ROLES OF NICKEL BINDING PROTEINS IN *HELICOBACTER* SPECIES

by

SUSMITHA SESHADRI

(Under the Direction of Robert J. Maier)

ABSTRACT

Proteins Hpn and Hpn-like of the gastric pathogen *H. pylori* were hypothesized to bind nickel since histidine residues make up 45% and 27% of their amino acid content, respectively. Characterization of an *hpn,* an *hpn-like* and an *hpn, hpn-like* double mutant revealed novel functions for these gene products in nickel detoxification and storage. Compared to the wild-type parent, mutant strains were more sensitive to elevated concentrations of nickel, cobalt, and cadmium, indicating roles for the two proteins in surviving metal toxicity. Under low nickel conditions, the mutants exhibited higher urease activities and had increased amount of Ni-associated with urease; but similar urease apo-protein levels to wild-type. The parent achieved mutant level urease activities under nickel supplementation and lower pH conditions while growth with a nickel chelator decreased mutant but not wild-type urease activities. These results strongly imply a role for these proteins as nickel reservoirs/storage proteins.

H. hepaticus colonizes a non-acidic niche, hence nickel metabolism may be different than in *H. pylori*. A *nikR* mutant exhibited higher urease and hydrogenase activities under all supplemental nickel conditions, but there was no change in urease expression, and NikR did not bind pUreA or pHydA. Higher total nickel levels (detected by ICP-MS) in the *nikR* mutant implied possible higher nickel transporter (NikA) levels, which was later verified by qRT-PCR

and binding of NikR to pNikA. Periplasmic nitrate reductase (NapA) was upregulated in the NikR strain. Wild-type *H. hepaticus* had increased H_2 oxidation levels in the presence of nitrate compared to O_2 provided as a terminal acceptor, suggesting the importance of anaerobic respiration for this bacterium. The NikR strain showed higher H_2 oxidation levels than the parent with either O_2/NO_3^- as electron acceptor. Also, the rate of nitrate disappearance was higher in a *nikR* mutant. Collectively, these results suggest NikR modulates the nickel-enzymes via regulating the nickel transporter and plays important roles other than in nickel metabolism.

My studies aimed at characterizing nickel binding proteins by studying the mutant strains in two different organisms *Helicobacter* species. The data indicate that these proteins play vital functions to specifically suit the needs of each pathogen.

INDEX WORDS: Nickel, Nickel Metabolism, Nickel homeostasis, Hpn, Hpn-like, Urease, Hydrogenase, NikA, NikR, *Helicobacter pylori*, *Helicobacter hepaticus*

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DEDICATION

I dedicate this work to my family- my parents, my husband, my brother, my grandmother and my daughter. I dedicate this dissertation to my dad, for instilling a love for science and books in me, for believing in me and for listening to me and for being a great friend. I dedicate this to my mother, for helping me when I needed it the most, and for encouraging me to make important life decisions- at the right time, that have brought me immense joy. I dedicate this work to my husband Ramesh, without whose support and encouragement, and numerous compromises, I could not have realized this lifelong ambition. I dedicate this work to my brother Krishna and my grandmother for cheering me up and showering me with their affection. Lastly, I dedicate this work to my daughter Shriya; whose arrival brought me indescribable happiness, the vigor and motivation to complete what I had started, when it was needed the most. I dedicate my dissertation to all of you for always being there for me and for unconditionally loving me.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

NICKEL METABOLISM AND HOMEOSTASIS

Nickel (Ni) is a silvery white, transition element and is found combined with oxygen and sulfur as oxides and sulfides naturally in the earth's crust in mineral ores like pentlandite, millerite, nickeline etc. It is one of the most abundant transition metals (0.02%) and is widely distributed in the environment. It is found in soils, in volcanic dust, and in ground and surface waters. Combustion of fossil fuels is one of the biggest anthropologic sources of nickel in the air (196). Ni is used in many metallurgical processes such as in the production of alloys, electroplating, welding and in Ni-cadmium batteries etc (63, 196). In the US, Ni is found in an average of 2-4.3 ppb concentration in the drinking water (51). Caffeine and nuts are also a good source of dietary Ni. In humans, most of the Ni is not absorbed and is excreted in the feces. The Ni that is absorbed is finally eliminated via the urine (51).

Like most transition metals, Ni is generally toxic to living organisms in elevated concentrations while being essential in smaller quantities. There is some evidence suggesting that Ni may be an essential trace element for mammals (249). Some studies have suggested that Ni is required for the absorption of iron in the mammalian intestines where iron was present in the unavailable ferric form, whereas this effect was not observed in the presence of available iron (51). Depending on the quantity, route of exposure (dermal, oral or inhalation) Ni toxicity in man causes systemic, immunological, developmental, or carcinogenic effects. Ni- induced toxicity in humans and animals is thought to be due to the depletion of glutathione and bonding to

sulphydryl groups of proteins (294). Exposure to Ni can also cause the formation of free radicals that can cause DNA damage and damage to proteins (51).

In archaebacteria, eubacteria, and fungi, Ni is an essential cofactor that is required for the catalysis of many enzymes (184, 215, 250, 306, 307). These Ni containing enzymes are a comparatively new and small class of metalloenzymes comprising only seven well characterized enzymes to date (307). These enzymes catalyze varied reactions including many redox and non-redox reactions and include urease, hydrogenase, CO-dehydrogenase, methyl-coenzyme M reductase, Ni-superoxide dismutase, glyoxylase I and cis-trans isomerase. Two other Ni-enzymes aci-reductone dioxygenase and methylenediurease have also been described. Since Ni is toxic and can cause extensive cellular damage- entry/import, distribution within the cell, and export/efflux of Ni are tightly regulated processes, and complex protein systems have evolved to carry out these functions and maintain Ni homeostasis in bacteria that produce the Ni containing enzymes.

MECHANISM OF NICKEL TOXICITY

Like most transition elements, nickel, though essential in minute concentrations for certain important metabolic processes, is toxic in larger quantities. Ni could be involved either directly or indirectly in the generation of reactive oxygen species (ROS) in cells, which in turn causes cellular damage. It was shown that in the presence of hydrogen peroxide, Ni^{2+} causes DNA strand breakage in purified *Salmonella typhimurium* DNA (150). In yeast systems, the presence of Ni inhibited histone acetylation leading to gene silencing (47). Many damaging effects of Ni are due to the interference with the metabolism of essential metals such as Fe^{2+}, Mn^{2+}, Ca^{2+}, Zn^{2+}, or Mg^{2+} (145). Many studies conducted in mammalian cells have shown that low, non-cytotoxic concentrations of Ni^{2+} (and other transition elements such as Co^{2+}, Cd^{2+},

As^{3+}) are able to inhibit DNA repair systems such as the nucleotide and base excision repair systems by interfering with certain zinc finger proteins that are involved in the repair mechanism (114). Ni^{2+} specifically interferes with the first step in the nucleotide excision repair- recognition of damaged nucleotides (113). Other studies have shown that though Ni directly does not cause oxidative damage, it inhibits removal of oxidative (damaged) DNA base modifications (50).

Despite the toxicity of nickel, Ni-enzymes catalyze important reactions required for colonization of specialized environmental niches. Considering the scope of damage capable by Ni, it is not very surprising to learn that bacteria have devised ingenious methods to limit the harmful effects of Ni where Ni is essential. First, bacteria are able to control the entry and import of Ni into the cells by regulating the proteins that are involved in Ni-transport. Second, once inside the cell, Ni is found in association with varied Ni-binding proteins. The binding of Ni to such proteins and other macromolecules within the cell limits accessibility and thereby prevents damage that could be caused by free Ni. Third, bacteria sometimes increase the expression of resistance elements comprising Ni-efflux pumps to eliminate excess Ni. Fourth, some bacteria are capable of chemotactically moving away from toxic concentrations of Ni. The next section provides a brief description of Ni-binding proteins including Ni import/efflux systems, Ni-enzymes, systems involved in the assembly and maturation of Ni-enzymes, Ni dependent gene regulators and Ni export and efflux systems in general. Ni metabolism in *Helicobacter* species is discussed in detail in a later section.

NICKEL TRANSPORT IN BACTERIA

Concentration of nickel in fresh and sea waters are in the 0.3-2 ppb range (nM concentrations). Nickel commonly occurs in nature as Ni (II) and therefore, Ni^{2+} is usually the substrate for transport. When present in excess, Ni can enter cells promiscuously via Mg^{2+}

transport systems or via Co^{2+} transport systems such as MgtAB, MgtE, CorA (125, 126, 256, 270, 307). Since Mg^{2+} occurs in a much higher concentration than Ni^{2+} in nature and excess concentrations of Ni^{2+} are usually toxic, the physiological occurrence of Ni^{2+} transport via a non-specific transporter is probably rare.

Since Ni^{2+} is found in low concentrations, Ni import into the cells occurs via proteins that have a high specificity and affinity for Ni. There are two major types of Ni transporters in bacteria. One is an ATP-binding cassette (ABC) type transporter while the other is a Ni-specific integral membrane protein, a permease (HoxN/NixA).

ATP-binding cassette- (ABC) type Ni transporters- *NikABCDE*

The NikABCDE system is best characterized in *Escherichia coli* (220, 314). The transport of Ni occurs with concomitant ATP hydrolysis. In this five component system, NikA is the soluble, periplasmic protein that binds Ni^{2+}, NikB and NikC form a hydrophobic transmembrane pore (have six membrane spanning regions) for Ni transport into the cytoplasm, and NikD and NikE are involved in ATP hydrolysis and couple the energy generated from ATP hydrolysis to Ni^{2+} transport. These five components NikA-E show significant similarity to dipeptide and oligopeptide transporters of gram-postive and gram-negative bacteria, rather than to other divalent cation transporters (220). The *nik* locus was identified in *E. coli* transposon mutants that lacked hydrogenase (a Ni-enzyme) activity. The absence of hydrogenase activity could be restored by addition of Ni to the growth medium and hence, this gene was initially annotated as the *hydC* region (314). Later, it was demonstrated that insertion mutations in the *nik* operon demolished hydrogenase activities under Ni-deficient conditions and affected Ni transport and accumulation based on $^{63}Ni^{2+}$ studies (220).

