_2 uptake-type hydrogenase (9) and the ammonia-producing urease, the latter being a key factor for virulence of the bacterium in the harsh acidic environment of the host (14). From analysis of the *H. pylori* genome (1, 22), it is clear that nickel-metabolizing accessory proteins that have been studied in other bacteria are present in *H. pylori*. For example, all of the accessory proteins thought to be needed to assemble a functional urease are present in *H. pylori*. Indeed, *H. pylori* even contains an additional accessory protein, UreI, not found in other urease-containing bacteria. To examine the hydrogenase accessory proteins (encoded by the *hyp* genes), we created gene-directed mutants in five specific *hyp* genes. Analysis of these mutants has led to the surprising finding that two Hyp proteins (HypA and HypB), previously assigned roles in mobilizing Ni^{+2} into hydrogenase (5, 7, 17, 20), are required not only for hydrogenase maturation, but also for urease maturation in *H. pylori*. The enzyme activities of both the Ni-enzymes in *hypA* and *hypB* mutants were partially restored by supplementing the medium with nickel. Partially purified urease from *hypA* and *hypB* mutants contained four-fold and five-fold less nickel content than that from the wild-type strain. Mutations in the other *hyp* genes, *hypD*, *hypE* and *hypF* did not affect the urease activity, although they affected the hydrogenase activity.

Materials and Methods

Bacterial strains and growth conditions. *H. pylori* ATCC strain 43504 was used as the parent strain. It was grown on *Brucella* agar (Difco) supplemented with 10% defibrinated sheep blood (BA). Kanamycin (15 μg/ml) or chloramphenicol (20 μg/ml) was added to the media as required. Cells were incubated in CampyPak Plus (Becton Dickinson) atmosphere in jars, or in a CO_2/O_2 incubator with 5% CO_2, 12 % O_2, and 95% relative humidity at 37˚C. DNA manipulations were done in *E. coli* strain DH5α (BRL) grown in LB broth or agar, supplemented with ampicillin (100 μg/ml), kanamycin (30 μg/ml) or chloramphenicol (20 μg/ml) as required.

Cloning of plasmids used in the disruption of *H. pylori* genes. Regions of *H. pylori* genomic DNA containing the individual *hypA*, *hypB*, *hypD*, *hypE* or *hypF* open reading frames (ORFs) were amplified by PCR using *Pfu* DNA polymerase (New England Biochemicals) and the primers listed in Table 2.1. These primers were chosen in order to amplify a fragment (~1 kb) of genomic DNA containing all of each *hyp* ORF plus flanking DNA (around 200 bp) on either side. The amplified product would also have a unique restriction site within each gene into which the kanamycin resistance cassette (*aphA3*) could be inserted. The PCR products were first phosphorylated with T4 polynucleotide kinase (Promega) and subsequently cloned into the *Sma*I site of pBluescript KS+ (Stratagene). The cloned plasmids were then verified to be correct by sequencing (Molecular Genetics Instrumentation Facility, University of Georgia). The gene was then interrupted by inserting the kanamycin resistance cassette, *aphA3*, into the unique restriction site located within the coding region of each gene (Table 2.1).

Transformation and mutagenesis of *H. pylori*. *H. pylori* competent cells were made by first harvesting 72 to 96 hrs-old cells from BA plates, followed by washing and centrifuging five times in the presence of ice-cold 9% sucrose plus 15% glycerol solution. Competent cells were either used immediately for transformation or flash frozen and stored at -80˚C. 2 μg of plasmid DNA (pHyp:kan) was added to 50 μl of competent cells and electroporated with a pulse of 2.5 kV in a transporator plus (BTX) apparatus. Cells were resuspended in 50 μl of Mueller-Hinton broth and spotted onto a cold non-selective BA plate, and incubated in CampyPak atmosphere. After 48 hours, the cells were swabbed out of non-selective media and plated onto selective plates with kanamycin. Resistant colonies appeared in 72 to 96 hours, and were confirmed by PCR amplification that the target genes in the chromosome had been disrupted by the antibiotic resistance cassette. That the insertion of the cassette took place within the gene was seen by the increased size of the PCR product on agarose gel electrophoresis (Figure 2.1). The hydrogenase and urease activities of the mutant and wild-type strains were assayed as described below.

Construction of the *hypA* and *hypB* complementation strains, HypA:kan [HA] and HypB:kan [HB]. The chloramphenicol resistance cassette was inserted into the polylinker of pHypA or pHypB to yield pHypA:cm or pHypB:cm respectively (Table 2.1). This clone contained the cassette adjacent to *hypA* or *hypB* (including 303 bases of *H. pylori* sequence upstream of the *hypA* start codon and 200 bases upstream of the *hypB* start codon). The fragment containing the chloramphenicol cassette along with the *hypA* or *hypB* genes was then excised from pHypA:cm or pHypA:cm as a single fragment and ligated into a *Sma*I site of a plasmid, pEU39, yielding pEU:HypA or pEU:HypB

respectively. The plasmid pEU39 contains a 2.04 kb fragment of *H. pylori* genomic DNA corresponding to bases 416433-418974 of the *H. pylori* 26695 genome. This region is distant from the *hypA* or *hypB* genes and its disruption had shown no obvious phenotype (data not shown). When pEU:HypA was used to transform the *hypA* mutant (HypA:kan), the entire *hypA* coding region (and no other complete ORF) is inserted into the chromosome. This procedure yielded a diploid strain, HypA:kan [HA], that contained the original interrupted copy of *hypA* and one good copy of *hypA* at an unrelated site. Similarly when pEU:HypB was used to transform the *hypB* mutant (HypB:kan), the entire *hypB* coding region is inserted into the chromosome, and a diploid strain, HypB:kan [HB], is obtained. This diploid has one interrupted copy of *hypB* and one good copy of *hypB* at an unrelated site. The hydrogenase and urease activities of these diploids were assayed.

Nickel supplementation. *H. pylori* wild-type and the *hyp* accessory gene mutant strains were grown on BA media supplemented with 1 and 5 μM Ni^{+2}. Cells were harvested after 72 hrs, washed once with 50 mM Hepes-OH buffer, pH 7.5, and centrifuged at 8000 x g for 10 min. The cell pellet was subjected to French press at 12,000 psi, followed by centrifugation at 28,000 x g for 10 min. The supernatant was used for quantitative urease assay by the phenol-hypochlorite method. Hydrogenase assays were carried out on whole cells resuspended in Mueller-Hinton broth.

Hydrogenase assay. Hydrogen-uptake activity was determined amperometrically on whole cells with O_2 as the final electron acceptor as previously described (9). The results reported are the mean ± standard deviation for five independent assays.

Urease assay. Qualitative urease assay was performed using the phenol red method as described (15). Quantitative urease activity was determined using the phenol-hypochlorite assay as previously described (23). Stated values are the mean ± standard deviation for five independent assays.

Results

Construction of mutant strains. The *hypA*, *hypB*, *hypD*, *hypE* and *hypF* genes were all disrupted individually by introduction of a kanamycin resistance cassette, *aphA3*. Inactivation of these genes yielded strains HypA:kan, HypB:kan, HypD:kan, HypE:kan and HypF:kan. Each *hyp* gene disruption was confirmed by PCR amplification of genomic DNA purified from the mutant strain using primers specific for each gene (Figure 2.1).

The *hyp* genes are required for hydrogenase activity in *H. pylori*. We have shown previously that *H. pylori* expresses a hydrogen-uptake hydrogenase (9). To explore the role of the hydrogenase accessory genes in *H. pylori*, we studied the phenotype of five *hyp* gene mutants. The *hyp* gene mutant strains were all found to have negligible hydrogenase activity while the wild-type strain had an activity of 0.6 ± 0.2 nmoles H_2 oxidized /min /10^8 cells (Figure 2.2).

***hypA* and *hypB* are required for urease activity in *H. pylori*.** Qualitative assays of urease activity from whole cells using the phenol red method (15) indicated that two of the mutant strains (HypA:kan and HypB:kan) were severely impaired in urease activity when compared to the parent strain. Nevertheless, these mutants were verified to be *H. pylori* by visual inspection (phase contrast microscopy) and by the characteristic *H. pylori* protein profile as determined by SDS-PAGE on whole cells. Quantitative phenol

hypochlorite urease assays performed on crude extracts of these strains confirmed that the HypA:kan and HypB:kan strains were severely deficient in urease activity, HypA:kan being 200-fold and HypB:kan being 40-fold less than the wild type (Figure 2.3). The SDS-PAGE analysis showed the presence of the UreA and UreB subunits in the wild-type, HypA:kan, and HypB:kan strains, indicating that the urease expression is not affected in these mutants (Figure 2.4). Hydrogenase and urease activities of these mutants can be complemented to almost wild-type levels by inserting a second (uninterrupted) copy of *hypA* or *hypB* at an unrelated site within the genome of the *hypA* and *hypB* mutants (Figure 2.5, 2.6). The complementation results confirm that the lowered urease activity in these mutants is solely due to the lack of HypA or HypB, and not the result of a polar effect on genes downstream of *hypA* or *hypB*. The urease activities of the *hypD*, *hypE* and *hypF* mutants was similar to that of the wild-type strain (Figure 2.3).

Nickel supplementation partially restores urease and hydrogenase activity. Because the *hyp* genes are generally considered to be required for maturation of hydrogenase, nickel supplementation can sometimes restore activity of the mutants (2, 10). Hydrogenase activity was restored to 20% of the wild-type activity in HypA:kan strain, and 10% of the wild-type activity in HypB:kan strain when media was supplemented with 5 μM $NiCl_2$. Hydrogenase activities of the *hypD*, *hypE* and *hypF* mutants was restored to around 7% of the wild type (Figure 2.2). Urease activity was restored to a much greater extent at 5 μM nickel, the urease activities for the HypA:kan and HypB:kan were as high as that for the wild-type strain (Figure 2.3). It should be noted however that at these concentrations of nickel, the wild-type strain only retained about 40% of the urease activity as compared to when there was no added nickel. We

have no explanation for this result, but it was reproducible. In the presence of 5 μM $NiCl_2$, the *hypD*, *hypE*, and *hypF* mutants retained only 25% of the urease activity as compared to that when there was no added nickel (Figure 2.3). From the nickel supplementation results, it can therefore be concluded that hydrogenase activity was restored partially and urease activity to a greater extent in the HypA:kan and HypB:kan strains.

It was also shown by atomic absorption spectrophotometry that the nickel content of partially purified urease from the *hypA* and *hypB* mutants had four-fold and five-fold less nickel respectively as compared to the wild-type urease (work done by J. W. Olson)

Discussion

The hydrogenase accessory proteins, HypA and HypB, are required for full urease activity in *H. pylori*. This is surprising given that there is virtually no homology between the enzymes in terms of their primary or secondary structure, and the nickel active sites differ in both number of nickel ions (two for urease, one for hydrogenase) and in the ligation of the nickel (nitrogen and oxygen for urease, sulfur for hydrogenase). Despite these differences, the enhancement of urease activity by HypA and HypB almost certainly involves an increase in the ability of *H. pylori* to incorporate nickel into the enzyme. One role of the *hyp* genes (especially *hypB*) in hydrogen-oxidizing organisms includes nickel mobilization into hydrogenase (5, 18). The *hypA* and *hypB* mutations do lead to the expected hydrogenase negative phenotype, but the lack of urease activity in these mutants cannot be attributed to the need for an active hydrogenase enzyme. This is because, neither HypD:kan, HypE:kan nor HypF:kan strains share the urease negative phenotype, yet all of them are hydrogenase negative. The UreA and UreB subunits of

urease were expressed by the *hypA* and *hypB* mutants strains as well as they were in the wild-type strain (Figure 2.4).

Although the urease gene cluster of *H. pylori* appears to contain all the factors required to produce a fully active urease, urease expressed in *E. coli* harboring the plasmid with the *H. pylori* urease genes was only partially active, as compared to that of *H. pylori* (13, 16). Expression of a high affinity nickel transporter, NixA (16), increased the activity of the *E. coli* expressed enzyme. The diminished urease activity seen in both the HypA:kan and HypB:kan strains implicate the HypA and HypB proteins as the missing factors required for full urease activity. Still, these proteins were not identified in an exhaustive screen for urease-enhancing factors in which individual clones containing *H. pylori* genomic DNA fragments were tested for their ability to enhance the *E. coli* expressed *H. pylori* urease activity (13). This would be explained if, as we believe, both the proteins act in concert. Furthermore, the physical distance between the *hypA* and *hypB* genes (over 32 kb in strain 26695) would prevent both from being expressed from the same clone (clones in the library used for identifying the urease-enhancing factors had an average size of only 6 kb).

This is the first report of accessory proteins from one nickel-containing enzyme (hydrogenase) having a pleiotropic effect on a different nickel enzyme (urease). To help explain this result, it is helpful to compare characteristics often associated with accessory proteins with those from other organisms. For both enzymes, there is an accessory protein which plays a role as a nicke- binding factor. For the hydrogenase system, this role is fulfilled by the HypB protein, which often contains a nickel binding histidine rich N-terminus (3, 19). Likewise for urease, the UreE protein often has a histidine rich C-

terminus, which also has been shown to bind nickel (6). These histidine-rich regions are absent from both the HypB and UreE proteins of *H. pylori*, indicating a diminished capacity to bind nickel in both these proteins. However, it should be noted that the histidine-rich region is not the only nickel-binding domain in HypB; *B. japonicum* HypB which has had 23 of its clustered 24 histidines deleted can still bind one Ni^{+2} ion per monomer (18). Another primary structural feature of the nickel enzyme accessory protein is the presence of a nucleotide-binding motif. The HypB proteins from *E. coli* and *B. japonicum* have been shown to have GTPase activity which is required for making a catalytically active hydrogenase (11, 18). The UreG protein of *K. aerogenes* possesses a nucleotide-binding motif, and GTP hydrolysis has been shown to be required for nickel insertion into urease (21).

Interestingly, the Ure proteins of *K. aerogenes* have been shown to act as a complex that can be readily purified. This complex is proposed to donate nickel to the nickel-free apourease in a GTP-dependent fashion (21). *H. pylori* encodes all the proteins that have been identified in the *K. aerogenes* Ure complex. If this complex exists in *H. pylori*, we would expect that it would also have a nickel-binding requirement and would depend on GTP hydrolysis. If the complex was deficient in nickel content or had insufficient GTPase activity, we would expect *H. pylori* to accumulate an inactive urease which lacked nickel. This is exactly the phenotype of both the *hypA* and *hypB* mutants. We propose therefore that *H. pylori* urease accessory gene complex has recruited HypA and HypB proteins to augment one (or both) of these requirements which are essential for the efficient nickel donation to the urease enzyme.

Bibliography

1. **Alm, R. A., L. S. Ling, D. T. Moir, B. L. King, E. D. Brown, P. C. Doig, D. R. Smith, B. Noonan, B. C. Guild, B. L. deJonge, G. Carmel, P. J. Tummino, A. Caruso, M. Uria-Nickelsen, D. M. Mills, C. Ives, R. Gibson, D. Merberg, S. D. Mills, Q. Jiang, D. E. Taylor, G. F. Vovis, and T. J. Trust.** 1999. Genomic-sequence comparison of two unrelated isolates of the human gastric pathogen *Helicobacter pylori*. Nature **397:**176-80.
2. **Du, L., and Tibelius, K. H.** 1994. *Azotobacter chrococcum* hydrogenase gene cluster is involved in nickel metabolism. Current Microbiol. **28.:**21-24.
3. **Fu, C., J. W. Olson, and R. J. Maier.** 1995. HypB protein of *Bradyrhizobium japonicum* is a metal-binding GTPase capable of binding 18 divalent nickel ions per dimer. Proc Natl Acad Sci U S A **92:**2333-7.
4. **Hausinger, R. P.** 1994. Nickel enzymes in microbes. Sci Total Environ **148:**157-66.
5. **Jacobi, A., R. Rossmann, and A. Böck.** 1992. The *hyp* operon gene products are required for the maturation of catalytically active hydrogenase isoenzymes in *Escherichia coli*. Arch Microbiol **158:**444-51.
6. **Lee, M. H., H. S. Pankratz, S. Wang, R. A. Scott, M. G. Finnegan, M. K. Johnson, J. A. Ippolito, D. W. Christianson, and R. P. Hausinger.** 1993. Purification and characterization of *Klebsiella aerogenes* UreE protein: a nickel-binding protein that functions in urease metallocenter assembly. Protein Sci **2:**1042-52.