NikA, the periplasmic component of the ABC type transport system, has been characterized well and crystal structures have been solved for the protein (42, 43, 56, 318). Initially NikA was thought to bind to Ni^{2+} with an $K_d < 0.1\mu M$. Studies conducted later have indicated that the affinity of NikA for Ni^{2+} is around 10 μM (261, 267). The values reported were also confirmed by other methods such as isothermal titration calorimetry (117, 261). In addition to Ni^{2+}, it has been reported that NikA can also bind Co, Cu, Zn and Fe, though with much lesser affinity than for Ni^{2+} (42, 56, 117). It has been shown that NikA does not directly bind Ni^{2+} but forms a hydrogen bond with a water molecule that coordinates to an arginine residue (Arg-137). Ni^{2+} in the Ni-NikA complex remains accessible to the solvents (117). Recent studies have also shown that NikA is able to bind heme in *E. coli* (267). This study has shown that, NikA is able to bind protoporphyrin IX and hemin in a Ni-independent manner. Also, a *nikA* mutant was not able to produce a newly characterized pigment P-547 produced during heme-biosynthesis. Hence, it is thought that under reducing conditions, NikA could be a heme-chaperone protein as well as a periplasmic Ni binding protein (267).

Nickel specific permeases

A nickel specific permease was first discovered in *Cupriavidus necator* H 16 (formerly *Alcaligenes eutrophus),* again, while studying hydrogen metabolism. It was discovered when strains carrying known deletions in hydrogenase genes on an indigenous plasmid were unable to grow autotrophicaly on hydrogen (70). This mutant was also deficient in urease and hydrogenase activities. These activities were restored when excess Ni was supplied to the growth medium or by complementing the mutant with a fragment of DNA containing a single gene- *hoxN,* introduced on another plasmid (70).

Today, we know that HoxN belongs to a family of Ni-cobalt (NiCo) secondary metal transporters (72). They are present in many gram-negative and gram-positive bacteria, and in archaea and many fungi. They are characterized by the presence of eight transmembrane domains and carry out high affinity Ni^{2+} and/or Co^{2+} transport into the cell. The metal specificity and affinity is dictated by a small conserved motif in the 2nd transmembrane domain of the protein- RHA(V/F)DADHI. The histidine residues and aspartate (H-X_4-D-H) of the conserved sequence contributed to the high affinity and specificity of HoxN for Ni (73). By conducting $^{63}Ni^{2+}$ studies, it was determined that Ni specific permeases concentrate Ni^{2+} only about ten-fold (57, 310). The source of energy for the transport of Ni^{2+} via the permeases still remains unknown. The K_t of HoxN for Ni^{2+} was found to be approximately 20 nM (310). This suggests that HoxN and similar permeases have a comparatively higher affinity for Ni^{2+} than the ABC-type Ni transporters.

HoxN (described earlier) and NixA of *Helicobacter pylori* are Ni- specific permeases (described in a later section). NhlF is a Ni- and cobalt- specific permease with a comparatively higher affinity for cobalt, and has been well characterized in *Rhodococcus rhodochrous* J1 (57, 71). The localization of these permease genes on the genome close to a Ni enzyme gene cluster or close to a cobalt enzyme gene cluster or close to enzymes involved in coenzyme B_{12} synthesis directed the substrate specificity to a certain extent (57, 71).

Other Nickel Transporters

HupE/UreJ- Both HupE and UreJ are present in many bacteria and the corresponding genes are present as a part of hydrogenase and urease gene clusters respectively (14, 194). HupE was initially identified in *Rhizobium leguminosarum* as a predicted transmembrane protein that was essential for hydrogenase activity (123). UreJ was discovered in a urease gene cluster from

Bordetella bronchiseptica (194). UreJ showed sequence similarity to HupE from *Rhizobium leguminosarum* and was suggested to be involved in Ni^{2+} transport. HupE and UreJ are related to the nickel permeases. They are predicted to have six transmembrane domains, with the 1st transmembrane domain containing the conserved histidine residues present in the canonical NiCo transporters that is required for Ni^{2+} binding. Ni accumulation studies conducted by expressing HupE and UreJ proteins from various bacteria in *E. coli* have confirmed the roles of HupE/UreJ in Ni transport. Mutations in the *hupE/ureJ* genes in *R. palustris* or *Cupriavidus necator* H16 showed that the Ni-enzyme activities in these enzymes were impaired, again confirming their roles in Ni metabolism (72).

UreH- UreH was discovered in *Bacillus* sp. when the urease gene cluster was characterized. UreH was shown to have sequence similarity to other NiCo transporters like HoxN (174). UreH contained six transmembrane domains and both the N- and C- terminal portions were found oriented to the cell exterior, though a signal sequence that would be required for such an orientation has not been discovered. To confirm the role of UreH in Ni metabolism, UreH from *R. palustris* CGA009 was cloned and expressed in *E. coli,* and subsequent Ni accumulation studies have confirmed the roles for these proteins in Ni transport (72). While checking the specificity of UreH for other metals, it was determined that only cobalt was a (minor) inhibitor of Ni transport (72).

Nickel export and efflux systems

An important feature in conferring bacterial resistance to toxic concentrations of metals is the presence of efflux and export systems. When the extracellular concentration of metals becomes high, the metals are able to enter the cells via non-specific transporters. For example, when the extracellular concentration of Ni is high, Ni is able to enter the cell via

magnesium/cobalt transporters. One method to deal with high intracellular concentrations of metals is by actively exporting the metals out of the cell, using efflux pumps. Presence of such efflux pumps can confer resistance in bacteria against high concentrations of metals. These resistance elements were initially found on plasmids, but mining the genome sequences of bacteria has shown that they also are widely distributed on bacterial chromosomes. There are four primary types of export systems for metals- 1) The proton driven, resistance-nodulation-cell division (RND) family of proteins that export many cations 2) The cation diffusion facilitators (CDF) family of proteins 3) P type ATPases 4) CHR family of proteins involved in chromium resistance (reviewed in (221)). Ni exporters were found among three of the four different categories of metal efflux systems. Ni efflux pumps from the P-type ATPase family of metal transporters have not been described thus far.

A protein CzcA from *Ralstonia metallidurans* was the first protein to be discovered that belonged to the RND family of proteins, followed by the discovery of a CnrA protein also from the same bacterium (259). These proteins form complexes with a membrane fusion protein (MFP), an outer membrane factor (OMF) that is often found in the same operon or on nearby genomic regions. Together, the protein complex performs the task of exporting the excess metal ion (139, 238). CnrCBA of *R. metallidurans* comprise the OMF, MFP and the RND components of the Ni-cobalt efflux pump and confer around a 16- fold increase in nickel resistance to the bacterium. Since the discovery of the CnrCBA system, many similar systems have been reported in other bacteria (223).

The proteins that belong to the cation diffusion facilitator family of metal exporters occur in all three domains of life (239, 258) and Zn^{2+} is the primary substrate for transport by this family, although Cd, Co, Ni and Fe transport by members of this group has also been described

in the literature (223). The only instance of Ni transport by a member of this family was described in a plant, *Thlaspi goesingense,* a closely related to a heavy metal hyperaccumulator plant (242). A gene identified from this plant when transcribed forms an unspliced and spliced mRNA that had differences in histidine residues that confer substrate specificity. The unspliced mRNA conferrs specificity to Cd^{2+}, Co^{2+} and Zn^{2+} while the spliced mRNA conferrs Ni^{2+} specificity (242).

There are two other nickel transport systems that are similar to the proteins that are involved in conferring resistance to the oxyion chromium of the CHR family of metal transporters- NreB, CnrT. *Synechocystis* sp., *Nostoc* sp., *S. meliloti* and *Magnetococus* all contain an NreB-like protein. It was characterized in R. metallidurans strain 31A. This protein was induced in the presence of Ni and conferred Ni resistance both in *R. metallidurans* as well as *E. coli.* Cells expressing the protein also showed decreased accumulation of Ni (105). These proteins contained 12 trans-membrane domain and a few had C-terminal regions rich in histidine residues, similar to a histidine-tag. The *cnrT* gene is located directly downstream of the *cnr* cobalt–nickel resistance determinant in *R. metallidurans* described in an earlier section and encodes a small degree of Ni resistance by itself.

NICKEL ENZYMES, THEIR ACCESSORY PROTEINS AND MATURATION FACTORS

Once Ni is imported into the cell, free Ni is mostly sequestered, presumably to limit toxic effects. Ni is bound by accessory proteins involved in Ni-enzyme maturation, in a complex process involving other proteins and non-protein components, or by Ni enzymes themselves. To date, nine unique Ni-containing enzymes have been described, namely- urease, NiFe hydrogenase, carbon monoxide dehydrogenase, acetyl CoA decarbonylase/synthase, methyl

coenzyme M reductase, certain superoxide dismutases, some glyoxylases, aci-reductone dioxygenase, and methylenediurease (reviewed in (116, 177, 215, 250, 307)) . Seven of these enzymes have been structurally characterized, confirming the presence of Ni at the metallocenter sites.

Nickel enzymes involved in the carbon cycle- Carbon monoxide dehydrogenases (CODHs), Acetyl CoA decarbonylase/synthase (ACS/ACDS), Methyl Coenzyme M Reductase (MCRs)

The carbon cycle involves the oxidation of energy-rich organic molecules to CO_2 and the reduction of CO_2 back to organic carbon. Oxidation of organic carbon by cells generates the energy required to sustain life through common oxidative processes like glycolysis that directly synthesize ATP through substrate-level phosphorylation or generate reducing equivalents that indirectly couple to ATP synthesis by oxidative phosphorylation (250). CO_2 reduction uses energy from light for phototrophs or inorganic sources for chemolithotrophs to fix carbon into the biomolecules of life (250). Many pathways exist that fix CO_2 into organic carbon molecules- the Calvin cycle, the reverse TCA cycle, the 3-hydroxypropionate pathway and finally the Wood- Ljungdahl pathway or the reductive acetyl-coA pathway. CODH and ACDS are key players in the reductive acetyl-coA pathway while MCR is responsible for most of the methane generated by methanogenic organisms and for anaerobic methane oxidation (250).

CODH is an oxidoreductase enzyme that catalyzes the reversible reaction $CO + H_2O \rightleftharpoons CO_2 + 2H^+ + 2e^-$ and allows organisms to use carbon monoxide as an energy source. Ni-CODH from *Rhodospirillum rubrum* has been very well studied and crystal structures from this organism are also available. Ni–CODHs are broken down into two classes: the monofunctional Ni CODH containing 10 Fe and 1 Ni per monomer; and the bifunctional CODH/ACS, containing 14 Fe and 3 Ni per monomeric unit. Besides Ni and Fe, CODHs contain another cofactor- Zn.

The monofunctional homodimer Ni-CODH per dimer contains two unusal [3Fe-4S] cluster bridged to a NiFe cluster called the C cluster. Besides the C cluster, each dimer contains two B and one D cluster made up of [4Fe-4S] subunits. The B and D clusters transfer electrons between the C clusters and other redox proteins such as ferredoxins.

The CODH is encoded in an *cooFSCTJ* operon (147) and it has been shown that the products of *cooCTJ* are required for the maturation of CODH, specifically for "Ni insertion". Studies of mutants lacking *cooC, cooT* or *cooJ* indicate a role for all of them in Ni insertion into CODH (148, 306). The CooC is thought to hydrolyze nucleotides that yield energy for Ni insertion into CODH. CooJ contains a C-terminal histidine rich region capable of binding up to 4 Ni atoms. Though insertional inactivation of these genes does not affect Ni transport, the mutant strains do accumulate lesser Ni based on $^{63}Ni^{+}$ studies (306) and require 50 fold (for *cooC* and c*ooT* mutants) and 10 fold (for *cooJ)* higher external Ni for CO- based growth. There results suggest important functions for these proteins in CODH maturation (148).

In nature the acetyl coA synthase (ACS)/decarbonylase (ACDS) almost always occurs in a complex with CODH. Such ACS/CODHs are found in selected methanogenic, sulfate reducing, and acetogenic microorganisms. ACS catalyzes the biosynthesis of acetyl coA while the ACDS catalyzes the decarbonylation of acetyl coA, a reversible reaction catalyzed by two forms of the same enzyme (250). The ACS/ACDS contains only one metallocenter- the A cluster. Hence, a CODH/ACS complex would contain one A, two B, two C and a D cluster. The A cluster comprises a unique NiFeS metallocenter where a binuclear NiNi center is bound to a [4Fe-4S] cluster via a cysteine residue. It is thought that a molecular tunnel exists through which the CO generated by CO_2 reduction is channeled to the other active site where the synthesis of acetyl-CoA occurs from CO, a methyl group attached to another protein, and CoA (191).

Studies are yet to be conducted to identify accessory proteins involved in ACS/ACDS maturation. Although, it is reasonable to expect the involvement of at least two accessory proteins required for the formation of the holo-enzyme based on the knowledge of CODH accessory proteins. The operon that encodes the acetyl-CoA synthase in *Clostridium thermoaceticum* is the *acsABCDE* operon. There is some evidence to indicate that in the genomic region between the *acsC* and *acsD* genes an open reading frame of 0.7 kb exists that could encode a putative *acsF* gene whose product is thought to be involved in ACS maturation (170). It is able to catalyze the hydrolysis of nucleotides and is similar to CooC, an accessory protein required for CODH maturation. Nevertheless, a role for *acsF* in maturation of ACS has not been confirmed as AcsF is not required for the production of active ACS in *E.coli* (170)

Methyl-coenzyme M reductase (MCR) is thought to catalyze the formation of all the methane that is biologically produced on earth, mostly by methanogenic archaea that oxidize H_2 using a Ni-containing hydrogenase and use the electrons produced to reduce carbon compounds that results in the formation of methane. The same enzyme or an isozyme of MCR is thought to catalyze anaerobic methane oxidation (192). All the pathways that reduce carbon compounds converge at the intermediate methyl-S-CoM and MCR catalyzes the final step in the reduction of the methyl group in methyl-S-CoM to methane (60, 61, 308).

MCR contains a Ni-cofactor called F_{430} that is unique to and present in all methanogenic archaea. This cofactor was identified independently by two groups in two *Methanobacterium* species by observing that Ni was essential for growth and that $^{63}Ni^{+}$ copurified with MCR (60, 308). The Ni cofactor is present in a tetrapyrrolic hydrocorphin structure, which is quite similar to a heme structure of cobalamine of vitamin B_{12}. There is no evidence in the literature that

suggests the presence of accessory proteins that are involved in mobilizing Ni into the active centers of MCR.

Glyoxylase I

Glyoxylase I is a member of the metalloglutathione (GSH) transferase superfamily, and plays a critical detoxification role in cells by catalyzing the conversion of methylglyoxal that is cytotoxic in millimolar concentration to a non-toxic product D-lactate (49, 284). In humans and higher organisms, glyoxylase I is a Zn- metalloenzyme while glyoxylase I from *E. coli* is a Ni^{2+} metalloenzyme (284). The Ni^{2+} binding site comprises His-5A, Glu-56A, His-74B, Glu-122B and two water molecules (284, 307). Extensive X-ray crystallographic studies have been carried out to study the details of structure and to understand the mechanism of the enzyme activity (52). Again, there is no evidence to indicate the presence of accessory proteins required for the maturation of glyoxylase I.

Superoxide dismutase

Superoxide dismutases (SODs) are important antioxidant enzymes that guard against superoxide toxicity. Various SOD enzymes have been characterized that employ either a copper, manganese, iron or Ni cofactor to carry out the conversion of two superoxide radicals and protons to form water and oxygen. Ni-SODs do not have any apparent homology to the other SODs. Ni-SODs were first purified from cytosolic fractions of *Streptomyces* sp. IMSNU-1 and *Streptomyces coelicolor* ATCC 10147 respectively (320). The Ni-SOD is a tetrameric protein that contains a His-Cys-X-X-Pro-Cys-Gly-X-Tyr N-terminal hook that provides almost all interactions critical for metal binding and catalysis (15). The Ni in the Ni-SOD from *S. coelicolor* is in the Ni^{3+} state of oxidation, which is quite unique (15). It has also been shown that in the presence of Ni, Ni-SOD is induced while the Fe-SOD is repressed (151). An

interesting feature of Ni-SODs is that in the absence of Ni, the N-terminal region is unfolded and proper folding occurs only in the presence of Ni (15). Little information is available to assess whether accessory proteins are needed for the insertion of Ni into the metallocenter. However, in one study, a metal binding protein CbiXhp, a homolog to the CbiX protein of *Bacillus megaterium,* was identified in *S. seoulensis;* when overexpressed, CbiXhp increased the SOD activity in the presence or absence of added Ni (152).

Hydrogenase

Hydrogenases are a functionally diverse group of enzymes that catalyze the reversible activation of molecular hydrogen into two protons and two electrons and hence play a central role in the energy metabolism in bacteria. Hydrogenases predominantly exist in bacteria (3) and archaea (7), though their presence has been described in some subcellular organelles of eukaryotes- from hydrogenosomes of protozoa (214, 236) and chloroplasts of algae (111, 112, 330). Depending on whether hydrogen is utilized or produced, these enzymes are described as H_2 uptake or H_2 evolving hydrogenases. Depending on the metal content, hydrogenases are classified as [NiFe]-hydrogenases (including the [NiFeSe] type), [Fe]-hydrogenases or metal-free/FeS-free hydrogenases (reviewed in (298, 313)). These enzymes assume varied functions depending on their cellular localization and on the metabolic mode of bacteria (i.e. necessity basis). In fermentative clostridia, conversion of protons to H_2 is an approach to remove excess reducing equivalents produced during fermentation (3). In certain aerobic and anaerobic bacteria, H_2 is used as an electron source for energy generation (including certain nitrogen fixers, knallgas bacteria, certain photosynthetic bacteria) (32, 39, 298). In many microaerophilic/facultative anaerobic intestinal pathogens, H_2 is an important energy substrate when H_2 oxidation is coupled with a respiratory pathway usually involving quinine- or heme- containing proteins (176).

Hydrogenase was shown to be important for virulence and long term host colonization in several human pathogens (176). In nitrogen fixing bacteria (*Rhizobium leguminosarum, Bradyrhizobium japonicum, Azotobacter vinelandii, Azotobacter chroococcum)*, uptake type hydrogenases recycle H_2 produced in the nitrogenase reaction (39, 298). H_2 may also be directly involved in proton translocation across the cytoplasmic membrane via H_2 cycling (229) or recycling (63). The reaction catalyzed by hydrogenase causes the generation or utilization of protons either in the cytoplasm/periplasm, thereby leading to the alkalization/acidity of either the cytoplasm/periplasm and also directly affecting the proton gradient across the cytoplasmic membrane (13, 292). Based on the function hydrogenases carry out, they are membrane bound, localized in a trans-membrane fashion or are present in the cytosol (298, 313).

The NiFe-hydrogenases are a large group of hydrogenases that are comprised of four different phylogenetic groups. They are (1) the H_2 uptake [NiFe] hydrogenases (group 1); (2) the cyanobacterial uptake hydrogenases and the cytoplasmic H_2 sensors (group 2); (3) the bidirectional cytoplasmic hydrogenases able to bind soluble cofactors (group 3); and (4) the membrane-associated, energy-converting, H_2 evolving hydrogenases (group 4) (297, 298). A single bacterium can have up to 10 different isozymes of hydrogenases that are regulated differently, that utilize varied substrates as electron acceptors, are expressed in different phases of growth etc. This section deals with the NiFe H_2 uptake hydrogenases, some of their structural features and their maturation.

Crystal structures of NiFe hydrogenases are available from many *Desulfovibrios* sp. (*D. gigas, D. vulgaris Miyazaki, D. fructosovorans, D. baculatum)*. The diffraction data reveal the presence of a binuclear active site containing Ni and Fe (81, 89, 90, 287, 301). The NiFe site is buried deep in the large subunit of the hydrogenase (39). Some common features of these

structures are that the Ni^{2+} is coordinated by four conserved cysteine residues (or in *D. baculatum* by three cysteine residues and a selenocysteine residue) (39). Two of these cysteine residues also bind Fe, and Fe is found liganded to other non-protein ligands such as CO, CN (or SO, CO, and CN) (39). The [Fe-S] clusters are present elsewhere in the hydrogenase structure and form channels/conduits for the transfer of electrons to appropriate electron carriers (39).