7. **Lutz, S., A. Jacobi, V. Schlensog, R. Bohm, G. Sawers, and A. Böck.** 1991. Molecular characterization of an operon (*hyp*) necessary for the activity of the three hydrogenase isoenzymes in *Escherichia coli*. Mol Microbiol **5:**123-35.

8. **Magalon, A., and A. Böck.** 2000. Dissection of the maturation reactions of the [NiFe] hydrogenase 3 from *Escherichia coli* taking place after nickel incorporation. FEBS Lett **473:**254-8.

9. **Maier, R. J., C. Fu, J. Gilbert, F. Moshiri, J. Olson, and A. G. Plaut.** 1996. Hydrogen uptake hydrogenase in *Helicobacter pylori*. FEMS Microbiol Lett **141:**71-6.

10. **Maier, T., and A. Böck.** 1996. Nickel incorporation into hydrogenases. In Mechanisms of Metallocenter Assembly. Hausinger, R. P., Eichhorn, G. L., and Marzilli, L. G. (eds). New York: VCH Publishers.**:**173-192.

11. **Maier, T., F. Lottspeich, and A. Böck.** 1995. GTP hydrolysis by HypB is essential for nickel insertion into hydrogenases of *Escherichia coli*. Eur J Biochem **230:**133-8.

12. **Maroney, M. J.** 1999. Structure/function relationships in nickel metallobiochemistry. Curr Opin Chem Biol **3:**188-99.

13. **McGee, D. J., C. A. May, R. M. Garner, J. M. Himpsl, and H. L. Mobley.** 1999. Isolation of *Helicobacter pylori* genes that modulate urease activity. J Bacteriol **181:**2477-84.

14. **McGee, D. J., and H. L. Mobley.** 1999. Mechanisms of *Helicobacter pylori* infection: bacterial factors. Curr Top Microbiol Immunol **241:**155-80.

15. **Mobley, H. L., M. J. Cortesia, L. E. Rosenthal, and B. D. Jones.** 1988. Characterization of urease from *Campylobacter pylori*. J Clin Microbiol **26:**831-6.

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

17. **Olson, J. W., C. Fu, and R. J. Maier.** 1997. The HypB protein from *Bradyrhizobium japonicum* can store nickel and is required for the nickel-dependent transcriptional regulation of hydrogenase. Mol Microbiol **24:**119-28.

18. **Olson, J. W., and R. J. Maier.** 2000. Dual roles of *Bradyrhizobium japonicum* nickelin protein in nickel storage and GTP-dependent Ni mobilization. J Bacteriol **182:**1702-5.

19. **Rey, L., J. Imperial, J. M. Palacios, and T. Ruiz-Argüeso.** 1994. Purification of *Rhizobium leguminosarum* HypB, a nickel-binding protein required for hydrogenase synthesis. J Bacteriol **176:**6066-73.

20. **Rey, L., J. Murillo, Y. Hernando, E. Hidalgo, E. Cabrera, J. Imperial, and T. Ruiz-Argüeso.** 1993. Molecular analysis of a microaerobically induced operon required for hydrogenase synthesis in *Rhizobium leguminosarum* biovar viciae. Mol Microbiol **8:**471-81.

21. **Soriano, A., and R. P. Hausinger.** 1999. GTP-dependent activation of urease apoprotein in complex with the UreD, UreF, and UreG accessory proteins. Proc Natl Acad Sci U S A **96:**11140-4.

22. **Tomb, J. F., O. White, A. R. Kerlavage, R. A. Clayton, G. G. Sutton, R. D. Fleischmann, K. A. Ketchum, H. P. Klenk, S. Gill, B. A. Dougherty, K. Nelson, J. Quackenbush, L. Zhou, E. F. Kirkness, S. Peterson, B. Loftus, D. Richardson, R. Dodson, H. G. Khalak, A. Glodek, K. McKenney, L. M. Fitzegerald, N. Lee, M. D. Adams, J. C. Venter, and et al.** 1997. The complete genome sequence of the gastric pathogen *Helicobacter pylori.* Nature **388:**539-47.

23. **Weatherburn, M. W.** 1968. Phenol-hypochlorite reaction for determination of ammonia. Anal. Chem. **39:**971-974.

Table 2.1. Strains, Plasmids and Primers

Name	Description	Source
Strains		
H. pylori		
43504	Parent strain for all *H. pylori* mutations	ATCC
HypA:kan	*aphA3* insertion within *hypA*	This study
HypB:kan	*aphA3* insertion within *hypB*	This study
HypD:kan	*aphA3* insertion within *hypD*	This study
HypE:kan	*aphA3* insertion within *hypE*	This study
HypF:kan	*aphA3* insertion within *hypF*	This study
HypA:kan [HA]	diploid *hypA* strain	This study
HypB:kan [HB]	diploid *hypB* strain	This study
E. coli		
DH5α	Cloning strain	BRL
Plasmids		
pBluescript KS+	Cloning vector	Stratagene
pHypA	*hypA* inserted into *Sma*I site of pBluescript KS+	This study
pHypA:kan	*aphA3* inserted into *Sph*I site of pHypA	This study
pHypB	*hypB* inserted into *Sma*I site of pBluescript KS+	This study
pHypB:kan	*aphA3* inserted into *Sac*I site of pHypB	This study
pHypD	*hypD* inserted into *Sma*I site of pBluescript KS+	This study
pHypD:kan	*aphA3* inserted into *Eco*47III site of pHypD	This study
pHypE	*hypE* inserted into *Sma*I site of pBluescript KS+	This study
pHypE:kan	*aphA3* inserted into *Eco*47III site of pHypE	This study
pHypF	*hypF* inserted into *Sma*I site of pBluescript KS+	This study
pHypF:kan	*aphA3* inserted into *Nsi*I site of pHypF	This study
pHypA:cm	cm^r cassette inserted adjacent to *hypA* in pHypA	This study
pHypB:cm	cm^r cassette inserted adjacent to *hypB* in pHypB	This study
pEU39	416433-418974 bps of *H. pylori* 26695 DNA in pUC19	TIGR
pEU:HypA	*hypA* coding region + cm^r cassette in pEU39	This study
pEU:HypB	*hypB* coding region + cm^r cassette in pEU39	This study
Primers (5'→ 3')		
hypAF	CGGGCTTACAGGCTTTAG	IDT
hypAR	CGCATTAGAGCTCGCTTC	IDT
hypBF	ATAAAGCTCAATGGATTCTAG	IDT
hypBR	AAATAAGGAAAATGAAATGATG	IDT
hypDF	TTCTTTGGAGGTTTTGTGGC	IDT
hypDR	GGGCGATTATGTGCTACTACAC	IDT
hypEF	CCCGCTTTTTTGTAACCCACC	IDT
hypER	TCCCCCCTAATGACAGCAGTATC	IDT
hypFF	TTTGCCCCCCTTTACCTCAC	IDT
hypFR	CTGCCTCTTTAGCCCTCTCAATC	IDT

Figure 2.1. Agarose gel electrophoresis of PCR products used to verify cassette insertion: DNA standard ΦX174 digested with *Hae*III (Lane 1 and 11); DNA standard lambda digested with *Hind*III (Lanes 10 and 14). Lanes 2 to 9 and Lanes 12, 13 contain fragments amplified from *H. pylori* genomic DNA from the following strains and primers with the approximate expected size of the fragment in parenthesis.

Lane 2: Wild-type (WT) using HypAR and HypAF (1,100 bp); Lane 3: HypA:kan using HypAR and HypAF (2,400 bp); Lane 4: WT using HypBF and HypBR (1175 bp); Lane 5: HypB:kan using HypBF and HypBR (2,475 bp); Lane 6: WT using HypDF and HypDR (1,000 bp); Lane 7: HypD:kan using HypDF and HypDR (2,300bp); Lane 8: WT using HypFF and HypFR (1,000 bp); Lane 9: HypF:kan using HypFF and HypFR, (2,300 bp); Lane 12: WT using HypEF and HypER (1,200 bp); Lane 13: HypE:kan using HypEF and HypER (2,500).

Figure 2.1

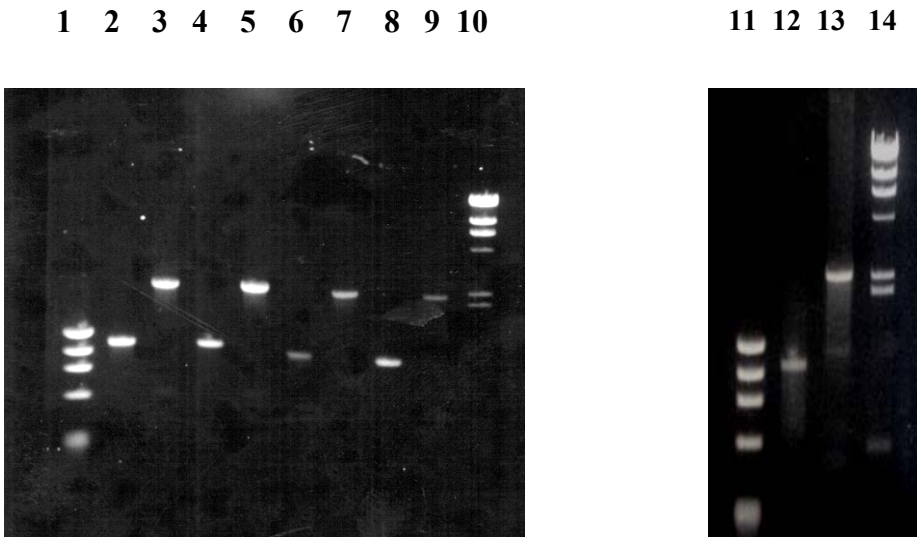


Figure 2.2. Hydrogenase activity of wild-type and *hyp* mutant strains grown in the presence and absence of added nickel: Hydrogenase activities of wild-type (WT) and mutant strains (*hypA*, *hypB*, *hypD*, *hypE*, and *hypF*) were measured amperometrically using whole cells. In the absence of added nickel, the mutants had negligible hydrogenase activities. In presence of 3 and 5 μM $NiCl_2$ the activities were restored partially.

Figure 2.2

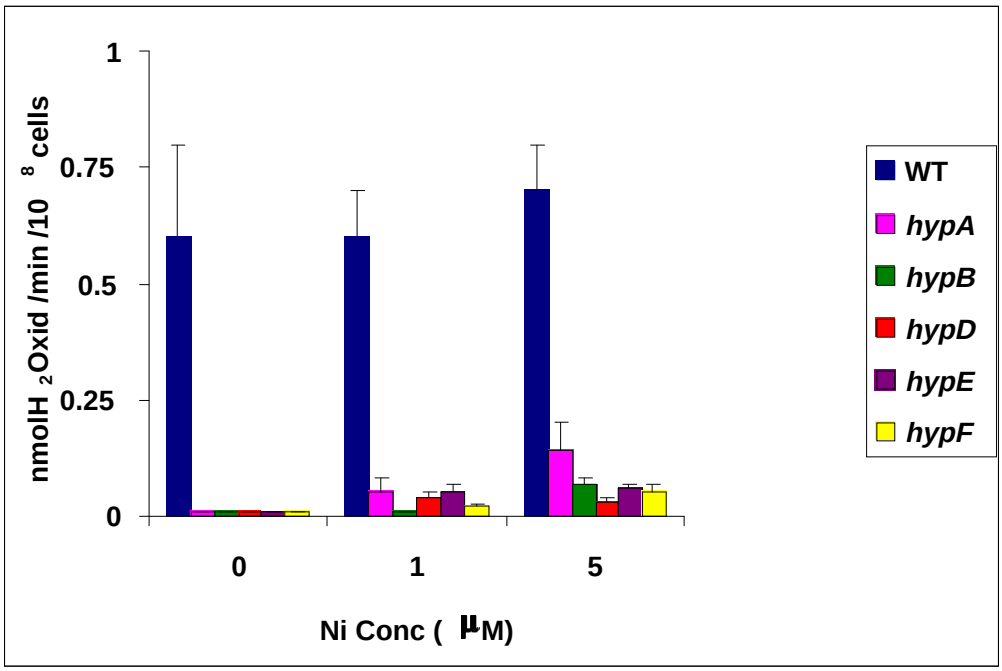

1
0.75
0.5
0.25
0
nmolH 2Oxid /min /10 8 cells
0
1
5
Ni Conc (µM)
WT
hypA
hypB
hypD
hypE
hypF

Figure 2.3. Urease activity of wild-type and *hyp* mutant strains grown in the presence and absence of added nickel: Urease activities were severely impaired in the *hypA* and *hypB* mutant strains. Activity was partially restored in the *hypA* (20%) and *hypB* (10%) mutant strains when grown in the presence of 3 and 5 μM $NiCl_2$. Urease activities of *hypD*, *hypE* and *hypF* mutant strains were similar to that of wild-type even in the absence of added $NiCl_2$.

Figure 2.3

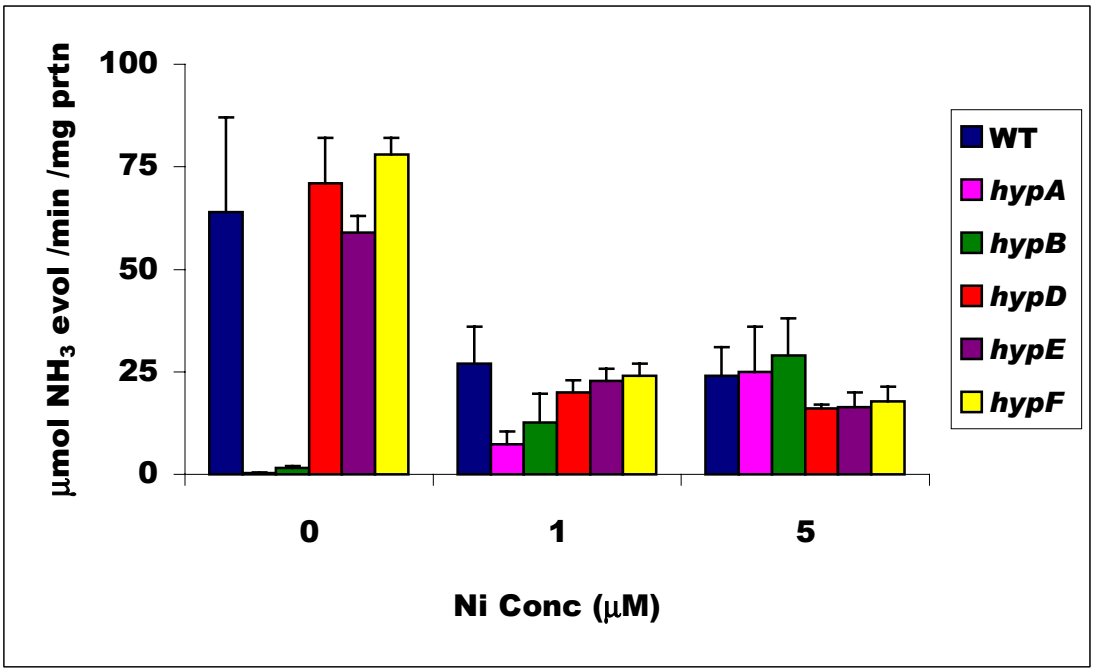

µmol NH3 evol /min /mg prtn
100
75
50
25
0
0
1
5
Ni Conc (µM)
WT
hypA
hypB
hypD
hypE
hypF