In *E. coli,* the structural genes that encode the large (*hyaB,hybC,hycE)* and small subunits (*hyaA, hybO, hycG)* of hydrogenase enzymes are usually present in the beginning of a multi-cistronic operon that also encode other '*cis*-acting' genes that are essential for hydrogenase activity (the *hya, hyb, hyc* operons). The '*cis*-acting' elements in the same operon as the structural genes only affect the maturation of that specific isozyme. There are other '*trans*-acting' elements present at different locations on the genome that seem to involved in the maturation of more than one isozyme; that is the hydrogenase pleotropic gene region or the *hypA-F* operon (39). Analysis of the NiFe uptake hydrogenases from different organisms suggests that depending on the organisms in which they are found, they have different nomenclatures (*hya, hox, hup)*, yet a functional homology for each exists in all bacteria (39).

This paragraph describes the formation/maturation of an *E.coli* hydrogenase HycGE, which has been very well characterized and can serve as the basis for our understanding of the maturation and assembly of other hydrogenases. Initially, HypC forms a complex with HypD and binds Fe by an unknown mechanism. Then, the Fe in the HypC-Fe is liganded to CO and CN in a reaction that requires the presence of HypEF and ATP and carbamoyl phosphate. The entire HypC complex binds to the unprocessed large subunit (pre-HycE) and HypD is removed from the complex. The next step, insertion of Ni into the complex requires the presence of HypA and HypB in a step that requires GTP hydrolysis. Once the Ni is inserted into the active site of the

pre-HycE-HypC complex, HypC disassociates and allows the carboxy terminal of pre-HycE to be processed by a specific protease (Hycl). Then, the processed large subunit associates with the HycG (that is processed by many accessory genes), the small subunit of hydrogenase, that contains FeS clusters and forms the functional holo-enzyme.

HypA and HypB are the key accessory proteins that are involved in Ni-insertion into hydrogenase. In *E. coli,* mutational analyses have indicated that mutants lacking intact HypA and HypB are deficient in hydrogenase activities that can be complemented by the addition of excess Ni in the growth medium (131, 138, 173, 179). The mechanism of their involvement in Ni insertion has been very well described (10, 162). Both HypA (or its homologue HybF) and HypB can bind Ni (10, 163, 196). HypA or HypF can bind Ni with micromolar affinity (binds Zn at a different site with a higher affinity) and HypA is thought to form a bridge between hydrogenase and HypB (10, 28, 196). Hence, HypA is thought of as a scaffold for the assembly of other components required for Ni-insertion. HypB can bind one equivalent of Ni and possess one high affinity (K_d sub pico-molar range) and a low affinity Ni binding site. Mutational analyses have indicated that an N-terminal CXXCGC motif comprises the high affinity site and the low-affinity metal-binding site localizes to the GTPase domain. Mutations in the GTPase domain of HypB have indicated that energy from GTP hydrolysis is required for Ni-insertion into hydrogenase (180).

Some organisms, such as *R. leguminosarum*, *A. vinelandii* or *B. japonicum*, have evolved a second function for HypB, that of Ni storage (231, 232). This function is accomplished by histidine-rich domains, which are present in the amino terminus. In the case of *B. japonicum*, a genetically altered version, lacking the histidine-rich tail is capable of promoting hydrogenase maturation, but only at increased Ni concentrations (232). Both HypA and HypB are associated

with the maturation of hydrogenase as well as that of urease in *Helicobacter* species. This is described in detail in a later section.

It is not surprising that HypB interacts with HypA (10, 196). Another interesting interaction of HypB has also been described- interaction with SlyD, a protein with peptidyl-prolyl activity, possessing a histidine rich C terminus, capable of binding Ni (163, 329). Mutational studies, affecting different domains of SlyD show that SlyD facilitates the release of Ni from HypB during Ni insertion into hydrogenase, while the peptidylprolyl activity is not essential for hydrogenase maturation (163, 329).

Urease

Urease is an enzyme that much interesting history associated with it. It was the first enzyme proven to also be a protein, the first protein ever to be purified, the first protein to be crystallized, the first Ni enzyme to be identified, and the first Ni enzyme to be crystallized (though the fact that urease was a Ni-enzyme was not known until 1975 (62)) . This magnificent work was carried out as early as 1926 when James B. Sumner was working with jack bean (*Canavalia ensiformis)* urease with the idea of isolating enzymes in their pure forms. Sumner and Northrop (who crystallized the enzyme pepsin in 1929) together won the Nobel Peace Prize in 1946 for Chemistry for their achievements.

Urea amidohydrolase or urease is the enzyme that catalyzes the hydrolysis of urea to yield ammonia and carbamate. Carbamate undergoes spontaneous hydrolysis to yield another molecule of ammonia and carbonic acid. Urease has been identified and described in plants (124, 246), algae (209) , fungi (48, 283, 321), archaea (25) and many prokaryotes (208, 209). The best characterized urease is from plants- the jack bean urease. Urea is the common nitrogenous waste product of terrestrial mammals, and uric acid is the common waste product of birds. Uric acid is

further broken down into urea in nature (299). Serum concentrations of urea in human are between 1 and 10 mM while urine concentrations can go upto 0.5 M (106). Urea is also the primary component of most industrial fertilizers used in the agricultural industry, and there is some evidence that activity of the enzyme urease increases urea accessibility for the plant (208, 309). Urea is also generated during the degradation of purines and pyrimidines (246) and urease allows organisms to use external or internal urea as a source of nitrogen for growth. Ni-deficient plants grown on urea show stunted growth and accumulate large amounts of urea in the cell that can become toxic (268). Similar phenotypes were observed in wild-type plants grown in the absence of Ni and in urease mutant plants (268). Urea accumulation caused leaf-tip necrosis called 'urea-burn' (268). Recent findings also indicate urease has roles beyond the scope of its function as an ureolytic enzyme- urease was found to possess anti-insecticidal, haemagglutination activities (79, 82, 254, 275). Canatoxin, an isoform of the jack bean urease has been demonstrated to cause convulsions (by causing hypoxia) and indirect haemagglutination in rats and mice (82, 254). Urease was also shown to contribute to plant defense by inhibiting α-amylase, proteinases in Coleptera (beetles) and Hemiptera (bugs) (79, 275) .

Bacterial urease has been studied extensively, and reviews are available that have described this enzyme in detail (34, 44, 208, 209). Briefly, jack bean urease is encoded by a single gene. In bacteria, urease structural genes are encoded by two (*Helicobacter* sp.) or three subunits (example-urease from *Proteus* sp., *Klebsiella* sp., *Yersinia* sp.). Though the organization of the structural genes seems different at first glance, sequence analysis shows that urease is well conserved among eukaryotes and prokaryotes. The N-terminal region of the jack bean urease is similar in sequence to UreA and UreB and the C-terminal region that is sequentially similar to

UreC of most bacteria (exception- N terminal similar to *Helicobacter* sp. UreA, that possesses homology to both UreA and UreB of other microbes and a C terminal region similar to UreB that shows homology to UreC of other bacteria) (34, 44, 208, 209).

The active site of urease constitutes a dinuclear Ni center, and crystal structures are available for urease from *Klebsiella aerogenes, Bacillus pasteurii, and H. pylori* (21, 110, 137). Structures indicate that the two Ni^{2+} ions are carbamylated to a lysine residue and a water molecule also bridges the two Ni^{2+} ions. These structures also reveal that the NiNi center is buried deep inside the protein and is inaccessible to chelators (21, 110, 137). The assembly of urease is a complex process that requires UreDGEF accessory proteins usually encoded within the same operon containing the structural genes, CO_2 for carbamylation and GTPase activity for carbamylation of the active lysine residue. Mutations from deletions in UreDGEF lead to the formation of inactive urease devoid of Ni (165). This was achieved by expressing the *K. aerogenes* gene cluster in *E. coli* from a plasmid and testing which of those genes affected urease maturation (164). In the *K. aerogenes* urease maturation system UreD was shown to interact with apo-urease and cause a conformational change required for subsequent assembly steps, although its function is not very clear (237) . Other studies also show that UreF plays a role in bringing about the conformational change needed for providing access to CO_2 and Ni (211, 262). UreG is responsible for the GTP hydrolysis that provides the energy required for the carbamylation of the lysine residue, and UreF was proposed to be an activator of this UreG GTPase activity based on modeling studies (262). Recent studies have indicated that UreG from *B. pastureii* and *H. pylori* is intrinsically disordered and binds Zn^{2+} that is required for the dimerization of UreG (325, 326). This is evidence to show that Zn^{2+} could play a role (although it may be indirect) in Ni^{2+} insertion into apo-urease.

UreE is a Ni-binding protein that acts as a metallo-chaperone for the delivery of Ni into the apo-enzyme (164, 165). UreE from *K. aerogenes* contains a His-rich C-terminal region where 10 of the last 15 amino acids are histidine residues (165). This protein is capable of forming a homodimer and can also exist as higher mass oligomers. A dimer of UreE was shown to bind to around 6 Ni^{2+} ions with an affinity of around 10 μM (165). A conserved histidine residue (His-96) is important for Ni binding in *K. aerogenes.* Not all UreEs have a C-terminal regions rich in histidine residues, *B. pastuerii* UreE does not have a His-rich C-terminus, and similarly, UreE from certain *Proteus* and *Helicobacter* sp. contain lesser number of histidine residues in their C-terminus. In fact, truncated versions of UreE, lacking the His-rich region are still able to Ni-activate urease, suggesting that the Ni-binding regions of UreE that contribute to Ni activation is present elsewhere (33). Recent studies (and unpublished results from S.L.Benoit and R.J.Maier) indicate that UreE interacts with UreG (326). It is thought that Ni from UreE binds to the apo-urease, followed by a Zn induced UreE-UreG interaction that acts as a signal for the GTPase activity. The latter is required for the carbamylation of the lysine residue which leads to the final active holo-urease formation (326). *H. pylori* UreE and the HypA and HypB proteins that were discussed earlier play critical roles in the maturation of urease and will be discussed in a later section.