Figure 2.4. SDS-PAGE analysis of *H. pylori* crude extracts: SDS-PAGE analysis of crude extracts of wild-type (Lane 2), *hypA* (Lane 3), and *hypB* mutant (Lane 4) strains show the presence of the large (UreB) and small (UreA) subunits of urease. Molecular weight markers (Lane 1) were composed of phosphorylase b (97.4-kDa), bovine serum albumin (66.2-kDa), ovalbumin (45-kDa), carbonic anhydrase (31-kDa), soybean trypsin inhibitor (21.5-kDa), and lysozyme (14.4-kDa). The presence of the UreA and UreB subunits was also confirmed by immunoblot analysis using antiUreA and antiUreB antibodies from *H. pylori* (Data not shown, work done by Jon Olson)

Figure 2.4

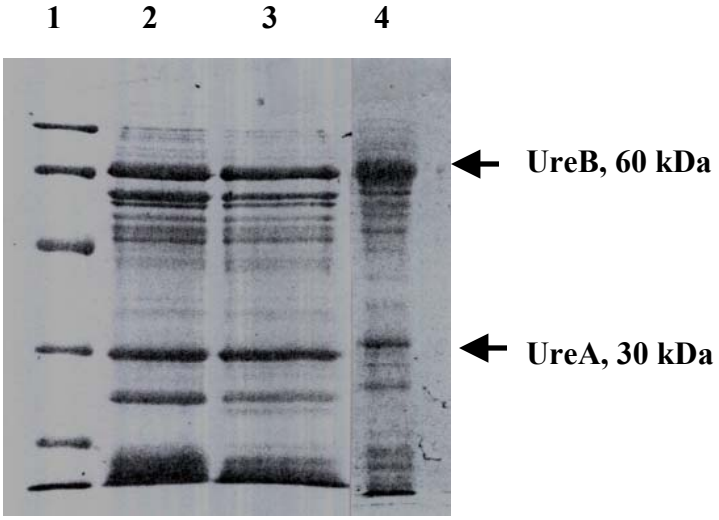


Figure 2.5. Hydrogenase activity of complemented strains of *hypA* and *hypB* mutations: *hypA* (A MT) and *hypB* (B MT) mutant strains were complemented by inserting a second uninterrupted copy of the corresponding gene at an unrelated site. Complemented *hypA* (Comp AMT) and *hypB* (Comp BMT) strains showed hydrogenase activity similar to the wild-type (WT) strain. Strains were tested after growth in media without added nickel.

Figure 2.5

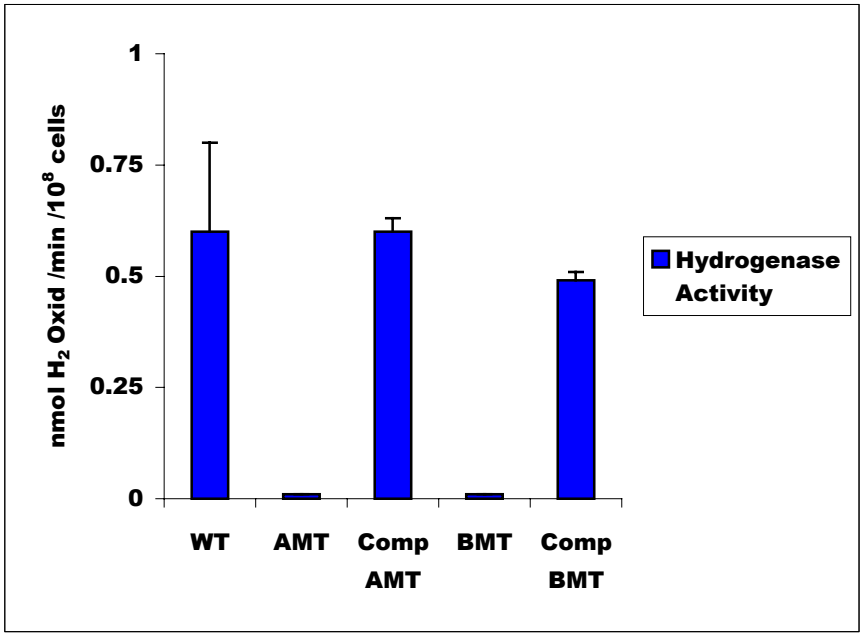
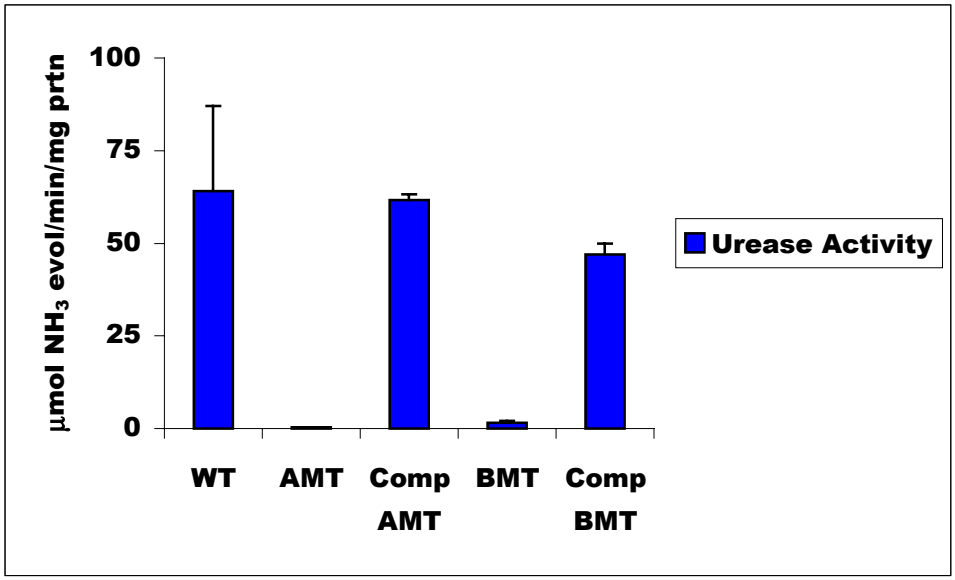

nmol H_2 Oxid /min /10^8 cells
1
0.75
0.5
0.25
0
WT
AMT
Comp AMT
BMT
Comp BMT
Hydrogenase Activity

Figure 2.6. Urease activity of complemented strains of *hypA* and *hypB* mutations: *hypA* (A MT) and *hypB* (B MT) mutant strains were complemented by inserting a second uninterrupted copy of the corresponding gene at an unrelated site. Complemented *hypA* (Comp AMT) and *hypB* (Comp BMT) strains showed urease activity similar to the wild-type (WT) strain. Strains were tested after growth in media without added nickel.

Figure 2.6


µmol NH3 evol/min/mg prtn
100
75
50
25
0
WT
AMT
Comp AMT
BMT
Comp BMT
Urease Activity

Chapter 3

CHARACTERIZATION OF *HELICOBACTER PYLORI* NICKEL METABOLISM ACCESSORY PROTEINS NEEDED FOR THE MATURATION OF BOTH UREASE AND HYDROGENASE [1]

[1] Mehta, N. S., J. W. Olson and R. J. Maier. 2002. J. Bacteriol. In Press. Reprinted here with the permission of the publisher (11/12/02).

Abstract

Previous studies demonstrated that two accessory proteins, HypA and HypB, play a role in nickel-dependent maturation of both hydrogenase and urease in *H. pylori*. Here, the two proteins were purified and characterized. HypA bound two Ni^{+2} ions per dimer with positive cooperativity (Hill coefficient approximately 2.0). The dissociation constants, K_1 and K_2 for Ni^{+2} were 58 and 1.3 μM respectively. Studies on purified site-directed mutant proteins in each of the five histidine residues within HypA, revealed that only one histidine residue (His2) is vital for nickel binding. NMR analysis showed that this purified mutant protein (H2A) was similar in structure to that of the wild-type HypA protein. A chromosomal site-directed mutant of *hypA* (in the codon for His2) lacked hydrogenase activity and possessed only 2% of the wild-type urease activity. Purified HypB had GTPase activity of 5 nmol GTP hydrolyzed per nmol HypB per min. Site-directed mutagenesis within the lysine residue in the conserved GTP-binding motif of HypB (Lys59) nearly abolished the GTPase activity of the mutant protein (K59A). In size exclusion chromatography, both HypA and HypB excluded as homodimers with molecular masses of 25.8 kDa and 52.4 kDa respectively. However, a 1:1 molar mixture of HypA plus HypB gave rise to a 43.6 kDa species composed of both proteins. A 43-kDa heterodimeric HypA-HypB complex was also detected by crosslinking. The cross-linked adduct was still observed in the presence of 0.5 mM GTP or 1 μM nickel, or when mutant HypA (H2A) and HypB (K59A) were tested. Individually, HypA and HypB formed homodimeric cross-linked adducts. An interaction between HypA and HP0868 protein (encoded by gene downstream to HypA) could not be detected by crosslinking, although such an interaction was predicted by yeast two-hybrid studies. In addition, the

phenotype of an insertional mutation within the HP0868 gene indicated that its presence is not critical for either the urease or hydrogenase activity.

Introduction

Helicobacter pylori is a spiral, Gram-type negative, microaerophilic bacterium that has been shown to be the etiological agent of gastritis and peptic ulcer disease (3, 4). It expresses two nickel-containing enzymes, both of which are important for its virulence. These are a membrane bound [Ni-Fe] hydrogen-uptake hydrogenase which permits respiratory-based energy production for the bacteria in the mucosa (19, 26), and an enzyme critical for early steps in colonization, the urease (23). Synthesis of metal-containing enzymes often requires the participation of accessory proteins, and the maturation of hydrogenase and urease are no exceptions. Indeed, the complete genome sequence of *H. pylori* reveals the presence of a full complement of urease (*ureIEFGH*) and hydrogenase (*hypABCDEF*) accessory genes (34). A number of studies exist that address these accessory genes in other bacteria and their role in the Ni-dependent maturation of urease and hydrogenase apoenzymes (7, 9, 13-15, 20, 25, 27, 32, 37). Although the specific role of each of these proteins has not been clarified to date, the existing data indicate that Ni-enzyme maturation involves concerted effort of the accessory proteins, likely utilizing sequential Ni-metabolizing steps.

By studying gene-directed mutants in *H. pylori*, it was found that two of the hydrogenase accessory genes, *hypA* and *hypB*, are required for both hydrogenase and urease activities (28). That *hypA* plays a role in *H. pylori* urease maturation was confirmed by another research group (39), also by use of a gene-directed mutation approach. The lack of urease activity could not be attributed to the lack of hydrogenase

activities in these two (*hypA* and *hypB*) mutants, since a hydrogenase structural gene mutant, *hydB,* and other *hyp* accessory mutants, *hypD, hypE* and *hypF* showed wild-type levels of urease activity although they were all deficient in hydrogenase activity. Also the expression levels of the urease apoenzyme in the *hypA* and *hypB* mutants strains were comparable to that in the wild-type strain; however the nickel content of the urease from the *hypA* mutant was four-fold less, and that from the *hypB* mutant was five-fold less than that of the wild-type enzyme (28). Therefore, it was proposed that in addition to being involved in the maturation of the hydrogenase apoenzyme, HypA and HypB are also involved in the maturation of the urease apoenzyme into its Ni-containing active form.

Both hydrogenase and urease maturation independently require an accessory protein with a functional nucleotide-binding domain and also one that is capable of binding and/or donating nickel to the active site (10, 11, 14, 16, 20, 24, 27). Mutation in the GTP-binding domain of *ureG* (*Klebsiella aerogenes*), and *hypB* (*Bradyrhizobium japonicum*) resulted in the production of an inactive urease and hydrogenase respectively (24, 27). The UreDFG-apourease complex from *K. aerogenes* could be activated *in vitro* only if GTP (200 μM) and nickel (20 μM) were present (32). UreE from *K. aerogenes* binds approximately 6 Ni^{+2} ions per dimer and this was attributed to the presence of a histidine-rich C- terminus. However later studies with a truncated UreE, lacking fifteen C- terminal residues, showed that in the absence of the histidine-rich region, the protein could still bind 2 Ni^{+2} ions per dimer (6). Similar to *K. aerogenes,* the nickel metabolizing accessory complexes of *H. pylori* would be expected to have both nickel sequestering and GTP hydrolyzing proteins for the mobilization of nickel into the active site of the nickel enzymes. *H. pylori* HypB has the characteristic GTP-binding domain, but lacks the

histidine-rich region. There are no such domains in HypA, but there are five scattered histidine residues within the protein. Since a mutation in *hypA* or *hypB* resulted in a deficiency in urease and hydrogenase activities in *H. pylori* (28), it is possible that these mutants are deficient in nickel sequestering, GTP hydrolysis, or in both functions.

In the present study we have purified and characterized the HypA and HypB proteins from *H. pylori*, determined their GTP hydrolyzing and nickel-binding abilities, and their ability to interact *in vitro*. This is the first report showing that a HypA protein is capable of binding nickel, and that HypA and HypB intimately interact *in vitro*. Site-directed mutagenesis of *hypA* was done in five histidine residues, and the nickel binding ability of each of the five purified mutant proteins was investigated. A site-directed mutant protein in the conserved lysine residue within the GTP-binding motif of HypB was also purified, and its GTPase activity was determined. A chromosomal site-directed mutation was also introduced into the *hypA* gene (codon for His2 replaced by that for alanine). Urease and hydrogenase activities of the mutant strain were measured.

Hydrogenase and urease activities of the HP0868 (gene downstream to *hypA*) mutant were measured, and crosslinking studies between HypA and HP0868 protein were also carried out.

Materials and methods

Bacterial strains and growth conditions. *E. coli* DH5α (BRL) was used for all genetic manipulations. *E. coli* BL21 (DE3) RIL (Stratagene) was used as host for expression of the recombinant proteins. *E. coli* XL1-Blue supercompetent cells (Stratagene) were used as host for site-directed mutagenesis. *E.coli* DH10B (BRL) was used as host for p*hypA*:KSF construct. *H. pylori* strain ATCC 43504 was used as wild-

type and parent strain for obtaining the HP0868 and the *hypA*:KSF mutant strains. *H. pylori* mutant strain, *hypA* :KSF was used as host for obtaining the *hypA* -H2A chromosomal site-directed mutant strain (Table 3.1).

E. coli strains were grown in Luria- Bertani (LB) medium or plates, supplemented with 100 μg/ml ampicillin or 30 μg/ml kanamycin as required. *H. pylori* was routinely grown on *Brucella* agar plates supplemented with 10% defibrinated sheep blood (BA plates), with and without 25 μg/ml kanamycin. For chromosomal site-directed mutants, BA plates with either 25 μg/ml kanamycin or 5% sucrose were used.

Construction of plasmids for over expression of wild-type HypA, HypB and HP0868 proteins. The *hypA, hypB* and HP0868 genes were PCR amplified in the presence of appropriate primers, HypA F1 and HypA R1; HypB F1 and HypB R1; 868 F1 and 868 R1 (IDT, Table 3.1) using genomic DNA from wild-type *H. pylori* as template. These primers engineer an *Nde*I restriction site at the 5' end prior to the start codon, and a *Bam*HI site at the 3' end immediately following the stop codon for each gene. The PCR products were then cloned into the *Sma*I site of pBluescript KS+, yielding pKS-*hypA*, pKS-*hypB*, and pKS-868. The pKS-*hypA,* pKS-*hypB* and pKS-868 were each digested with *Nde*I and *Bam*HI, and the fragment containing the coding region for each gene was purified and ligated into *Nde*I and *Bam*HI digested pET-21A, yielding pET-*hypA*, pET-*hypB* and pET-868 respectively (Table 3.1). These recombinant pET plasmids were then transformed into electrocompetent *E. coli* BL21 (DE3) RIL cells using a pulse of 2.5 kV in a transporator plus (BTX) apparatus.