REGULATORS OF NI METABOLISM

A major player in Ni metabolism and homeostasis in bacteria are the transcriptional factors or the regulators, which play important functions in activating, de-repressing, or repressing the other players in Ni metabolism, namely the Ni transport and efflux systems, the Ni binding storage/sequestering/detoxifying proteins, and Ni enzymes. Often times they also self-regulate their own expression. In different bacteria, these regulators bind Ni, and regulate varied

genes in response to the availability of Ni and the need for Ni and other environmental factors. Ni dependent regulators are found in various families of DNA binding transcriptional factors. Based on the structures, metal sensing transcriptional regulators have been classified into seven major families (96). An excellent review discusses the features and characteristics of each families (96). Ni sensing regulators have been identified in four of the seven families and are briefly discussed in this section (96). These regulators are 1) NikR, the only metallo-regulator of the Ribbon-Helix-Helix family of DNA binding proteins (RHH), 2) Nur, the Ni uptake regulator, belonging to the Fur family of transcriptional regulators, 3) SrnR, KmtR and NmtR, belonging to the ArsR/SmtB family of metalloregulators 4) RcnR, a Ni regulator, belonging to a novel class CsoR family of transcriptional regulators.

NikR, as mentioned earlier is a member of the β-α-α RHH family of transcriptional regulators (55). Genome searches indicate that a NikR homologue is present in many bacteria and has been well characterized in *E. coli, H. pylori* and *P. horikoshii* . NikR of *E. coli* has a central tetrameric oligomerization domain that is flanked by the RHH DNA binding domains. The NikR tetramer can bind 4 Ni ions and is bound by three conserved histidine residues and a cysteine residue. This site is the high affinity site, binding Ni with pM affinity and thus activating DNA binding. Interestingly, *E. coli* NikR also has another Ni binding site that binds Ni with much lower affinity, and binding of Ni to this site is thought to further enhance NikR-DNA binding affinity and the structure of a NikR-DNA complex (29, 58, 59, 166). In *E. coli*, the physiological role of NikR is to repress the NikABCDE Ni transport system in the presence of excess Ni (55). NikR binds to a perfect inverted repeat upstream of the *nikABCDE* promoter to repress this operon.

Nur is a Ni uptake regulator belonging to the Fur family of transcriptional regulators. It was identified in *Streptomyces coelicolor* as the repressor of the Fe-SOD and the activator of Ni-SOD. It also represses the transcription of a *nikABCDE* operon in a Ni dependent fashion (5). A recent study speculates that Nur could bind one Ni ion per monomer and probably exist as an asymmetric dimer (8).

SrnR is a Ni dependent repressor of the ArsR family of transcriptional regulators and in *Streptomyces grisius*, SrnR represses the *soF* expression in a Ni dependent manner. SrnR was found in a complex with SrnQ, a Ni sensor, which binds Ni and is thought to improve SrnR-DNA binding (153). NmtR and KmtR are two Ni and cobalt responsive regulators characterized in *Mycobacterium tuberculosis* (38, 41). KmtR, a recently discovered protein, was found to repress a CDF family of metal efflux pump in a Ni and cobalt dependent manner (38). NmtR, a dimeric Ni binding protein, is the other Ni responsive regulator of *Mycobacterium tuberculosis* that derepresses NmtA, a nickel-cobalt efflux pump of the P-type ATPase family, in a predominantly Ni dependent manner. However, KmtR binds both nickel and cobalt with higher affinities and hence is active under lower concentrations of nickel and cobalt, while NmtR becomes active at a much higher concentration of these two metal ions (38).

RcnR is a recently indentified Ni- responsive regulator identified in *E. coli.* RcnR regulates a nickel-cobalt efflux pump (RcnA) by directly binding to and repressing the promoter (134). Nickel or cobalt inhibits RcnR interaction with DNA. RcnR regulation of the Ni efflux pump is closely linked with NikR activity levels *in vivo* and NikR repression of NikA. NikABCDE is speculated to be a hydrogenase specific Ni uptake system (134). There are speculations that there is another unidentified Ni importer in *E. coli* that provides Ni associated

with the glyoxylase I and the RcnA efflux pumps and that this Ni pool is regulated by RcnR. NikR is thought to be the bridge between these two distinct Ni pools (134).

Studies of such metal-sensing transcriptional regulators suggests that bacteria are able to utilize metal binding to dictate structural features in a protein that in turn allows them to adapt continually to varying environmental pressures.

HELICOBACTER PYLORI **AND** ***HELICOBACTER HEPATICUS*****- THE MODEL ORGANISMS FOR STUDY**

History

Since the time spiral shaped bacteria were first observed in the human gastric mucosa in the early 20th century to the identification and discovery of *Campylobacter pylori* (later renamed *Helicobacter pylori*,(1)) in the 1980's (187, 189), our knowledge of the genus *Helicobacter* has come a long way. There were many reports of spiral shaped bacteria in the gastric mucosa of animals such as dogs, cats, and monkeys dating as early as the late 19th century (26, 109, 136, 168). In 1906, Krienitz of Germany first described various spiral shaped microorganisms in the fresh stomach contents of a patient suffering from stomach cancer (155). Throughout the 20th century there were many more reports of "Spirochaetes" and spiral bacteria in gastrectomy specimens and in gastric mucosa samples from patients with gastritis respectively (16, 135, 276). Finally, in 1984, Barry Marshall and Robin Warren reported the presence of curved bacilli in patients with gastric and peptic ulcerations (189). For their pioneering work in fulfilling Koch's postulates and establishing *Campylobacter pylori* (now *Helicobacter pylori)* as the etiological agent of peptic and gastric ulcers, they received the 2005 Nobel Prize in Physiology/Medicine.

Since the 1980s, extensive research conducted in numerous laboratories across the world has been aimed at understanding every aspect of *H. pylori* biology, ranging from the studies of

morphology and physiology, to vaccine development and eradication. Since the discovery of *H. pylori,* 30 or more *Helicobacter* species have been identified and characterized. Today, searching the online database at the U.S. National Library of Medicine and National Institute of Health (popularly known as PubMed) with the word *Helicobacter* retrieves over 28,124 results. Considering the medical significance of helicobacters and the growing number of species identified, it will not be surprising to see this figure grow over time.

Class Epsilonproteobacteria and Genus *Helicobacter*

Helicobacter pylori and *Helicobacter hepaticus* both belong to the class Epsilonproteobacteria of the phylum *Proteobacteria*. ε- Proteobacteria is a comparatively small class consisting of two orders- *Campylobacterales, and Nautiliales* (107). They comprise medically and ecologically significant genera. The representative genera are *Campylobacter, Arcobacter, Sulfurospirillum, Sulfurimonas, Helicobacter,* and *Wolinella* (149). The medically important genera are *Campylobacter* and *Helicobacter.* From certain estimates, it is likely that greater than 50% of the world human population is infected by *H. pylori,* the causative agent of gastric ulcers and certain types of stomach carcinomas (67, 285). *Campylobacter jejuni* is the major causative agent of food borne diarrhea. In addition to these genera, there are other unclassified members like *Nitratiruptor, Sulfurovum,Thiovulum,* and *Thioreductor* to name a few (149).

The ε-Proteobacteria are currently distinguished from other bacteria based on the branching in the 16 S rRNA tree (107). With respect to the 16 S rDNA sequence similarity, all members of the ε- subdivision are very closely related (160), yet these bacteria are very diverse with respect to their habitat, morphology, and metabolism. They comprise human and animal pathogens, host associated symbionts, free living organisms in sulfidic habitats, water reservoirs,

sewage, and oil field and episymbionts and endosymbionts of deep sea hydrothermal vent invertebrates (107, 149, 160, 218). In fact, recent studies have indicated that ε-Proteobacteria are by far the most prevalent group in deep sea vent habitats (37, 282). The free living and marine ε-Proteobacteria are important players in various elemental cycles in their respective habitats.

The majority of organisms in this subdivision are curved to spiral shaped, Gram- negative microaerophiles or anaerobes capable of mesophilic to thermophilic growth. This group comprises chemolithotrophs, autotrophs and heterotrophs that are very versatile in their energy metabolism. They are able to utilize many alternate electron donors such as H_2, formate, elemental sulfur, sulfide and thiosulfate and several electron acceptors such as sulfite, elemental sulfur, nitrate, and oxygen.

The genus *Helicobacter*

The genus *Helicobacter* (meaning a spiral rod) was established in 1989, to help distinguish this genus from the genus *Campylobacter* (100, 296). The most important features required for an organism to be classified as a *Helicobacter* were (i) cell motility by means of sheathed flagella; (ii) an external glycocalyx produced *in vitro* in liquid media; (iii) menaquinone-6 (MK-6) present as the major isoprenoid quinone; (iv) G+C content of chromosomal DNA of 35-44 mol% ; and (v) absence of hexadeconoic acids from the major fatty acid profiles (100, 296).

Today, the genus *Helicobacter* comprises about 40 different species that have been isolated from various host gastric, enteric or enterohepatic tissues. There are numerous candidate and unvalidated species. Helicobacters generally are Gram-negative, curved, spiral or fusiform rods and are motile by means of flagella. The flagella in most species are sheathed and are observed in various numbers and configurations. The presence of flagella and their spiral shape

are thought to aid in the colonization of the mucus layer of the gastrointestinal tract. Most helicobacters are microaerophilic in nature while a few genera are anaerobes (no growth is observed aerobically). They are non-spore forming, non-pigmented bacteria. All species exhibit oxidase activity and most species produce catalase. They show a respiratory type of metabolism and are mostly chemoorganotrophic with respect to their energy requirements. Most helicobacters are asaccharolytic when tested for sugar catabolism under standard laboratory conditions, though there are exceptions (*H. pylori)*.