Construction of plasmids for over expression of HypA and HypB mutant proteins with single amino acid substitutions. Site-directed mutagenesis of *hypA*

(histidine at positions 2, 17, 24, 79 and 95) and *hypB* (lysine at position 59 located within the GTP-binding motif) was done using the QuikChange Site-Directed mutagenesis protocol (Stratagene). Each of these amino acid residues was replaced by alanine. Primers containing the desired mutation were purchased commercially (IDT, Table 3.1). For *hypA* and *hypB* mutations, pET-*hypA* and pET-*hypB* were used as templates respectively. These recombinant pET plasmids with site-directed mutation in *hypA* or *hypB* gene (Table 3.1) were sequenced at Molecular Genetics and Instrumentation Facility (MGIF, University of Georgia) and transformed into electrocompetent BL21 (DE3) RIL cells for over expression of the mutant proteins.

Over expression of HypA, HypB and HP0868 proteins. BL21 (DE3) RIL cells containing the recombinant pET plasmid were grown at 25 ^{o}C to an OD_{600} of 0.6 in 1 L of LB medium with 100 μg/ml ampicillin. Expression was then induced by addition of 0.1 mM IPTG followed by incubation for 3 hrs at 25 ^{o}C. The cells were harvested by centrifugation at 8000 x g for 10 min at 4 ^{o}C. All subsequent steps were carried out at 4 ^{o}C. The cell pellet was washed once with buffer containing 10 mM Tris-Cl (pH 7.5) plus 25 mM NaCl (TN buffer), and re-pelleted by centrifugation. The pellet was resuspended in approximately 10 ml of the same buffer. The cells were broken by two passages through a French pressure cell at 12,000 psi and the lysate was centrifuged at 28,000 x g for 10 min to remove the cell debris. The supernatant was collected and subjected to ultracentrifugation at 100,000 x g for 1.5 hr. The membrane-free supernatant was used as a source for purification of these proteins.

Purification of HypA, HypB and HP0868 proteins. Purification of HypA and HypB (wild-type and mutant versions) was achieved in two steps, Q-Sepharose anion

exchange followed by Sephacryl S-100 gel filtration chromatography. TN buffer was used as the start buffer and 400 mM NaCl plus 10 mM Tris-Cl (pH 7.5) was used as the elution buffer for anion exchange chromatography. For gel filtration chromatography, TN buffer was used.

HP0868 protein was purified by SP-Sepharose cation exchange, followed by a gel filtration chromatography. 50 mM Na_2HPO_4 (pH 7.5) was used as the start buffer and 50 mM Na_2HPO_4 (pH 7.5) plus 1 M NaCl was used as the elution buffer for cation exchange chromatography. Gel filtration chromatography was performed in the presence of 20 mM Na_2HPO_4 (pH 7.5) plus 25 mM NaCl buffer.

Fractions were analyzed by SDS-PAGE to determine extent of purity. Protein concentration was estimated using the Coomasie Plus protein assay reagent and the protocol followed was that suggested by the manufacturer (Pierce). Pure fractions were then pooled and dialyzed for 48 hrs against different buffers depending on the type of assay.

N- terminal amino acid sequencing and determination of molecular mass. N-terminal sequencing of purified HypA, HypB and HP0868 proteins was carried out at MGIF (UGA). The molecular mass was determined by LC-MS at the Chemical and Biological Sciences Mass Spectrometry facility (UGA).

Size exclusion chromatography. The native size of HypA and HypB wild-type proteins was determined using a FPLC Superose 6 HR 10/30 column (Pharmacia). The column was pre-equilibrated with 50 mM Tris-Cl (pH 7.5) plus 100 mM KCl. Calibration was done using the MW GF 200-kit markers (SIGMA): β amylase, alcohol dehydrogenase, bovine serum albumin, carbonic anhydrase, and cytochrome C (*Mr* =

200,000, 150,000, 66,000, 29,000, and 12,400 respectively). Buffer used for protein elution was the same as that used for equilibration. A 1:1 molar mixture of wild-type HypA and HypB proteins (after a 10 min pre incubation at 4 ^{o}C) was also loaded onto the column to look for any interaction under native conditions.

Crosslinking assay. A 1:1 molar mixture of HypA and HypB wild-type proteins was incubated for 30 min at 25 ^{o}C in the presence of 5 mM dimethyl suberimidate (DMS, Pierce). After 30 mins, the reaction was quenched by adding 1 M Tris-Cl (pH 7.5) followed by incubation for 15 min at 25 ^{o}C. Crosslinking studies were also done for wild-type HypA and HypB proteins in the presence of 1 μM nickel or 0.5 mM GTP. The ability of the mutant HypA protein (H2A) to crosslink with the mutant HypB protein (K59A) was also investigated. Protein concentrations tested are shown in Figure 3.2A, 3.2B and 3.2C. Controls (proteins + BSA in a 1:1 molar ratio) were treated identically. After quenching, the samples were subjected to SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane as described (35). The membrane was blocked by incubating in the presence of 5% non-fat milk prepared in 10 mM Tris-Cl (pH 7.5) plus 150 mM NaCl, (BLOTTO). This was followed by a 3 hr incubation along with a 1:1000 dilution of either anti-HypA or anti-HypB antibody raised in rabbits against purified *H. pylori* HypA or HypB proteins (Cocalico Biologicals, PA). Following incubation, the membrane was washed five times with buffer containing 10 mM Tris-Cl (pH 7.5) plus 150 mM NaCl and incubated for 1 hr with a 1:1000 dilution of the secondary antibody (goat anti-rabbit immunoglobulin G complexed with alkaline phosphatase). The membrane was again washed five times with the same buffer. Bound antibody was detected by the addition of the chromogenic reagents, Nitroblue tetrazolium

(0.25 mg/ml) and 5'-bromo-4-chloro-3-indolyl phosphate (0.125 mg/ml) (SIGMA). A 1:1 molar mixture of wild-type HypA and HP0868 protein was also tested similarly.

Nickel-binding assay. The ability of the purified HypA and HypB proteins to bind nickel was determined by equilibrium dialysis followed by electrothermal atomic absorption spectrophotometry (Shimadzu). Briefly, 6-7 μM protein was dialyzed versus increasing concentrations of $NiCl_2$ (concentrations shown in Figure 3.3A and 3.3B) taken in 1 L of 50 mM NaCl (pH 8.25) for 48 hrs. Following dialysis, the nickel concentration of the protein solution (bound plus free Ni^{+2}) and the dialysis buffer (free Ni^{+2}) was determined. The bound nickel was estimated by subtracting the two values. The nickel binding ability of the HypA mutant proteins (H2A, H17A, H24A, H79A and H95A) was determined similarly. A 1:1 molar mixture of wild-type HypA plus HypB proteins was also assayed for nickel binding.

NMR spectroscopy for HypA (wild-type and H2A mutant proteins). NMR analysis was performed at the Chemical Sciences Magnetic Resonance Facility (University of Georgia). The NMR data were collected on a Varian Inova 500 spectrometer (499.8 MHz, ^1H). Water signal suppression was achieved using flip-back pulses (12) and pulse field gradients (29). The data were processed using vnmr (Varian, Inc.) without solvent subtraction.

GTPase assay. The ability of the purified HypA and HypB proteins to hydrolyze GTP was measured colorimetrically using previously described protocol (1, 11). A 1:1 molar ratio of HypA: HypB was also tested similarly. The ability of HypB to hydrolyze ATP or dGTP was determined similarly to that described for GTP. GTPase activity was also measured for the HypB mutant protein (K59A).

Construction of *H. pylori* chromosomal site-directed mutant of *hypA*. This mutant was constructed using a previously described protocol (8). Briefly, a *kan-sacB-flaA* fragment (KSF) was excised from pKSF-II (kindly provided by M. Copass), and inserted into a *Sph*I site within the *hypA* gene, previously cloned into pBluescript KS+ (p*hypA*) (Table 3.1). The KSF fragment has the *aphA3* kanamycin resistance cassette from *Campylobacter coli* (36), a promoterless *sacB* gene from *Bacillus subtilis* (33), and the *flaA* promoter from *H. pylori* (17). The resulting construct (p*hypA*:KSF) was transformed into *H. pylori* ATCC 43504 wild-type strain by natural transformation. The *hypA* gene was interrupted by insertion of the KSF fragment due to allelic exchange giving rise to the *hypA*:KSF mutant strain. This strain was kanamycin resistant and sucrose sensitive. The desired mutation was confirmed by PCR amplification of chromosomal DNA from *hypA*:KSF strain using primers, HypAF and HypAR (Table 3.1) followed by agarose gel electrophoresis (data not shown). This mutant was used as parent strain for transformation of p*hypA*-H2A construct.

The p*hypA*-H2A construct (with a site-directed mutation in the codon for His2) was prepared using the QuikChange Site-Directed mutagenesis protocol (Stratagene) and p*hypA* as the template. Primers (HypA: H2A F1 and HypA: H2A R1) were specifically designed to introduce the desired mutation (Table 3.1).

The transformation of the *H. pylori* mutant strain *hypA*:KSF with the construct p*hypA*-H2A was done by natural transformation. A site-directed mutant of *hypA* (*hypA*-H2A) was obtained as a result of allelic exchange. This mutant was sucrose resistant and kanamycin sensitive. Mutation was confirmed by PCR amplification of the chromosomal DNA using primers HypAF and HypAR (Table 3.1), followed by sequencing of the

product at MGIF (UGA). Hydrogenase and urease activities were assayed for the wild-type and mutant strains.

Insertional mutagenesis of HP0868 gene. Mutagenesis of the HP0868 gene was done using previously described protocol (28). Primers 868 F and 868 R were used for PCR amplification. The construct p868: kan (Table 3.1) was electrotransformed into *H. pylori* ATCC 43504 wild-type strain. Allelic exchange resulted in insertion of the kanamycin resistant cassette (*aphA3*) into the HP0868 gene. Mutation was confirmed by PCR amplification of chromosomal DNA using primers 868 F and 868 R followed by agarose gel electrophoresis to confirm the increased size of the interrupted gene (data not shown). Hydrogenase and urease activities were assayed for the wild-type and mutant strains.

Hydrogenase assay. Hydrogen-uptake activity was determined amperometrically for whole cells with O_2 as the final electron acceptor. The protocol followed was that described previously (19).

Urease assay. Cells were harvested after 72 hrs of growth, washed twice with 50 mM HEPES buffer pH 7.5, and broken by two passages through a French pressure cell at 12,000 psi. The lysate was centrifuged at 28,000 x g to remove the cell debris, and the supernatant was assayed for urease activity using the phenol-hypochlorite method (22, 38).

Results and Discussion

Purification of HypA, HypB and HP0868 proteins. Earlier observations that HypA and HypB proteins were involved in urease maturation prompted us to purify and characterize these two proteins. The HypA and HypB proteins (wild-type and mutant

versions) were over expressed as soluble proteins, and purified to near homogeneity. The yield for all these proteins was between 25 to 30 mg/L. On SDS-PAGE, wild-type HypA and HypB migrated at approximately 13- and 30-kDa respectively (Figure 3.1A, 3.1B). Mutant HypA and HypB proteins also migrated at 13- and 30-kDa respectively (Figure 3.1C). The molecular mass of HypA almost matched its predicted mass of 13.2-kDa. However HypB ran as a slightly larger polypeptide since its predicted mass is 27.5-kDa.

A yeast two-hybrid study had predicted a strong interaction of HypA with another protein, HP0868 (encoded by a gene downstream of *hypA*) (30). To address any interaction, the HP0868 protein was over expressed and purified to near homogeneity. The yield was approximately 20 mg/L. The HP0868 protein migrated at approximately 21-kDa on SDS-PAGE (predicted mass is 18.5-kDa) (Figure 3.1D).

N- terminal sequence and molecular mass determination. The N-terminal sequences of wild-type HypA, HypB and HP0868 proteins exactly matched the deduced amino acid sequence as seen in the TIGR database for *H. pylori*. The amino terminal methionine was present in all three proteins. The molecular masses for HypA, HypB, and HP0868 proteins as determined by mass spectroscopy were 13.2 ± 0.0025, 27.3 ± 0.034 and 18.7 ± 0.029 kDa respectively.

Size exclusion chromatography. The native molecular masses of HypA and HypB as determined by gel filtration chromatography were around 25.8-kDa and 52.4-kDa, which is consistent with them existing as dimers in solution. Interestingly a 1:1 molar mixture of HypA and HypB gave rise to a species with a molecular mass of 43.6-kDa. Immunoblot analysis of this species with anti-HypA and anti-HypB antibodies from *H. pylori* showed the presence of both proteins (data not shown). This indicates the

existence of an intermolecular interaction between the two proteins in native solution. This conclusion is further supported by crosslinking studies.

Crosslinking assay. Since a mutation in either *hypA* or *hypB* resulted in a phenotype in which both hydrogenase and urease activities were markedly reduced, we proposed that these two proteins might be acting in a co-ordinated fashion for the activation of both these enzymes (28). To investigate this possibility, crosslinking studies were carried out in the presence of 5 mM dimethyl suberimidate, a homobifunctional crosslinker. Mixing equimolar concentrations of wild-type HypA and HypB resulted in the formation of a unique 43-kDa heterodimeric complex, which was not seen when either of these two proteins was tested individually (Figure 3.2A). This size corresponds to a 1:1 molar amount of HypA:HypB. This observation is in agreement with the size exclusion results. It therefore appears that HypA and HypB may be forming a complex to aid in nickel enzyme maturation. The wild-type HypA plus HypB mixture also showed formation of both monomer and dimer in the case of HypA, and monomer, dimer and perhaps an additional larger oligomer in the case of HypB. These latter results matched with that observed when the two proteins were tested individually; HypA showed formation of monomer (13-kDa) and dimer (30-kDa), and HypB showed formation of monomer (30-kDa), dimer (55-kDa) and a larger species (83-kDa) (Figure 3.2A). Although HypA and HypB are individually capable of forming dimers, together they did not give rise to a heterotetrameric complex, which might indicate that the strength of the interaction between HypA and HypB monomers supercedes that between the dimers. Addition of equimolar concentrations of bovine serum albumin in the reaction mixture did not perturb the interactions (data not shown).

Crosslinking reactions involving wild-type HypA, HypB and DMS in the presence of 0.5 mM GTP or 1 μM nickel also gave rise to this 43-kDa heterodimeric complex, with no additional complexes detected (Figure 3.2B). A mixture of the HypA mutant (H2A) and HypB mutant (K59A) proteins formed a similar complex (Figure 3.2C). So did a mixture of HypA mutant protein (H2A), wild-type HypB protein plus 1 μM nickel or a mixture of HypB mutant protein (K59A), wild-type HypA protein and 0.5 mM GTP (Figure 3.2C). No new complexes were seen in any of these reactions. These results indicate that the contact point for forming the HypA:HypB heterodimer does not involve the nickel-binding residue in HypA (His2) nor the conserved lysine residue (Lys59) within the GTP-binding motif of HypB.

Since a strong interaction was predicted between HypA and its downstream gene (HP0868) in yeast two-hybrid studies (30), a crosslinking reaction was attempted between HP0868 and HypA proteins in the presence of 5 mM DMS. HypA did not show any cross-linked products with the HP0868 protein (data not shown).