MICROBIOLOGY OF *HELICOBACTER PYLORI*

Morphology and Physiology of *H. pylori*

Shape and Flagella- *H. pylori* is a Gram-negative 'S' or a spiral shaped bacterium with 2-3 turns and is 0.5- 5 μm in length and about 0.5- 1 μm in width (103, 140). It possesses 5-7 polar flagella and an interesting feature of these flagella is that they are covered by a sheath. The flagella extend 3-5 μm in length and the tip is often present as a club-like thickening. There is some evidence to suggest that the sheath is a protective covering (continuation of the outer membrane) that allows the flagellar proteins to withstand the acidity in the stomach and it is likely made up of lipopolysaccharide and some proteins (94). The flagellar filament has been well studied and it is composed of two major proteins flagellin A (FlaA) and flagellin B (FlaB) proteins and is attached to the basal body via a flagellar hook protein FlaE (reviewed in (226)). The flagella biogenesis and regulation of flagella synthesis involves numerous proteins and sigma factors and has been well described (271). Many studies have discussed the importance of flagella for gastric colonization as they allow the bacterium to actively move towards the gastric mucosal surface where the pH is closer to neutral than in the gastric lumen (141, 226, 319). In fact, certain studies conducted with non-motile mutants showed that they were less efficient in

colonizing the gastric epithelium of gnotobiotic piglets compared to their motile counterparts. It was also observed that the degree of infectivity correlated directly with the degree of motility (69).

Normal, actively growing *H. pylori* cells are in the typical spiral shape while cells that have aged or have been exposed to stress condition assume coccoid shapes (64, 158). The coccoid forms are non-cultivable though are alive. The cells in the coccoid form possess lesser activities of major enzymes such as urease, SOD and catalase and contain a higher percentage of DNA damage (219). *H. pylori* cells produce an external glycocalyx that can be visualized by electron microscopy and it is thought to be important for cell adhesion (101).

Oxygen requirements and factors combating oxidative stress- *H. pylori* is a microaerophile. It is routinely grown in 4-10% oxygen tensions. However, there are some studies that indicate that many strains of *H. pylori* have been successfully grown in regular CO_2 incubators with concentrations of up to 20% oxygen (193, 204, 260). It is a strict capnophile, requiring elevated partial pressures of CO_2 (around 5-10%) for growth. In our lab, we grow *H. pylori* in the presence of 4-5% O_2, 5% CO_2, and the balance gas as N_2. Including H_2 in the atmosphere aids growth. A recent study indicates that *H. pylori* is a microaerophile, capable of growth at "high" oxygen concentrations, but only at high cell densities and only under high pCO_2 (35). It also grows better under microaerophilic conditions compared to aerobic conditions (35). *H. pylori* does not grow under anoxic conditions (64, 203). *H. pylori* produces an active fumarate reductase and it was thought that fumarate reductase activity could generate energy for anaerobic respiration, though this has never been shown experimentally (199). In fact, even in the presence of fumarate, *H. pylori* could not grow anaerobically.

Though it is a microaerophile, *H. pylori* is possibly able to grow well under higher pO_2 because it possesses extremely efficient and competent systems to combat the reactive oxygen species (ROS) that are formed as a by-product of oxygenic reactions. Superoxide radicals (O_2^-) and hydrogen peroxide (H_2O_2) are formed when flavoproteins undergo auto-oxidation (133). The H_2O_2 can react with Fe^{2+} and cause the production of hydroxyl radicals OH^- and hydroxyl ions (OH^-) in the Fenton reaction (108). OH^-, the most toxic ROS is capable of damaging DNA and other macromolecules in the cell (133). H_2O_2 can attack [4Fe-4S] clusters of proteins and the sulfur atoms present in cysteines and methionines, causing loss of key enzymes for the cells (133). The O_2^- radicals can also damage enzymes by damaging the [Fe-S] clusters (86).

As a result of colonizing the gastric mucosal surface, *H. pylori* elicits a strong inflammatory response mediated by host neutrophils and macrophages leading to the production of large amounts of ROS. *H. pylori* has a large variety of enzymes and systems to combat this oxidative burst and persist in the host mucosal surface. The SOD of *H. pylori* is the Fe-SOD and it can cause the dismutation of O_2^- into H_2O_2 (243, 273). H_2O_2 is subsequently eliminated by catalase or peroxidase. Other than these enzymes, *H. pylori* possesses robust DNA repair systems (*mutS*, *ruvC, nth)*, protein-repair mechanisms (*msr)*, free iron sequesteration systems (ferritin, *napA* (neutrophil activating protein), regulators of genes involved in ROS and Fe metabolism (Fur), alkyl hydroperoxidase reductase to detoxify ROOH and other reactive nitrogen intermediates (reviewed in (303)). All these systems function to sequester/detoxify toxic elements, repair the damage caused by ROS to macromolecules and regulate genes and responses to ROS. This repertoire allows *H. pylori* to have numerous and redundant systems to combat persistent ROS, the result makes *H. pylori* a formidable persistent pathogen.

Other growth requirements and Sugar Catabolism- Most *H. pylori* strains grow best at 37˚C. Some strains grow poorly at 30˚C while none grow at 25˚C. Growth at 42˚C has been reported for a very few strains from rhesus monkeys. It has been observed that moisture improves growth at least on solid growth media.

During *in vitro* growth, *H. pylori* can be cultured on complex media such as Muller-Hinton, Brain-Heart Infusion Media when supplemented with 5-10% sheep's blood or fetal-bovine serum. *H. pylori* can be grown in a defined medium as described by Reynolds and Penn when supplemented with various amino acids and β-cyclodextrin (253). *H. pylori* requires amino acids such as arginine, histidine, isoleucine, leucine, valine, methionine, phenylalanine, serine and alanine for growth (221, 253). *H. pylori* uses these amino acids as carbon and energy sources.

Initially, it was thought that *Helicobacter* sp. was asaccharolytic since it is very closely related to the campylobacters that do not utilize sugars. While it is true that most helicobacters are asaccharolytic, *H. pylori* is able to utilize glucose (oxidatively or fermentatively) via the pentose-phosphate pathway or the entner-doudoroff pathway to obtain energy (200-202). Studies conducted by one other group indicate that the enzymes required for the regular glycolytic pathway (the EMP pathway) are also present, however controversies exist in this area (127). The pentose phosphate pathway is thought to be important for the generation of NADPH and NADH and for the generation of the 5-carbon intermediates that are necessary for purine and pyrimidine biosynthesis (200). In fact, for *H. pylori*, NADPH serves as the major electron donor in the respiratory chain rather than NADH that serves as the major electron donor in most bacteria. The citric acid cycle of *H. pylori* is that of a branched non-cyclic type. The tricarboxylic branch proceeds until succinate but lack the succinyl-CoA synthase enzyme that converts succinyl-CoA

into succinate. Interestingly, α-ketoglutarate is converted to succinyl-CoA via the α-ketoglutarate: ferredoxin oxidoreductase enzyme rather than via the typical α-ketoglutarate dehydrogenase enzyme (146, 183). Succinyl-CoA is converted to succinate via a succinyl-CoA: acetoacetyl-CoA transferase (46). The tricarboxylic branch proceeds from oxaloacetate to fumarate. *H. pylori* lacks the malate dehydrogenase enzyme that converts malate to oxaloacetate, but contains a malate:quinone oxidoreductase that performs the same function of oxidizing malate to oxaloacetate (146).

All living organisms need nitrogen for growth. Analysis of the *H. pylori* genome indicates that this bacterium is capable of utilizing ammonia, urea, and amino acids as a source of nitrogen for growth (285). Urea is hydrolyzed to produce ammonia needed for the formation of glutamate from α-ketoglutarate by the action of the enzyme glutamate dehydrogenase (198). This is an important mechanism adopted by *H. pylori* to survive in an ammonia-rich environment. Other than using urea and ammonia via glutamine synthase and glutamate dehydrogenase, ^{13}C label studies indicate that amino acids act as important nitrogen source for the biosynthesis of various important metabolites such as formate, lactate, acetate, and succinate (198).

Iron is another important growth requirement for almost all organisms. Like in any other organism, iron is vital for *H. pylori* as iron containing proteins such as SOD, catalase, and many other iron or heme containing proteins play important roles in respiration, ROS resistance and the iron containing enzymes catalyze other important functions in the cell. However, iron like Ni can be toxic in higher quantities; thus its import is tightly regulated by 'Fur', the iron-dependent repressor protein. Genome analysis and experimental evidence shows that iron is/could be scavenged by many means- Fe^{3+} dicitrate transporters (Fec system), Fe^{2+} ferric siderophore

receptors (Feo), periplasmic iron binding proteins (Cue) and the cognate systems required for the active transport of iron into the cells (TonB, ExbB, ExbD systems) like in *E. coli.* Once inside the cell, free iron is sequestered by ferritin (Pfr), bacterioferritin (NapA) (reviewed and described in (183, 264)).

Genome and Strain Diversity and Plasmids

The genome sequences of three *H. pylori* strains are currently available - strain 26695, strain J99 and strain *HPAG1*. The genome size is approximately 1.6 Mb with each having 1630, 1491 and 1544 genes respectively (http://cmr.jcvi.org). The three genomes have two copies of 5S, 16S and 23S rRNA genes. Some strains carry one or more cryptic plasmids that have been used extensively as shuttle vectors and have allowed important complementation studies of various phenotypes (the pHel2 and pHel3 plasmids) (121, 122). These plasmids contain minimal genes necessary for replication in both *E. coli* and *H. pylori* permitting various molecular cloning experiments. There are some studies that indicate the presence of bacteriophages that are capable of infecting *H. pylori*, but this area needs further research (118). In fact, use of bacteriphages or their components as a treatment option for many bacterial infections, including *H. pylori* infections is being studied currently, but this treatment is still in its infancy and requires much fine tuning before it becomes a usable, viable treatment (30, 190).

Niche of Colonization, Disease, Pathogenesis and Virulence

Niche- Robin Warren and Barry Marshall disproved the myth that no organism could colonize the stomach because of the presence of concentrated hydrochloric acid in the gastric juices. *H. pylori* colonizes the gastric mucosal surface of humans (Figure 1.2). The acidity in the stomach ranges from a pH of 2 in the lumen to a pH close to 7 near the gastric epithelial surface. *H. pylori* is one of the only organisms capable of surviving and successfully colonizing such a

harsh environment. Factors other than acidity that make this environment inhospitable are- constant peristaltic movements in the stomach, periodic entry and emptying of stomach contents, and shedding of mucus and epithelial cells. In addition, there is constant immunologic response from the host comprising infiltrating neutrophils and macrophages that cause constant ROS stress and RNI stress on the bacterium.

H. pylori possesses a fleet of highly efficient and effective mechanisms to persist and infect the gastric mucosal lining and they are discussed in this section. Motility (discussed earlier) and the enzyme urease (discussed later) are among the factors that are absolutely essential for initial host-colonization. Other than those, Type IV secretion system (T4SS) - *cag pathogenicity* island (*cag* PAI), vacuolating cytotoxin (VacA), adhesins and certain outer membrane proteins and many other factors are involved in virulence (213). The Ni-Fe hydrogenase is important for the persistence of the infection and is required for H_2 dependent energy production (175).

***cag*PAI**- The *cag* pathogenicity island or *cag*PAI of *H. pylori* (reviewed in (12) encodes anywhere between 27-31 genes depending on the strains, 18 of which are involved in the formation of a syringe-like structure that is involved in the secretion of CagA, peptidoglycan or other bacterial factors into the host. This 40kb DNA locus encodes a cytotoxin associated gene A, which is highly immunogenic and potentially plays many roles as an effector molecule. CagA, peptidoglycan or unidentified factors that are secreted by this T4SS into the host are thought to cause a pro-inflammatory response, that also aids in the development of disease. CagA is thought to disrupt host-signaling pathways, specifically by interacting with host cell kinases (known oncogenes) to ultimately cause cell elongation and scattering, destabilizing the gastric epithelium, thereby contributing to pathogenesis (12). CagA also interacts with host tight

junction proteins and promotes loss of cell polarity and affects host cell gene expression. CagA is not present in all *H. pylori* strains ($CagA^-$ strains) and its presence ($CagA^+$ strains) is correlated with a more severe/chronic case of gastritis, peptic ulcer disease and gastric cancers. Still, some patients with severe gastritis or even gastric cancers are associated with $CagA^-$ *H. pylori* strains (12).

VacA- VacA or the Vacuolating cytotoxin produced by *H. pylori* is a potent cytotoxin encoded by the *vacA* gene (reviewed in (302). The gene product is an unprocessed 138kDa protein or a mature 87-95 kDa protein. The amino terminal region of VacA (or the mature VacA), excluding the signal sequence and the C-terminal region, is secreted into the extracellular space and is thought to produce a hexameric, flower-like structure that when in contact with host-membranes (such as mitochondrial, plasma or vacuolar membranes) forms an anion-selective, voltage dependent pore (302). This is damaging to the host cells as it affects osmotic balance across membranes and can cause the leakage of toxic components into the host cells. The mid-region (or the carboxy terminal region of the mature VacA) of the VacA protein is thought to be responsible for binding host-receptor cells. Numerous studies with HeLa cells have shown that VacA induces vacuolation and disrupts normal endo-lysosomal functions, thereby leading to apoptosis of host cells. VacA is also thought to be immunosuppressive in nature by inhibiting the proliferation of host T cells (302).

Adhesins, outer membrane proteins involved in virulence and other factors

H. pylori normally colonizes the mucous lining the gastric epithelial cells. Since it colonizes the mucous gel layer, it is protected from the acidity in the gastric lumen and is able to evade host immune response to a certain extent. Yet 10-15% of *H. pylori* cells are found adhering to the gastric epithelial cells (120). The same study also shows the degree of attachment

has a direct correlation with the progression of cellular damage. Nearly 4% of the *H. pylori* genome is thought to encode outer membrane proteins (OMPs) (285). Of the many OMPs, a few have been described to play roles in adherence to host cell surfaces and in pathogenesis. Some of them are BabA, SabA, OipA, Hsp60, AlpA/B, HopZ (reviewed in (159, 227, 265). BabA, a 78kDa protein encoded by the gene *babA* has been well characterized and is thought to mediate binding to fucosylated Lewis B antigens that act as receptors on the surface of gastric epithelial cells. Animal studies have indicated that BabA-dependent adhesion is important for animal colonization and is thought to be associated with the more severe form of disease (chronic gastritis and adenocarcinoma). However, *H. pylori* is also able to adhere to gastric epithelium in the absence of the Lewis B antigens. SabA is the sialic acid-binding adhesin (SabA) used by *H. pylori* to recognize Lewis X antigen of gastric epithelial cells. SabA is associated with nonopsonic activation of human neutrophils and is thought to bind the extracellular matrix protein laminin. SabA mediates binding to the sialylated glycoconjugates. The Lewis antigens tend to be more sialylated in *H. pylori* induced chronic inflammations and adenocarcinomas; therefore, SabA probably plays a role in the more severe forms of a *H. pylori* infection. Other outer membrane proteins, in various studies have been implicated in adherence. Mutants lacking AlpAB proteins that are co-transcribed (228) and HopZ another outer membrane protein (265), seem to show BabA independent decrease in adherence to various gastric epithelial cells and tissues, indicating a role for the three of them and a group of 32 similar outer membrane proteins in adherence. Hsp60 or heat-shock protein 60, a protein that is secreted by *H. pylori* is believed to be involved in adherence (316, 317). Monoclonal antibodies against Hsp60 inhibited binding of *H. pylori* to gastric epithelial cells, though this was not reported for all *H. pylori* strains. It is

hypothesized that exposure of *H. pylori* to a luminal pH of ~ 2, induces Hsp60 production, that primes *H. pylori* for adherence to host sulfatide receptors (316, 317).

Host factors

H. pylori colonizes more than 50% of the world population. Most of the people carrying *H. pylori* remain asymptomatic and never report any problems due to *H. pylori* colonization. Only a small subset of this population develops symptomatic disease like acute/chronic gastritis, peptic and duodenal ulcers (10-15%). Even within this group, only in a small subset of the population, the disease progresses to gastric malignancies (1-2%). A lot of this has to do with the strain of *H. pylori* one is infected with (as noted earlier, $VacA^+$, $CagA^+$, presence of certain OMPs may cause a more severe disease outcome). There are other factors that seem to affect colonization and the outcome of *H. pylori* infection in an individual, namely- ethnicity, socioeconomic status, host immune factors, dietary factors. It has been reported that in multi-ethnic regions such as Malaysia, people of Indian origin were more like to be *H. pylori* sero-positive than Malay or Chinese populations living in the same region (99). Other similar studies indicate ethnicity plays a role in predisposition for *H. pylori* infection (85). Other studies indicate that socioeconomic status and proper hygiene that is probably lower in developing countries is the reason for the higher incidence (up to 90% of the population) of *H. pylori* infection in those regions compared to that in developed countries (<50%) (reviewed in (181) . Among people in a lower socioeconomic status, the age of acquisition of the disease was another factor that affected *H. pylori* infection outcome. The longer a child/person is infected with *H. pylori*, the higher the chances of the disease progressing to a malignancy. Another confounding factor is the host immune response and other unknown host factors. In a study with twins- homozygotic twins, reared either in the same or different environments mostly showed the same *H. pylori* status

while heterozygotic twins, reared in the same environment and eating the same diet, showed different outcomes after infection with *H. pylori* (182). This clearly indicates that we need better understanding of host factors and *H. pylori* host interactions.

Disease and Pathogenesis

H. pylori infection is associated with active and chronic gastritis, peptic and duodenal ulcers, gastric dyspepsia, GERD, MALT lymphomas and gastric adenocarcinomas some of which are discussed in this section.

Acquisition and transmission- *H. pylori* infection usually occurs in early childhood and the bacterium is most likely to spread via human-to-human transmission likely by fecal-oral or oral-oral routes. There are some recent studies that suggest that drinking water, food and animals could be potential vectors for transmission of *H. pylori* (88, 95, 154, 241, 248). This hypothesis is based on routine detection of *H. pylori* DNA based on PCR studies of different drinking water, ocean water and other water source samples, especially in drinking water distribution systems where biofilms gave been found (95). Another hypothesis is that *H. pylori* could spread via food such as contaminated raw sheep, cow, and goat milk (248). Though, *H. pylori* has never been cultivated from the above mentioned water sources or dairy products as of today (88, 95, 154, 241, 248). Other studies have shown that a mother and her children are usually infected with the same genotype of *H. pylori.* In fact, a study showed that 29% of the mothers had the exact same genotype as their youngest child and a lower percentage of mothers (6 and 8%) of the mothers had the same genotype as their middle or eldest children(217). This, along with many other studies conducted, indicates towards an intra-familial mode of transmission for *H. pylori* with infection being acquired during early childhood (6, 66, 205, 217, 222) .

Acute Gastritis- Inflammation of the gastric mucosal layer, associated with gastric injury including epithelial cell damage and regeneration is called gastritis. Since *H. pylori* infection is mostly asymptomatic, at least in the initial stages of infection, there aren't many studies describing the symptoms and pathology of acute gastritis. Most of the data comes from deliberate or inadvertent *H. pylori* infections (104, 186, 272). A controlled study whereby healthy volunteers were infected with a well studied laboratory strain has shown that during the very early phase of infection, volunteers complained of fullness, nausea, vomiting (pangastritis) and pathology specimens indicated the presence of inflammation of proximal and distal mucosa (104). This phase of acute gastritis is also associated with hypochlorhydria and can last for months. Some people are able to clear the infection and while others are even able to prevent colonization (182).

Chronic Gastritis- Persistence infection with *H. pylori* leads to chronic gastritis. This can be characterized by the presence of persistent dull pain in the stomach, belching and bloating mostly after meals, unintentional loss of weight along with pangastritis. In most people, chronic gastritis is even asymptomatic. Persistent infection with *H. pylori* causes recruitment of host-immune responsive cells such as macrophages and neutrophils. The Presence of macrophages and neutrophils as well as *H. pylori* in the gastric mucosal layers affects acid secretion by the stomach and also affects secretion of gastrin. When acid secretion is unaffected, there is more antral gastritis, compared to a more even distribution of gastritis in both the antrum and corpus of the stomach in people with hypochlorhydria (159). Use of proton pump inhibitors (PPI) can also reduce acid secretion. Reduced acid secretion could predispose a person towards atrophic gastritis, intestinal metaplasia and gastric carcinomas (159).