Nickel-binding assay. Earlier studies had shown that nickel supplementation in the growth media restored the urease activity of the *hypA* and *hypB* mutants, and partially purified urease from *hypA* and *hypB* mutants had 4-fold and 5-fold lower nickel content respectively as compared to that from the wild-type (28). We determined the number of Ni^{+2} ions bound to wild-type HypA and HypB proteins over a range of nickel concentrations. Equilibrium dialysis of HypB showed that it is incapable of binding nickel (Figure 3.3A). This is in contrast to the documented nickel-binding ability of HypB from *B. japonicum* and *Rhizobium leguminosarum* which bound 9 and 3.9 Ni^{+2} ions per monomer respectively (11, 31). Equilibrium dialysis of HypA showed ability to

bind up to 2 Ni^{+2} ions per dimer (Figure 3.3A). Another plot of the data (Figure 3.3B) showed positive cooperativity in nickel binding with a Hill coefficient of approximately 2.0 (2). The dissociation constants K_1 and K_2 for nickel were 58 ± 6 and 1.3 ± 0.2 μM respectively, with half saturation at around 15 μM Ni^{+2} ion concentration (2). In the presence of equimolar concentrations of HypB, the nickel binding ability of HypA remained unaltered (data not shown). The ability of HypA to bind Ni^{+2} ions is a novel observation, although it may not be the only protein performing this function in *H. pylori*. Yet due to the presence of two Ni-dependent enzymes, urease and hydrogenase, this observation seems significant, especially in light of the inability of a HypA site-specific mutant protein to bind nickel (see below). HypA from *E. coli* has been over expressed and purified, but its nickel binding ability has not been addressed (18).

The TIGR database showed the presence of five histidine residues distributed within the HypA protein. Since the wild-type HypA protein bound Ni^{+2} ions, we carried out site-directed mutagenesis of each of the five histidine residues within *hypA* (His2, 17, 24, 79, 95). The sequence of the recombinant pET plasmids showed the presence of an intact initiation codon and also a site-directed mutation at the targeted location (codon for His to Ala) within the open reading frame of *hypA*. The nickel binding ability of each of the over expressed and purified mutant protein was investigated at different $NiCl_2$ concentrations. Four of the mutant proteins (H17A, H24A, H79A and H95A) could bind around 2 Ni^{+2} ions per dimer similar to the wild-type protein, with half saturation at 15 μM Ni^{+2} ion concentration (data not shown). However, the H2A mutant protein lacked the ability to bind nickel. Sequence comparison studies showed that this histidine residue (His2) is well conserved among other HypA proteins as well. The Ni^{+2} ions bound by

each of the HypA mutant proteins after equilibrium dialysis against 60 μM Ni^{+2} (which falls in the saturation range) is shown in Figure 3.4. From these results we can conclude that among the five histidine residues, only the His2 is critical for nickel binding by HypA.

NMR spectroscopy for HypA (wild-type and H2A mutant proteins). Since His2 is critical for nickel binding based on characteristics of the H2A mutant protein, both the wild-type HypA and H2A mutant proteins were compared for their NMR spectra. The 1-D NMR spectra for the two proteins in the fingerprint NH region (6 ppm to 10 ppm) were similar to each other, indicating that there is no significant difference between the two structures (Figure 3.5, Panel A and B). The spectra also indicated that the two proteins were in the folded state (Q. Teng, Personal communication, Chemical Sciences Magnetic Resonance Facility, UGA). The inability to bind nickel by the H2A mutant protein can therefore be attributed to the replacement of the His2 with alanine and not due to any structural changes.

GTPase assay. A large number of GTP-binding proteins show the presence of a well-conserved GTP-binding motif which has been speculated to bind and hydrolyze GTP (5). It has been therefore hypothesized that these proteins may be involved in a "switch mechanism", whereby some intracellular signal or reaction may be turned 'on' in the GTP bound form and 'off ' following GTP hydrolysis. The deduced amino acid sequence of HypB from *H. pylori* also shows the presence of a GTP-binding motif. Therefore, purified HypB was assayed for its GTPase activity. *H. pylori* HypB showed a GTPase activity of 5 nmol GTP hydrolyzed per nmol HypB per min and the rate of hydrolysis was linear over 140 min (Figure 3.6). Since *H. pylori* HypB did not possess

nickel binding ability, but the *hypB* mutant was deficient in urease activity (28), GTP hydrolyzing activity may be initiating some steps leading to the insertion of nickel into the urease apoenzyme. Since a lysine residue (Lys59) in the GTP-binding motif of HypB is also well conserved in other HypB proteins, a site-directed mutation was introduced that replaced this residue with alanine. Unlike the wild-type HypB protein, however, the HypB K59A mutant protein showed negligible GTPase activity (0.1 nmol GTP hydrolyzed per nmol HypB per min) (Figure 3.6). This result is not unexpected because it has been shown that substitution of the conserved lysine residue in the GTP-binding motif of HypB in both *B. japonicum* and *E. coli* also greatly lowered its GTPase activity (21, 27). HypB showed slight dGTPase and negligible ATPase activities (data not shown). Purified HypA showed negligible GTPase activity, and the GTPase activity of HypB remained unaltered in the presence of equimolar concentrations of HypA (data not shown).

Hydrogenase and urease activities of chromosomal site-directed mutant of *hypA*. Since the H2A mutant protein of HypA showed an inability to bind nickel, a site-directed mutation was introduced within the *hypA* gene so as to substitute the codon for His2 with that for alanine. The *H. pylori* wild-type and mutant strains were assayed for hydrogenase and urease activities. The mutant completely lacked hydrogenase activity (< 0.01 nmol H_2 oxidized per min per 10^8 cells) as compared to the wild-type (0.4 ± 0.2 nmol H_2 oxidized per min per 10^8 cells). The urease activity of the *hypA*-H2A mutant strain was around 2 % (0.85 ± 0.05 μmol NH_3 evolved per min per mg protein) of that of the wild -type (39 ± 8.1 μmol NH_3 evolved per min per mg protein). These results show the significant role played by His2 of the HypA protein in urease and hydrogenase

activities, and indicate that specific Ni- sequestering by a single protein is one of the molecular events common to the maturation of both nickel containing enzymes.

Hydrogenase and urease activities of HP0868 insertional mutant strain. The hydrogenase and urease activities for the wild-type and HP0868 mutant strains were around 0.35 ± 0.22 nmol H_2 oxidized per min per 10^8 cells and 40 ± 3.2 μmol NH_3 evolved per min per mg protein respectively. Since the activities were similar for the wild-type and mutant strains, the presence of the HP0868 gene is not critical for the hydrogenase and urease activities. This is in contrast to the results seen in case of the *hypA* mutant which was deficient in both activities (28). These results are in agreement with the crosslinking results that also do not support an interaction between HypA and the HP0868 protein as determined with the yease two-hybrid approach (30).

Conclusions

Our results constitute the first demonstration of the nickel-binding ability of HypA, and the significant role played by His2 of HypA for nickel-binding and also for the activation of hydrogenase and urease. Another significant observation was that of the ability of the HypA and HypB proteins to interact and form a heterodimeric complex in solution as well as in the presence of DMS. The size of this complex corresponds to a 1:1 molar amount of HypA: HypB. Although individually both proteins formed dimers, a heterotetrameric complex was not seen indicating that the strength of interaction between the monomers supercedes that between dimers. HypB lacked nickel binding, but was capable of hydrolyzing GTP. The lysine residue (Lys59) in the well-conserved GTP-binding motif of HypB was shown to be vital for the GTP hydrolyzing activity of HypB. These results are compatible with the role of nickel sequestration by HypA, and the

initiation of steps leading to nickel incorporation via the GTP-hydrolyzing ability of HypB. The histidine located in the extreme N-terminus of HypA (His2), and the lysine in the GTP-binding domain of HypB (Lys59) play vital roles in nickel binding and GTP hydrolysis respectively.

Bibliography

1. **Ames, B. N., and D.T. Dubin.** 1960. The role of polyamines in the neutralization of Bacteriophage deoxyribonucleic acid. J Biol Chem **235:**769-775.
2. **Bell, J. E., and E. T. Bell.** 1988. Proteins and Enzymes, Prentice-Hall, Inc., New Jersey, E. C.
3. **Blaser, M. J.** 1987. Gastric *Campylobacter*-like organisms, gastritis, and peptic ulcer disease. Gastroenterology **93:**371-83.
4. **Blaser, M. J.** 1990. *Helicobacter pylori* and the pathogenesis of gastroduodenal inflammation. J Infect Dis **161:**626-33.
5. **Bourne, H. R., D. A. Sanders, and F. McCormick.** 1991. The GTPase superfamily: conserved structure and molecular mechanism. Nature **349:**117-27.
6. **Brayman, T. G., and R. P. Hausinger.** 1996. Purification, characterization, and functional analysis of a truncated *Klebsiella aerogenes* UreE urease accessory protein lacking the histidine-rich carboxyl terminus. J Bacteriol **178:**5410-6.
7. **Colpas, G. J., and R. P. Hausinger.** 2000. In vivo and in vitro kinetics of metal transfer by the *Klebsiella aerogenes* urease nickel metallochaperone, UreE. J Biol Chem **275:**10731-7.
8. **Copass, M., G. Grandi, and R. Rappuoli.** 1997. Introduction of unmarked mutations in the *Helicobacter pylori vacA* gene with a sucrose sensitivity marker. Infect Immun **65:**1949-52.
9. **Drapal, N., and A. Böck.** 1998. Interaction of the hydrogenase accessory protein HypC with HycE, the large subunit of *Escherichia coli* hydrogenase 3 during enzyme maturation. Biochemistry **37:**2941-8.

10. **Fu, C., and R. J. Maier.** 1994. Nucleotide sequences of two hydrogenase-related genes (*hypA* and *hypB*) from *Bradyrhizobium japonicum*, one of which (*hypB*) encodes an extremely histidine-rich region and guanine nucleotide-binding domains. Biochim Biophys Acta **1184:**135-8.

11. **Fu, C., J. W. Olson, and R. J. Maier.** 1995. HypB protein of *Bradyrhizobium japonicum* is a metal-binding GTPase capable of binding 18 divalent nickel ions per dimer. Proc Natl Acad Sci U S A **92:**2333-7.

12. **Grzesiek, S., and A. Bax.** 1993. The importance of not saturating water in protein NMR-Application to sensitivity enhancement and NOE measurements. J.Am. Chem. Soc. **115:**12593-12594.

13. **Heimer, S. R., and H. L. Mobley.** 2001. Interaction of *Proteus mirabilis* urease apoenzyme and accessory proteins identified with yeast two-hybrid technology. J Bacteriol **183:**1423-33.

14. **Hube, M., M. Blokesch, and A. Böck.** 2002. Network of hydrogenase maturation in *Escherichia coli*: role of accessory proteins HypA and HybF. J Bacteriol **184:**3879-85.

15. **Jacobi, A., R. Rossmann, and A. Böck.** 1992. The *hyp* operon gene products are required for the maturation of catalytically active hydrogenase isoenzymes in *Escherichia coli*. Arch Microbiol **158:**444-51.

16. **Lee, M. H., H. S. Pankratz, S. Wang, R. A. Scott, M. G. Finnegan, M. K. Johnson, J. A. Ippolito, D. W. Christianson, and R. P. Hausinger.** 1993. Purification and characterization of *Klebsiella aerogenes* UreE protein: a nickel-

binding protein that functions in urease metallocenter assembly. Protein Sci **2:**1042-52.

17. **Leying, H., S. Suerbaum, G. Geis, and R. Haas.** 1992. Cloning and genetic characterization of a *Helicobacter pylori* flagellin gene. Mol Microbiol **6:**2863-74.

18. **Lutz, S., A. Jacobi, V. Schlensog, R. Böhm, G. Sawers, and A. Bock.** 1991. Molecular characterization of an operon (*hyp*) necessary for the activity of the three hydrogenase isoenzymes in *Escherichia coli*. Mol Microbiol **5:**123-35.

19. **Maier, R. J., C. Fu, J. Gilbert, F. Moshiri, J. Olson, and A. G. Plaut.** 1996. Hydrogen uptake hydrogenase in *Helicobacter pylori*. FEMS Microbiol Lett **141:**71-6.

20. **Maier, T., A. Jacobi, M. Sauter, and A. Böck.** 1993. The product of the *hypB* gene, which is required for nickel incorporation into hydrogenases, is a novel guanine nucleotide-binding protein. J Bacteriol **175:**630-5.

21. **Maier, T., F. Lottspeich, and A. Böck.** 1995. GTP hydrolysis by HypB is essential for nickel insertion into hydrogenases of *Escherichia coli*. Eur J Biochem **230:**133-8.

22. **McGee, D. J., C. A. May, R. M. Garner, J. M. Himpsl, and H. L. Mobley.** 1999. Isolation of *Helicobacter pylori* genes that modulate urease activity. J Bacteriol **181:**2477-84.

23. **McGee, D. J., and H. L. Mobley.** 1999. Mechanisms of *Helicobacter pylori* infection: bacterial factors. Curr Top Microbiol Immunol **241:**155-80.

24. **Moncrief, M. B., and R. P. Hausinger.** 1997. Characterization of UreG, identification of a UreD-UreF-UreG complex, and evidence suggesting that a nucleotide-binding site in UreG is required for in vivo metallocenter assembly of *Klebsiella aerogenes* urease. J Bacteriol **179:**4081-6.

25. **Mulrooney, S. B., and R. P. Hausinger.** 1990. Sequence of the *Klebsiella aerogenes* urease genes and evidence for accessory proteins facilitating nickel incorporation. J Bacteriol **172:**5837-43.

26. **Olson, J. W., and Maier, R. J.** 2002. Molecular hydrogen as energy source for *Helicobacter pylori*. Science. In Press.

27. **Olson, J. W., and R. J. Maier.** 2000. Dual roles of *Bradyrhizobium japonicum* nickelin protein in nickel storage and GTP-dependent Ni mobilization. J Bacteriol **182:**1702-5.

28. **Olson, J. W., N. S. Mehta, and R. J. Maier.** 2001. Requirement of nickel metabolism proteins HypA and HypB for full activity of both hydrogenase and urease in *Helicobacter pylori*. Mol Microbiol **39:**176-82.

29. **Piotto, M., V. Saudek, and V. Sklenar.** 1992. Gradient-tailored excitation for single-quantum NMR spectroscopy of aqueous solutions. J Biomol NMR **2:**661-5.

30. **Rain, J. C., L. Selig, H. De Reuse, V. Battaglia, C. Reverdy, S. Simon, G. Lenzen, F. Petel, J. Wojcik, V. Schächter, Y. Chemama, A. Labigne, and P. Legrain.** 2001. The protein-protein interaction map of *Helicobacter pylori*. Nature **409:**211-5.

31. **Rey, L., J. Imperial, J. M. Palacios, and T. Ruiz-Argüeso.** 1994. Purification of *Rhizobium leguminosarum* HypB, a nickel-binding protein required for hydrogenase synthesis. J Bacteriol **176:**6066-73.

32. **Soriano, A., and R. P. Hausinger.** 1999. GTP-dependent activation of urease apoprotein in complex with the UreD, UreF, and UreG accessory proteins. Proc Natl Acad Sci U S A **96:**11140-4.

33. **Steinmetz, M., D. Le Coq, H. B. Djemia, and P. Gay.** 1983. Genetic analysis of *sacB*, the structural gene of a secreted enzyme, levansucrase of *Bacillus subtilis* Marburg. Mol Gen Genet **191:**138-44.

34. **Tomb, J. F., O. White, A. R. Kerlavage, R. A. Clayton, G. G. Sutton, R. D. Fleischmann, K. A. Ketchum, H. P. Klenk, S. Gill, B. A. Dougherty, K. Nelson, J. Quackenbush, L. Zhou, E. F. Kirkness, S. Peterson, B. Loftus, D. Richardson, R. Dodson, H. G. Khalak, A. Glodek, K. McKenney, L. M. Fitzegerald, N. Lee, M. D. Adams, J. C. Venter, and et al.** 1997. The complete genome sequence of the gastric pathogen *Helicobacter pylori*. Nature **388:**539-47.

35. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci U S A **76:**4350-4.

36. **Trieu-Cuot, P., G. Gerbaud, T. Lambert, and P. Courvalin.** 1985. In vivo transfer of genetic information between gram-positive and gram-negative bacteria. Embo J **4:**3583-7.

37. **Vignais, P. M., B. Billoud, and J. Meyer.** 2001. Classification and phylogeny of hydrogenases. FEMS Microbiol Rev **25:**455-501.

38. **Weatherburn, M. W.** 1968. Phenol-hypochlorite reaction for determination of ammonia. Anal. Chem. **39.:**971-974.

39. **Wöhl, F., L. Otto, B. Waidner, U. Klönne, S. Phadnis, M. Kist, and S. Bereswill.** 2001. CHRO Abst. K-22. Mutational analysis of the hydrogenase accessory protein HypA in *Helicobacter pylori* strain 26695. IJMM **291:**100.

Table 3.1 Strains, plasmids and primers

Name	Description	Source
Strains		
H.pylori 43504	Mutagenesis host	ATCC
E.coli DH5α	Cloning host	BRL
E.coli BL21 (DE3) RIL	Over expression host	Stratagene
E.coli XL1-Blue	Site directed mutagenesis host	Stratagene
E.coli DH10B	Cloning host for p*hypA*:KSF	BRL
hypA:KSF	KSF fragment inserted into *Sph*I site of *hypA in H. pylori*	This study
hypA-H2A	Codon for His2 replaced by Ala within *hypA* in *H. pylori*	This study
Plasmids		
pBluescript KS +	Cloning vector	Stratagene
pKS-*hypA*	*hypA* inserted into the *Sma*I site of pBluescript KS+	This study
pKS-*hypB*	*hypB* inserted into the *Sma*I site of pBluescript KS+	This study
pKS-868	HP 0868 gene inserted into the *Sma*I site of pBluescript KS+	This study
pET-21A	Cloning vector	Novagen
pET-*hypA*	*hypA* introduced between the *NdeI* and *BamHI* site of pET-21A	This study
pET-*hypA*-H2A	pET-*hypA* with the His2 replaced by Ala	This study
pET-*hypA*-H17A	pET-*hypA* with the His17 replaced by Ala	This study
pET-*hypA*-H24A	pET-*hypA* with the His24 replaced by Ala	This study
pET-*hypA*-H79A	pET-*hypA* with the His79 replaced by Ala	This study
pET-*hypA*-H95A	pET-*hypA* with the His95 replaced by Ala	This study
pET-*hypB*	*hypB* introduced between the *Nde*I and *Bam*HI site of pET-21A	This study
pET-*hypB*-K59A	pET-*hypB* with the Lys59 replaced by Ala	This study
pET-868	HP 0868 gene introduced between the *Nde*I and *Bam*HI site of pET-21A	This study
p868	HP 0868 gene inserted into the *Eco*RI site of pBluescript KS+	This study
p868:kan	*aphA3* inserted into *Bse*RI site within HP 0868 gene	This study
pKSFII	*Kan-sacB-flaA* fragment (KSF) within pEK	Ref 8
p*hypA*	*hypA* with flanking region inserted into *Sma*I site of pBluescript KS+	Ref 28
p*hypA*:KSF	KSF fragment inserted into *Sph*I site of *hypA* in p*hypA*	This study
p*hypA*-H2A	p*hypA* with His2 replaced by Ala	This study

Table 3.1 Strains, plasmids and primers (contd)

Primers (5'→3')		
868 F	GGAATTCCGGCTAAGGGGATATTAAAAG	IDT
868 R	GGAATTCCGCGAAGAAGAATCAAG	IDT
868 F1	GGCATATGCAAGAAGAATTGAACGCTTAC	IDT
868 R1	CGGATCCTTATTTGTCTTTCAAAAGGTTTTGGA	IDT
HypA F1	GGCATATGCATGAATACTCGGTCG	IDT
HypA R1	CCGGATCCTTATTCCGCTAACATTTC	IDT
HypB F1	GGCATATGAGCGAACAACGACAAG	IDT
HypB R1	CGGATCCTTAAAACGAATGCGTGGAC	IDT
HypA:H2A F	GAAGGAGCATATACATATGGCTGAATACTCGGTCGTTTC	IDT
HypA:H2A R	GAAACGACCGAGTATTCAGCCATATGTATATGCTCCTTC	IDT
HypA:H17A F	CGCTCTTTGCGAAGAGGCTGCGAAGAAAAATCAAGCC	IDT
HypA:H17A R	GGCTTGATTTTTCTTCGCAGCCTCTTCGCAAAGAGCG	IDT
HypA:H24A F	GCGAAGAAAAATCAAGCCGCTAAGATTGAAAGAGTCGTG	IDT
HypA:H24A R	CACGACTCTTTCAATCTTAGCGGCTTGATTTTTCTTCGC	IDT
HypA:H79A F	GAATGCAAGGATTGTTCGGCCGTTTTTAAGCCTAACGCATTAG	IDT
HypA:H79A R	CTAATGCGTTAGGCTTAAAAACGGCCGAACAATCCTTGCATTC	IDT
HypA:H95A F	GATTATGGGGTGTGTGAGAAATGCGCTAGCAAGAATGTTATTATCAC	IDT
HypA:H95A R	GTGATAATAACATTCTTGCTAGCGCATTTCTCACACACCCCATAATC	IDT
HypB:K59A F	CTCCTGGCAGCGGTGCAACCACGATGCTAG	IDT
HypB:K59A R	CTAGCATCGTGGTTGCACCGCTGCCAGGAG	IDT
HypAF	CGGGCTTACAGGCTTTAG	IDT
HypAR	CGCATTAGAGCTCGCTTC	IDT
HypA:H2AF1	GCTAATTTAAAGATTAAGGTTTAGTATGGCTGAATACTCGGTCGTT TCTTC	IDT
HypA:H2AR1	GAAGAAACGACCGAGTATTCAGCCATACTAAACCTTAATCTTTAAA TTAGC	IDT

Figure 3.1A. SDS/PAGE analysis of purification steps for HypA (wild-type) protein: 5 μg (each) of membrane-free supernatant (Lane 2), pooled anion exchange fractions (Lane 3), and pooled gel filtration fractions (Lane 4) were loaded. Molecular weight markers (Lane 1) were composed of phosphorylase b (97.4-kDa), bovine serum albumin (66.2-kDa), ovalbumin (45-kDa), carbonic anhydrase (3-kDa), soybean trypsin inhibitor (21.5-kDa), and lysozyme (14.4-kDa).

Figure 3.1A

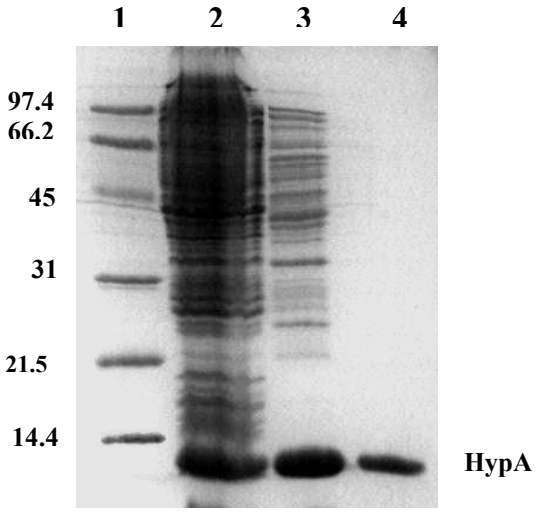


Figure 3.1B. SDS/PAGE analysis of purification steps for HypB (wild-type) protein: 5 μg (each) of membrane-free supernatant (Lane 2), pooled anion exchange fractions (Lane 3), and pooled gel filtration fractions (Lane 4) were loaded. Molecular weight markers (Lane 1) were composed of phosphorylase b (97.4-kDa), bovine serum albumin (66.2-kDa), ovalbumin (45-kDa), carbonic anhydrase (31-kDa), soybean trypsin inhibitor (21.5-kDa), and lysozyme (14.4-kDa).

Figure 3.1B

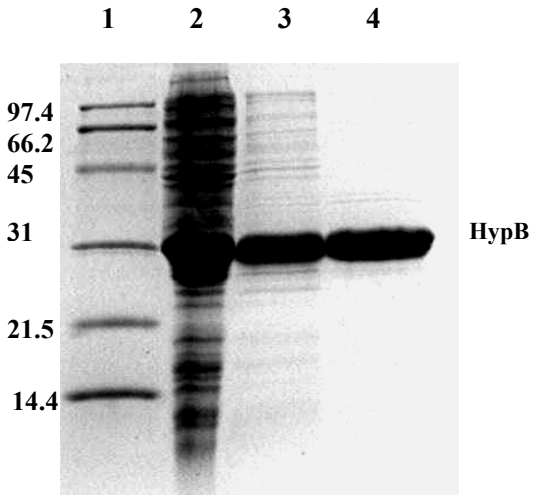


Figure 3.1C. SDS/PAGE analysis of purified HypA and HypB (wild-type and mutant) proteins: 5 μg (each) of pooled gel filtration fractions of wild-type HypA (Lane 2), and mutant HypA-H2A (Lane 3), H17A (Lane 4), H24A (Lane 5), H79A (Lane 6), H95A (Lane 7), wild-type HypB (Lane 8) and mutant HypB-K59A (Lane 9) proteins. Molecular weight markers (Lane 1) were composed of phosphorylase b (97.4-kDa), bovine serum albumin (66.2-kDa), ovalbumin (45-kDa), carbonic anhydrase (31-kDa), soybean trypsin inhibitor (21.5-kDa), and lysozyme (14.4-kDa).

Figure 3.1C

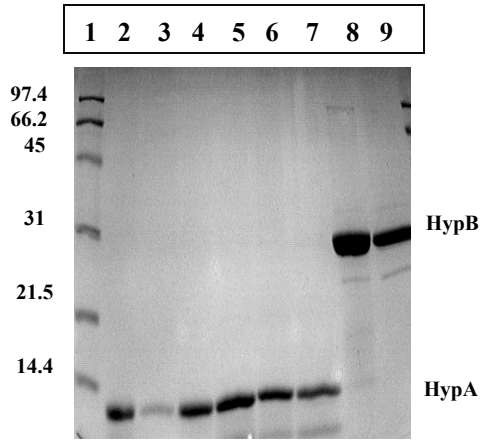


Figure 3.1D. SDS/ PAGE analysis of purified HP 0868 (wild-type) protein: 5 μg of pooled gel filtration fractions (second step of purification, Lane 2). The first purification step was Q- sepharose anion exchange chromatography (not shown). Molecular weight markers (Lane 1) were composed of phosphorylase b (97.4-kDa), bovine serum albumin (66.2-kDa), ovalbumin (45-kDa), carbonic anhydrase (31-kDa), soybean trypsin inhibitor (21.5-kDa), and lysozyme (14.4-kDa).

Figure 3.1D

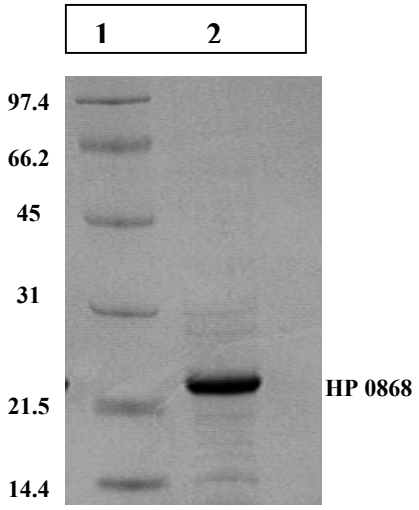


Figure 3.2A. Western blot of cross-linked products arising from a mixture of wild-type HypA and HypB proteins: Reaction mixture includes: HypB + DMS (Lane 1)**,** HypA + HypB + DMS (Lane 2)**,** HypA + HypB + DMS (Lane 4) and HypA + DMS (Lane 5). The concentration of each protein in the reaction mixture was 26 μM.
A 43-kDa heterodimeric complex was seen only when the reaction mixture had both proteins (Lane 2 and 4). HypA and HypB tested individually and as a mixture gave rise to monomers and dimers.

Panel I was probed with anti-HypB antibody, and **Panel II** was probed with anti-HypA antibody. A 1:1 molar mixture of proteins was used for these studies. Prestained protein markers (Lane 3) were composed of phosphorylase B (101-kDa), bovine serum albumin (79-kDa), ovalbumin (50 kDa), carbonic anhydrase (34.7-kDa), soybean trypsin inhibitor (28.4-kDa), and lysozyme (20.8-kDa).

Figure 3.2A

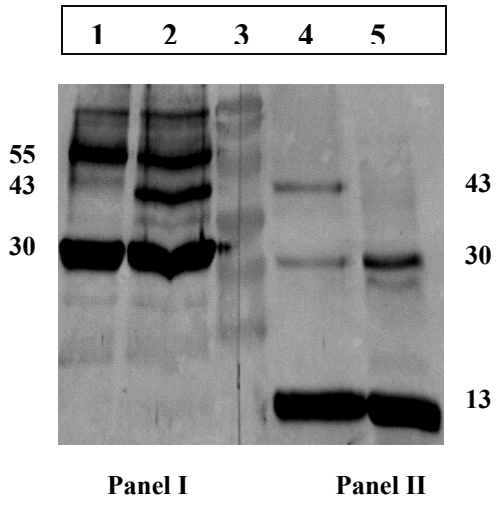


Figure 3.2B. Western blot of cross-linked products arising from a mixture of wild-type HypA and HypB proteins in presence and absence of 1 μM $NiCl_2$ or 0.5 mM GTP: Reaction mixture includes: HypA + HypB + 0.5 mM GTP + DMS (Lane 1), HypA + HypB + DMS (Lane 2), HypA + HypB + 1 μM $NiCl_2$ + DMS (Lane 3), HypA + HypB + 1 μM $NiCl_2$ + DMS (Lane 5), HypA + HypB + DMS (Lane 6), and HypA + HypB + 0.5 mM GTP + DMS (Lane 7). The concentration of each protein in the reaction mixture was 31 μM. The 43-kDa heterodimeric complex was seen in all the lanes.

Panel I was probed with anti-HypB antibody and **Panel II** was probed with anti-HypA antibody. A 1:1 molar mixture of proteins was used for these studies. Prestained protein markers (Lane 4) were composed of phosphorylase B (101-kDa), bovine serum albumin (79 -kDa), ovalbumin (50-kDa), carbonic anhydrase (34.7-kDa), soybean trypsin inhibitor (28.4-kDa), and lysozyme (20.8-kDa).

Figure 3.2B

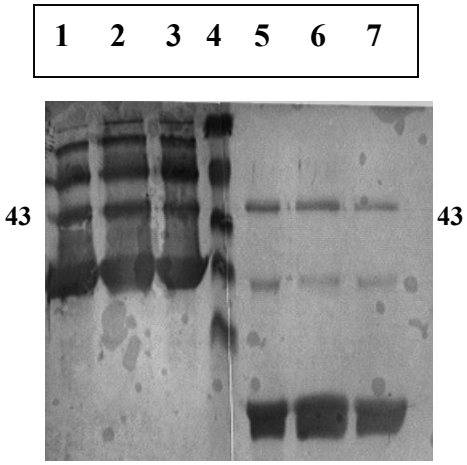


Figure 3.2C. Western blot analysis of cross-linked products arising from a mixture of wild-type (WT) and mutant HypA (H2A) and HypB (K59A) proteins: Reaction mixture includes: K59A + HypA (WT) + DMS (Lane 1), K59A + HypA (WT) + 0.5 mM GTP + DMS (Lane 2), K59A + DMS (Lane 3), K59A + H2A + DMS (Lane 4), K59A + H2A + DMS (Lane 6), H2A + DMS (Lane 7), H2A + HypB (WT) + 1 μM $NiCl_2$ + DMS (Lane 8), and H2A + HypB (WT) + DMS (Lane 9). The concentration of each protein in the reaction mixture was 29 μM. The 43-kDa heterodimeric complex was seen in all lanes except when the mutant proteins were tested individually (Lane 3 and 7).

Panel I was probed with anti-HypB antibody and **Panel II** was probed with anti-HypA antibody. A 1:1 molar mixture of proteins was used for these studies. Prestained protein markers (Lane 5) were composed of phosphorylase B (101-kDa), bovine serum albumin (79-kDa), ovalbumin (50-kDa), carbonic anhydrase (34.7-kDa), soybean trypsin inhibitor (28.4-kDa), and lysozyme (20.8-kDa).

Figure 3.2C

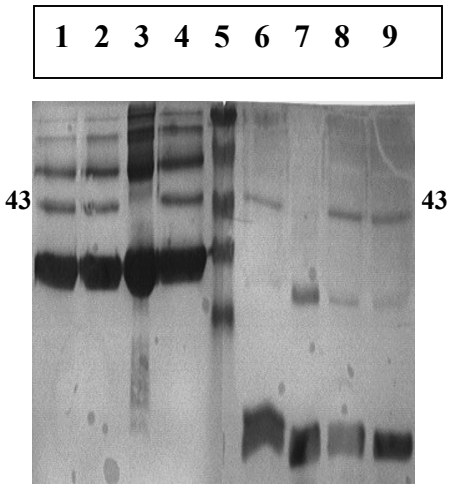


Figure 3.3A: Ni^{+2} binding ability of HypA and HypB (wild-type) proteins: Nickel-binding ability was determined by equilibrium dialysis using 6-7 μM of HypA (◆) or HypB (◇) against different concentrations of $NiCl_2$ as indicated on the X-axis, followed by atomic absorption spectrophotometry. HypA bound 2 Ni^{+2} ions per dimer, HypB lacked Ni^{+2} binding ability.

Figure 3.3A

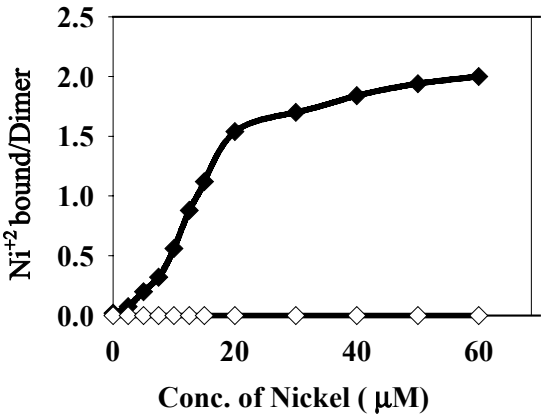


Figure 3.3B. Sigmoidal curve for Ni^{+2} binding by HypA (wild-type): A sigmoidal ligand-binding curve was obtained with the same set of data as shown in Figure 3A for HypA when a graph of % fractional saturation (**Y**) was plotted against log of Ni^{+2} ion concentration (**Log** [**L**]). This plot indicated the presence of positive cooperativity for Ni^{+2} binding by HypA. A SigmaPlot 8.0 graphical program was used to fit this curve, using the equation: $y = \min + (\max\text{-}\min / 1+10^{(\log EC50 - x)\ Hillslope})$.

Figure 3.3B

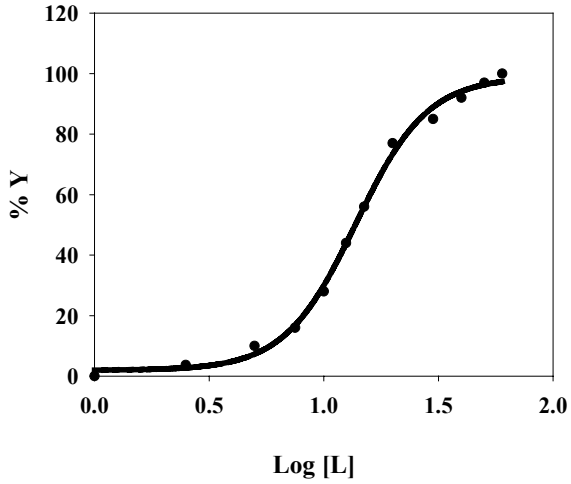


Figure 3.4: Ni^{+2} binding ability of HypA (wild-type and mutant) proteins: Nickel binding ability was determined for wild-type and mutant HypA proteins after equilibrium dialysis against 60 μM $NiCl_2$ concentration. Proteins indicated on X-axis are HypA wild-type (WT), and H2A, H17A, H24A, H79A and H95A mutant proteins. Except for the H2A mutant protein, all others proteins are capable of binding approximately 2 Ni^{+2} ions per dimer.

Figure 3.4

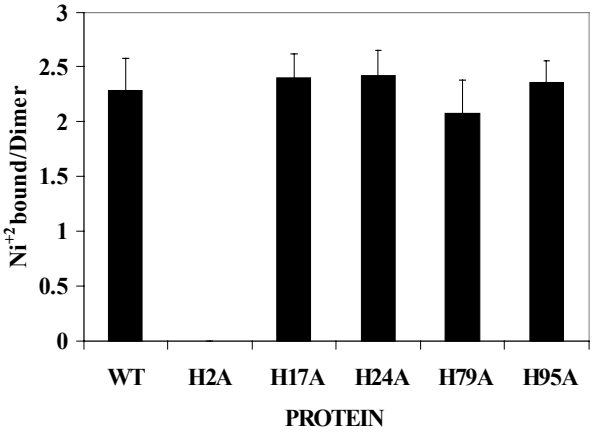

3
2.5
2
1.5
1
0.5
0
Ni+2 bound/Dimer
WT
H2A
H17A
H24A
H79A
H95A
PROTEIN

Figure 3.5. 1-D NMR spectral analysis for wild-type HypA (Panel A) and H2A mutant (Panel B) proteins: Spectra for H2A mutant protein in the fingerprint NH region (6ppm to 10 ppm) looked similar to that of the wild-type, indicating similarity in their structures.

Figure 3.5

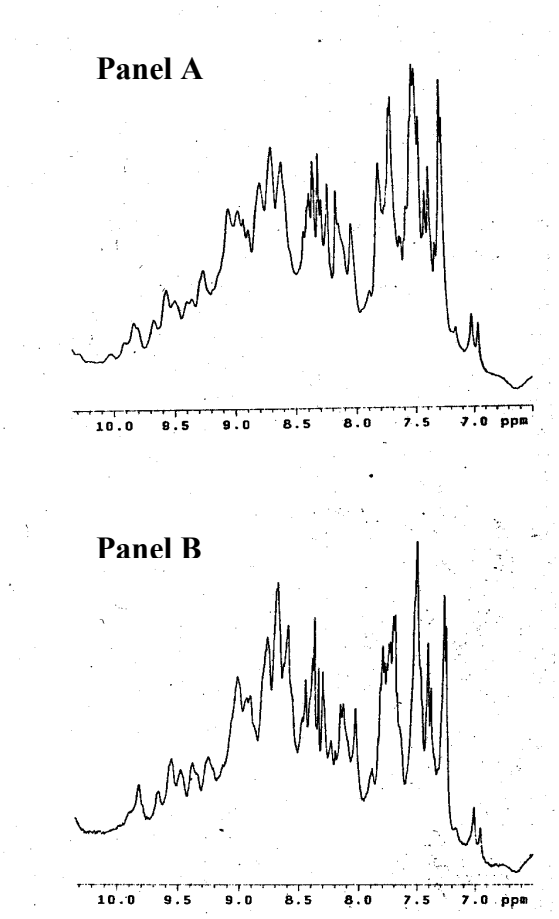
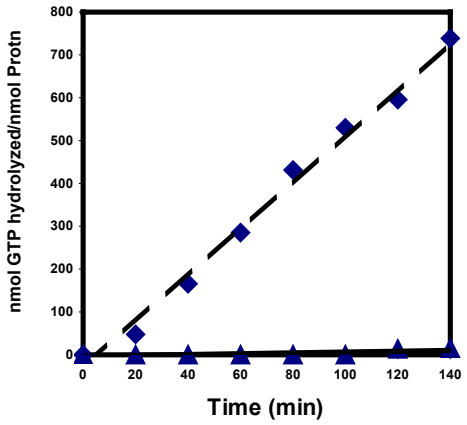


Figure 3.6. GTPase assay of HypB (wild-type and mutant) proteins: Time course assay of GTP hydrolysis by HypB wild-type (◆) and HypB K59A mutant (▲) proteins. 7 μM protein was used for this assay. The PO_4^{-3} released was measured at different time intervals as indicated on the X- axis. HypB wild-type protein showed 5 nmol GTP hydrolyzed /nmolHypB /min. The HypB K59A mutant protein had negligible GTPase activity.

Figure 3.6


nmol GTP hydrolyzed/nmol Protn
800
700
600
500
400
300
200
100
0
0
20
40
60
80
100
120
140
Time (min)

Chapter 4

CONCLUSION

Helicobacter pylori is a spiral, Gram-type negative, microaerophilic, flagellated bacterium. It was first cultured from a gastric biopsy tissue in 1982 by Drs. Marshall and Warren. Interest in this bacterium has arisen due to its ability to cause various types of gastric ulcer diseases. It is present as a chronic infection in almost two-thirds of the world's population, although most people do not manifest symptoms of any kind. In some people however, it has been shown to cause acute and chronic peptic ulcer disease (1-4, 13, 14). Prolonged untreated gastritis may progress in few cases to chronic atropic gastritis which is a predisposing factor for gastric carcinoma and B-cell mucosa associated lymphoid tissue lymphoma (7, 20, 23, 24, 26). The extraordinary ability of *H. pylori* to survive and colonize the extremely acidic conditions within the gastric mucosa has forced researchers to explore the factors which protect this pathogen under such conditions. One important factor is its ability to produce a potent multisubunit enzyme, urease. It is a nickel containing metalloenzyme which can hydrolyze urea to give two moles of ammonia, the latter contributing to an increase in the pH of the mucosa (6, 15). Another nickel-containing metalloenzyme, hydrogenase, allows the bacterium to use H_2 in a respiratory manner for energy conservation. Hydrogenase has recently been shown to aid in mouse colonization, because insertional mutants in the hydrogenase structural genes showed only 24% colonization as compared to 100% colonization by the wild-type strain (16).

Analysis of the genome sequence show the presence of structural and accessory genes for both enzymes. Accessory genes play a very crucial role for maturation of these enzymes. Mutations in two hydrogenase accessory genes, *hypA* and *hypB*, showed that they were not only required for the activation of hydrogenase, but also for urease (19). This was a surprising observation given the fact that this bacterium has the entire repertoire of urease accessory genes. The urease activity of these mutants was restored to almost wild-type levels by supplementing the media with 5 μM nickel. It was also shown by immunoblotting that these two mutants were not impaired in their ability to express urease, however their urease had four- to five-fold less nickel content when measured by atomic absorption spectrophotometry, as compared to that from the wild-type strain. Therefore, in order to further understand the role of the HypA and HypB proteins, these proteins were purified and characterized. Since accessory proteins have been shown to play vital roles such as nickel-binding or GTP hydrolysis in other bacteria (5, 8-12, 17, 18, 21, 22, 25), both HypA and HypB were tested for these activities. HypB from *H. pylori* did not bind nickel, which is quite unexpected since its role in other bacteria is to bind nickel (8, 11, 12, 17, 18, 21). *H. pylori* HypA, for which no role has been assigned yet in other bacteria, did bind two Ni^{+2} ions per dimer. This is a novel observation. HypA also showed positive cooperativity (based on a sigmoidal-ligand binding curve) in binding to nickel with a Hill coefficient around 2.0. Two dissociation constants K_1 and K_2 for nickel were calculated from the nickel binding data of HypA with values of 58 and 1.3 μM, respectively. This means that although the binding affinity for Ni^{+2} is initially weak, it increases after first nickel is bound. In other words, binding of the first nickel promotes the binding of the second nickel with greater affinity. Five widely distributed

histidine residues in HypA were each replaced by alanine and the purified mutant proteins were tested for nickel-binding ability. Only the mutant protein lacking His2, which is conserved among many HypA proteins, was shown to be important for nickel binding. Therefore, a chromosomal site-directed mutant of *H. pylori* was obtained within *hypA* in which the codon for His2 was replaced by that for alanine. This mutant lacked detectable hydrogenase activity, and also showed reduced urease activity (2% of that shown by the wild-type protein). The His2 residue of HypA thus proved critical for nickel-binding and for both Ni-enzyme activities.

HypB of *H. pylori* possessed significant GTPase activity of 5 nmol GTP hydrolyzed per nmol HypB per min. HypA, however lacked GTPase activity. A lysine (Lys59) in the well-conserved GTP-binding motif of HypB was replaced with alanine by site-directed mutagenesis and the mutant protein was assayed for GTPase activity. The GTPase activity of the mutant HypB protein was nearly abolished. This indicates that the Lys59 residue is critical for GTPase activity.

On SDS-PAGE HypA and HypB ran with molecular masses of 13- and 30-kDa, respectively, which matched with their predicted molecular weights. HypA and HypB exist as dimers in native solution with a molecular mass of 25.8- and 52.8-kDa, respectively. A 1:1 mixture of both proteins gave rise to a new complex with a molecular mass of 43.6-kDa when run through a native gel. This complex was shown to contain both proteins by immunoblotting using antisera directed against HypA or HypB. Interaction studies were done for the wild-type HypA and HypB proteins in the presence of a crosslinker dimethyl suberimidate (DMS). A 43-kDa heterodimeric complex was also seen in these studies, which was not seen when the two proteins were tested

individually. This complex showed presence of both HypA and HypB proteins by immunoblotting. Individually, both proteins formed dimers in the presence of DMS. These results indicate that HypA and HypB proteins can interact *in vitro*, and would be consistent with a role for a HypA/HypB complex aiding in nickel sequestration and GTP hydrolysis, culminating in maturation of both the nickel enzymes. Nevertheless, accessory proteins specific to each of the two Ni-enzyme maturation processes would play roles too. In conclusion, HypA and HypB proteins might be compensating for some missing functions of the urease accessory proteins, or may be functioning along with the urease accessory proteins for activation of the urease apoenzyme (Figure 4.1).

Bibliography

1. **Blaser, M. J.** 1987. Gastric *Campylobacte*r-like organisms, gastritis, and peptic ulcer disease. Gastroenterology **93:**371-83.
2. **Blaser, M. J.** 1990. *Helicobacter pylori* and the pathogenesis of gastroduodenal inflammation. J Infect Dis **161:**626-33.
3. **Blaser, M. J.** 1992. Hypotheses on the pathogenesis and natural history of *Helicobacter pylori*-induced inflammation. Gastroenterology **102:**720-7.
4. **Blaser, M. J.** 1997. Not all *Helicobacter pylori* strains are created equal: should all be eliminated? Lancet **349:**1020-2.
5. **Brayman, T. G., and R. P. Hausinger.** 1996. Purification, characterization, and functional analysis of a truncated *Klebsiella aerogenes* UreE urease accessory protein lacking the histidine-rich carboxyl terminus. J Bacteriol **178:**5410-6.
6. **Eaton, K. A., and S. Krakowka.** 1994. Effect of gastric pH on urease-dependent colonization of gnotobiotic piglets by *Helicobacter pylori.* Infect Immun **62:**3604-7.
7. **Eidt, S., M. Stolte, and R. Fischer.** 1994. *Helicobacter pylori* gastritis and primary gastric non-Hodgkin's lymphomas. J Clin Pathol **47:**436-9.
8. **Fu, C., J. W. Olson, and R. J. Maier.** 1995. HypB protein of *Bradyrhizobium japonicum* is a metal-binding GTPase capable of binding 18 divalent nickel ions per dimer. Proc Natl Acad Sci U S A **92:**2333-7.
9. **Lee, M. H., S. B. Mulrooney, M. J. Renner, Y. Markowicz, and R. P. Hausinger.** 1992. *Klebsiella aerogenes* urease gene cluster: sequence of ureD and

demonstration that four accessory genes (*ureD, ureE, ureF,* and *ureG*) are involved in nickel metallocenter biosynthesis. J Bacteriol **174:**4324-30.

10. **Lee, M. H., H. S. Pankratz, S. Wang, R. A. Scott, M. G. Finnegan, M. K. Johnson, J. A. Ippolito, D. W. Christianson, and R. P. Hausinger.** 1993. Purification and characterization of *Klebsiella aerogenes* UreE protein: a nickel-binding protein that functions in urease metallocenter assembly. Protein Sci **2:**1042-52.

11. **Maier, T., A. Jacobi, M. Sauter, and A. Bock.** 1993. The product of the *hypB* gene, which is required for nickel incorporation into hydrogenases, is a novel guanine nucleotide-binding protein. J Bacteriol **175:**630-5.

12. **Maier, T., F. Lottspeich, and A. Bock.** 1995. GTP hydrolysis by HypB is essential for nickel insertion into hydrogenases of *Escherichia coli*. Eur J Biochem **230:**133-8.

13. **Marshall, B. J., J. A. Armstrong, D. B. McGechie, and R. J. Glancy.** 1985. Attempt to fulfil Koch's postulates for pyloric *Campylobacter*. Med J Aust **142:**436-9.

14. **Marshall, B. J., and J. R. Warren.** 1984. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. Lancet **1:**1311-5.

15. **Mobley, H. L., and R. P. Hausinger.** 1989. Microbial ureases: significance, regulation, and molecular characterization. Microbiol Rev **53:**85-108.

16. **Olson, J. W., and Maier, R. J.** 2002. Molecular hydrogen as energy source for *Helicobacter pylori*. Science. In Press.

17. **Olson, J. W., C. Fu, and R. J. Maier.** 1997. The HypB protein from *Bradyrhizobium japonicum* can store nickel and is required for the nickel-dependent transcriptional regulation of hydrogenase. Mol Microbiol **24:**119-28.

18. **Olson, J. W., and R. J. Maier.** 2000. Dual roles of *Bradyrhizobium japonicum* nickelin protein in nickel storage and GTP-dependent Ni mobilization. J Bacteriol **182:**1702-5.

19. **Olson, J. W., N. S. Mehta, and R. J. Maier.** 2001. Requirement of nickel metabolism proteins HypA and HypB for full activity of both hydrogenase and urease in *Helicobacter pylori*. Mol Microbiol **39:**176-82.

20. **Parsonnet, J., S. Hansen, L. Rodriguez, A. B. Gelb, R. A. Warnke, E. Jellum, N. Orentreich, J. H. Vogelman, and G. D. Friedman.** 1994. *Helicobacter pylori* infection and gastric lymphoma. N Engl J Med **330:**1267-71.

21. **Rey, L., J. Imperial, J. M. Palacios, and T. Ruiz-Argueso.** 1994. Purification of *Rhizobium leguminosarum* HypB, a nickel-binding protein required for hydrogenase synthesis. J Bacteriol **176:**6066-73.

22. **Soriano, A., G. J. Colpas, and R. P. Hausinger.** 2000. UreE stimulation of GTP-dependent urease activation in the UreD-UreF-UreG-urease apoprotein complex. Biochemistry **39:**12435-40.

23. **Strickland, R. G., and I. R. Mackay.** 1973. A reappraisal of the nature and significance of chronic atrophic gastritis. Am J Dig Dis **18:**426-40.

24. **Walker, I. R., R. G. Strickland, B. Ungar, and I. R. Mackay.** 1971. Simple atrophic gastritis and gastric carcinoma. Gut **12:**906-11.

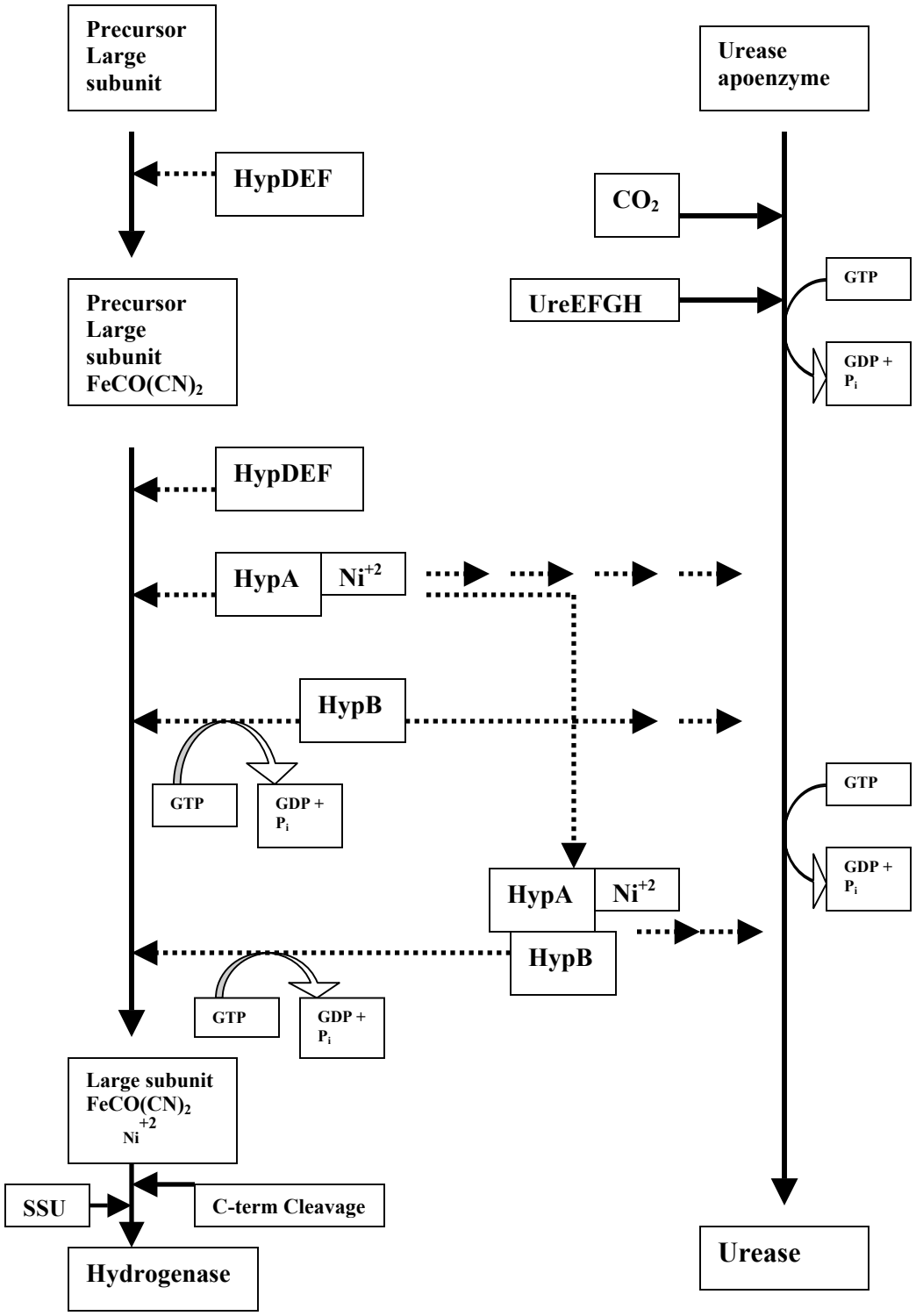
25. **Waugh, R., and D. H. Boxer.** 1986. Pleiotropic hydrogenase mutants of *Escherichia coli* K12: growth in the presence of nickel can restore hydrogenase activity. Biochimie **68:**157-66.

26. **Wotherspoon, A. C., C. Ortiz-Hidalgo, M. R. Falzon, and P. G. Isaacson.** 1991. *Helicobacter pylori*-associated gastritis and primary B-cell gastric lymphoma. Lancet **338:**1175-6.

Figure 4.1. Proposed model for hydrogenase and urease maturation in *H. pylori*. Steps involving hydrogenase maturation: The initial step involves the insertion of the CO and CN ligands to the Fe in the active site of the precursor of the large subunit of hydrogenase. The nickel insertion step involves HypA which may be functioning as the nickel donor, the reaction being catalyzed by HypB via its GTP hydrolyzing ability. Hydrogenase accessory proteins, HypD, HypE and HypF may be either involved during the liganding steps or during the mobilization of Ni^{+2} into the active site of the large subunit. HypA and HypB may be either functioning individually or as a complex during the maturation process. After nickel insertion, the large subunit may undergo C-terminal processing followed by interaction with the small subunit (SSU), thus giving rise to the mature hydrogenase.

Steps involving urease maturation: The urease accessory proteins, UreE, UreF, UreG and UreH may be either functioning individually or may be interacting with HypA, HypB or the HypA: HypB complex to bring about activation of the urease apoenzyme. Nickel insertion and GTP hydrolysis are two important maturation steps. HypA and HypB may not be interacting directly with the urease apoenzyme, but may be interacting indirectly via one or more of the urease accessory proteins. Carbon dioxide is the source of the lysine carbamate bridge between the two Ni^{+2} ions. The dotted arrows indicate the tentative steps. Thus HypA and HypB may be the proteins common to both pathways.

Figure 4.1


Precursor Large subunit
HypDEF
Precursor Large subunit FeCO(CN)2
HypDEF
HypA
Ni+2
HypB
GTP
GDP + Pi
HypA
Ni+2
HypB
GTP
GDP + Pi
Large subunit FeCO(CN)2 Ni+2
SSU
C-term Cleavage
Hydrogenase
Urease apoenzyme
CO2
UreEFGH
GTP
GDP + Pi
GTP
GDP + Pi
Urease

APPENDIX

CONSERVED LYSINE RESIDUE IN NUCLEOTIDE-BINDING DOMAIN OF HYPB REQUIRED FOR UREASE ACTIVATION IN *HELICOBACTER PYLORI*

Helicobacter pylori **produces a nickel containing enzyme, urease, which is believed to play a role in the pathogenesis of gastric ulcer disease. Its main role is protection of the bacterium in the extremely acidic conditions within the gastric mucosa. Animal model studies have shown that urease is an important factor for colonization and also for causing damage to the mucosa. Recent studies with another nickel containing enzyme, hydrogenase, has shown that this enzyme also aids in the colonization. The annotated *H. pylori* genome sequence shows the presence of an entire repertoire of structural and accessory genes needed for the synthesis and maturation of both Ni-containing enzymes. A surprising finding that two hydrogenase accessory genes, *hypA* and *hypB,* are required for urease activation prompted us to purify and study the role of these two proteins. HypB was shown to possess significantly high GTP hydrolyzing activity. A site-directed mutant protein of HypB (in which the lysine residue within the conserved GTP-binding motif was replaced with alanine) lacked GTP hydrolyzing activity. Hence a site-directed mutation was introduced into the chromosome of *H. pylori* in which the codon for the lysine59 was replaced with that for alanine. This mutant lacked hydrogenase activity and also showed markedly reduced (<1.0% of the wild-type levels) urease activity. This observation is an additional proof of the role played by HypB in the**

activation of urease, and also indicates that the Lys59 residue is vital for GTP hydrolysis as well as for urease activation.

The gastric pathogen, *H. pylori*, produces a potent nickel-containing multi-subunit enzyme urease which has been proven to be an essential colonization and virulence factor in gastric ulcer (2, 3, 4). Recently another nickel containing enzyme, hydrogenase, was also shown to be vital for mouse colonization, since a hydrogenase structural gene mutant was shown to be less efficient in colonization (8). Although an entire cluster of genes required for expression and maturation of urease are present in the *H. pylori* genome, two hydrogenase accessory genes were shown to be required for urease activation (1, 9, 10). The two proteins, HypA and HypB, encoded by these genes were over expressed and purified. HypA bound to 2 μM Ni^{+2} per dimer and lacked GTP hydrolyzing activity. HypB did not bind nickel, but had a specific activity of 5 nmol GTP hydrolyzed per nmol HypB per min. A site-directed mutant protein of HypA (H2A) lacked nickel-binding ability and a site-directed mutant protein of HypB (K59A) lacked GTP hydrolyzing ability. Thus these two residues played critical roles in each protein. In order to study their *in vivo* role, a chromosomal site-directed mutation was introduced by replacing the codon for each of these residues with alanine. As reported earlier, the mutant in the His2 codon of *hypA* was devoid of hydrogenase activity and retained only 2% of the urease activity of the parental strain. Here, a chromosomal site-directed mutant in the codon for Lys59 within *hypB* was constructed and assayed for hydrogenase and urease activities. The protocol followed for the mutagenesis was similar to that followed for making a *hypA* mutant (7). The mutation within the *hypB* gene was confirmed by sequencing the

PCR amplified product. Urease and hydrogenase assays were performed for the mutant using previously described protocols (5, 6, 11).

Results showed that the site-directed *hypB* mutant strain (Lys59) lacked hydrogenase activity and had less than 1.0% of the urease activity of the parental strain. These results indicate the significant role played by Lys59 of HypB in hydrogenase and urease activity. Since this residue has also been shown to be important for GTP hydrolyzing activity, it seems that there is a correlation between the GTPase activity of HypB and both hydrogenase and urease activation.

Bibliography

1. **Alm, R. A., L. S. Ling, D. T. Moir, B. L. King, E. D. Brown, P. C. Doig, D. R. Smith, B. Noonan, B. C. Guild, B. L. deJonge, G. Carmel, P. J. Tummino, A. Caruso, M. Uria-Nickelsen, D. M. Mills, C. Ives, R. Gibson, D. Merberg, S. D. Mills, Q. Jiang, D. E. Taylor, G. F. Vovis, and T. J. Trust.** 1999. Genomic-sequence comparison of two unrelated isolates of the human gastric pathogen *Helicobacter pylori*. Nature **397:**176-80.
2. **Eaton, K. A., C. L. Brooks, D. R. Morgan, and S. Krakowka.** 1991. Essential role of urease in pathogenesis of gastritis induced by *Helicobacter pylori* in gnotobiotic piglets. Infect Immun **59:**2470-5.
3. **Ferrero, R. L., S., S. L. Hazell, and A. Lee.** 1991. The importance of urease in acid protection for gastric -colonising bacteria *Helicobacter pylori* and *Helicobacter felis* sp. nov. Microb. Ecol. Health Dis. **4:**121-134.
4. **Hazell, S., and A. Lee.** 1986. *Campylobacter pyloridis*, urease, hydrogen ion back diffusion, and gastric ulcers. Lancet. **ii:**15-17.
5. **Maier, R. J., C. Fu, J. Gilbert, F. Moshiri, J. Olson, and A. G. Plaut.** 1996. Hydrogen uptake hydrogenase in *Helicobacter pylori*. FEMS Microbiol Lett **141:**71-6.
6. **McGee, D. J., C. A. May, R. M. Garner, J. M. Himpsl, and H. L. Mobley.** 1999. Isolation of *Helicobacter pylori* genes that modulate urease activity. J Bacteriol **181:**2477-84.

7. **Mehta, N. M., J. W. Olson, and R. J. Maier.** 2002. Characterization of *Helicobacter pylori* nickel metabolism accessory proteins needed for the maturation of both urease and hydrogenase. J Bacteriol. In Press.

8. **Olson, J. W., and Maier, R. J.** 2002. Molecular hydrogen as energy source for *Helicobacter pylori*. Science. In Press.

9. **Olson, J. W., N. S. Mehta, and R. J. Maier.** 2001. Requirement of nickel metabolism proteins HypA and HypB for full activity of both hydrogenase and urease in *Helicobacter pylori*. Mol Microbiol **39:**176-82.

10. **Tomb, J. F., O. White, A. R. Kerlavage, R. A. Clayton, G. G. Sutton, R. D. Fleischmann, K. A. Ketchum, H. P. Klenk, S. Gill, B. A. Dougherty, K. Nelson, J. Quackenbush, L. Zhou, E. F. Kirkness, S. Peterson, B. Loftus, D. Richardson, R. Dodson, H. G. Khalak, A. Glodek, K. McKenney, L. M. Fitzegerald, N. Lee, M. D. Adams, J. C. Venter, and et al.** 1997. The complete genome sequence of the gastric pathogen *Helicobacter pylori*. Nature **388:**539-47.

11. **Weatherburn, M. W.** 1968. Phenol-hypochlorite reaction for determination of ammonia. Anal. Chem. **39:**971-974.