Peptic and Duodenal Ulcers- Ulcers, defined as areas where there is a break in mucosal tissues or as areas of mucosal tissue erosions, occur mostly in areas with severe inflammation. There is a strong association between occurrence of duodenal and peptic ulcers with *H. pylori* infection. Nearly 90% of the people with duodenal ulcers and 70% of the people with peptic ulcers are infected with *H. pylori* (102, 185, 188, 225). Patients with *H. pylori* infection have increased gastrin levels, decreased gastric mucus production and duodenal mucosal bicarbonate secretion, all of which favor ulcer formation (128, 290, 291). The same studies also indicate that for small ulcers, eradication of *H. pylori* along with administration of PPIs is sufficient for management of disease. Severe forms of peptic ulcers or peptic ulcer complications include conditions such as bleeding, perforation of tissue, or stricture formation. In cases of bleeding ulcers, endoscopic administration of adrenalin, coagulation with a heater probe or clipping off bleeding vessels is performed to stop bleeding. This is followed by suppression of acid secretion by PPIs. In the case of perforations, surgeries are performed followed by acid suppression. In cases where non-malignant strictures associated with *H. pylori* occur, eradication of *H. pylori* alone is often sufficient to manage the condition (98, 159, 266). Administration of non-steroid anti-inflammatory drugs (NSAIDs) when infected with *H. pylori* is thought to be a reason for the induction of duodenal ulcers though this association between NSAIDs and *H. pylori* is still controversial (159).

Gastric cancer and MALT lymphoma- Chronic gastritis can lead to atrophic gastritis and intestinal metaplasia characterized by the loss of normal gastric mucosal architecture, loss of gastric glands and parietal cells. Replacement of the lost gastric glandular cells followed by their replacement by intestinal-type epithelial cells is called intestinal metaplasia. Atrophic gastritis again is associated with decreased acid secretion (157). The association of *H. pylori* with the

occurrence of gastric cancer was supported by the findings of numerous studies and led to *H. pylori* being declared a class I carcinogen by WHO (132). Numerous studies indicated that gastric malignancies often occur in the anatomically same areas with severe gastritis and atrophic gastritis and intestinal metaplasia often caused by *H. pylori* infection (11, 76, 78, 130, 156, 244). A geographical distribution of gastric cancers was directly proportional to the prevalence of *H. pylori* positive subjects in the regions. Long term infections with *H. pylori* also increases the chances of developing cancers (1-2% of *H. pylori* positive patients in developed countries to 60% or higher in developing countries). Numerous ongoing studies are determining if treatment of *H. pylori* infections and eradication of *H. pylori* would reduce the risk of gastric cancers. Recent animal studies are questioning specifically if gastric atrophy and metaplasia are conditions beyond the point of no return in cancer formation.

Gastric MALT lymphomas almost always occur in *H. pylori* positive patients. MALT-lymphomas occur when B- cells in the gastric mucosal layers proliferate to form MALT-lymphomas (9, 257, 312). The gastric mucosa is normally devoid of immune cells, but in some rare cases, in response to *H. pylori* colonization, a monoclonal population of B-cells may arise in this region, leading to MALT lymphomas. In cases of low grade gastric MALT lymphomas, eradication of *H. pylori* infection causes regression of the malignancies in nearly 80% of the cases suggesting a close association between *H. pylori* and the malignancy (212).

Diagnosis, Treatment and Prevention- There are numerous methods that could be used to detect *H. pylori*. Physicians and hospitals throughout the world choose a combination of methods to detect the presence of *H. pylori* (reviewed in (169, 293, 322)). Some non-invasive methods that are routinely used include serology (testing for the presence of anti-*H. pylori* antibodies), urea breath test (checks for the presence of ^{13}C or ^{14}C labeled CO_2, 10-30 minutes after the

consumption of a labeled urea meal- urea would be hydrolyzed to produce CO_2 due to the action of urease), stool antigen tests (again detects presence of *H. pylori* antigens in stool). Invasive tests include histology (preferred 'gold-standard' test in hospitals, provides data on inflammation and atrophy), culture biopsy (allows antibiotic sensitivity screening) or rapid urease test. The latter is used during endoscopies and biopsies when a small specimen is directly placed in a solution with urea and phenol red to test for the presence of urease activity, commonly used in conjunction with another test. In many places around the world, rapid detection methods such as use of quantitative real-time PCR methods are also being used to detect the presence of *H. pylori* specific DNA and to test for sensitivity to antibiotics such as clarithromycin (230, 247).

There are numerous treatment regimens that are followed by physicians throughout the world for the eradication of *H. pylori* infection (reviewed in (87, 255, 263, 277)). Since *H. pylori* primarily colonizes the gastric mucosa, single drug therapies are usually inadequate for clearing the disease, as the drugs may not effectively reach the bacterium at the site of colonization. Hence, initially, a triple therapy including two antibiotics (amoxicillin, tetracycline, clarithromycin and metronidazole) and a proton pump inhibitor (PPI) such as omeprazole, lansoprazole, rabeprazole, esomeprazole, pantoprozole is prescribed for 1-2 weeks. After antibiotic treatement, a test is usually conducted to test for the efficacy of treatment and *H. pylori* eradication. If the treatment has failed and *H. pylori* is still detected, a second line of treatment is chosen that includes bismuth subsalicylate, or ranitidine bismuth citrate and a PPI, a combination of two different antibiotics. Use of other antibiotics such as ciprofloxacin, levofloxacin (fluroquinolones), rifampin and streptomycin, rifabutin and furazolidone have been reported in cases where initial therapy has failed or where *H. pylori* strains have been found resistant to the other antibiotics (87, 263). A combination of dosage, drugs, durations are used and efficacy of

treatment usually ranges between 90-95%. Failure in eradication usually has been observed in patients with other side effects, failure to adhere to treatment regimen and in cases where *H. pylori* drug resistance persists (87).

Since *H. pylori* infection in most people is asymptomatic, many times, infections are not diagnosed until chronic infections/malignancies have already set in. The highest number of cancer related deaths worldwide are due to stomach cancers (after lung cancers), though this number has been continually decreasing in developed nations. As treatment for *H. pylori* infections requires a multi-drug treatment that may involve taking up to 20 tablets per day for 2 weeks, problems with non-compliance to treatment regimen and side effects due to drug intake are common. This makes preventing *H. pylori* infections a very attractive option.

As of today, extensive research is being conducted to develop an effective vaccine against *H. pylori* (reviewed in (4, 159). *H. pylori* infection results in strong but not protective inflammatory response, mainly characterized by the presence of Th1 cells at the site of inflammation. It is currently believed that an effective vaccine would induce both a humoral and Th2 cell mediated immune response (159). Initial research focused on administration of whole bacterial sonicates or individual *H. pylori* proteins to obtain protective effects towards *H. pylori* infection in animals. Whole cell sonicates of *H. felis* was orally administered to mice and this induced IgG and IgA antibodies against *H. felis* antigens. Studies with purified proteins of *H. pylori* including UreA and UreB subunits of urease, GroES and GroEL heat shock proteins, neutrophil activating protein-NapA, CagA, VacA, catalase, and several other proteins have been attempted (142, 143, 172, 281, 304). While results have been promising, there are some areas of concern. *H. pylori* does not naturally infect mice and most of these studies have to be done with specific strains that are effective mouse colonizers. Also, the studies cannot proceed for a long

duration due to the short lifespan of the mice and lastly mice are vastly different from human beings. When attempted in humans, adjuvant related side effects have been observed and the trial vaccines proved to be only moderately efficient in conferring protection against *H. pylori* infection and in reducing *H. pylori* load in infected people.

DNA vaccines where fragments of either *kat* or *ureB* or *groES* have been in cloned into plasmids and introduced as naked plasmids or via attenuated carrier organisms such as *Salmonella* have been studied. While these vaccines were successful in being immunogenic (eliciting both humoral and cell mediated immunity), studies to check if these vaccines confer protection against *H. pylori* infections are lacking (115, 206, 315, 327). *H. pylori* UreA and UreB were expressed in different *Salmonella typhi* strains and were injected into mice and such a vaccine was successful in reducing *H. pylori* colonization in the stomach. Bacterial ghosts are obtained by treating Gram-negative bacteria with protein E from bacteriophage PhiX174. This causes leakage of cytoplasmic contents while leaving the surface antigenic structures intact. When such a bacterial ghost membrane for *H. pylori* was tried as a candidate vaccine and administered with an adjuvant (cholera toxin), complete protection of mice against *H. pylori* was observed. Vaccines with biodegradable microspheres containing *H. pylori* antigens are also being studied. There still is a long way to go in the development of prophylactic or therapeutic vaccines against *H. pylori*.

MICROBIOLOGY OF *HELICOBACTER HEPATICUS*

Helicobacter hepaticus was discovered in 1992 at the National Cancer Institute-Frederick Cancer Research and Development Center, when a high incidence of hepatocellular tumors were reported in A/JCr mice mice (305). Numerous rats and mice in this facility exhibited chronic active hepatitis and this was associated with the incidence of hepatocellular

carcinoma. Upon histological examination of infected hepatic tissues with Steiner's modification of Warthin-Starry stain and by electron microscopy, a helical organism with single bipolar flagella was identified. Further molecular analysis by 16 S rRNA studies lead to the classification of this microorganism as *Helicobacter hepaticus* (305).

H. hepaticus proved to be a valuable tool as it persistently colonizes the liver tissues and causes hepatitis and was associated with tumor development in those tissues. Hence, this proved to be a natural model to study and understand the effects of long term, persistent bacterial infections and their relationship with bacterium-associated carcinogenesis. Moreover, the similarity of *H. hepaticus* to *H. pylori* in causing hepatitis and hepatocellular carcinomas much like *H. pylori* associated gastritis and gastric adenocarcinomas made it a preferred model system for exploring the relationship between bacterium induced inflammation and cancers.

Morphology and Physiology

Shape and flagella- *H. hepaticus* cells are curved or spiral (with one or two turns) in shape. They are between 1.5- 5 μM in length and are about 0.2-0.3 μM in width. They are motile by single bipolar, sheathed flagella. They lack the periplasmic fibers that are present in other enterohepatic helicobacters. While the curved shape of *H. pylori* and the flagella of *H. pylori* were shown to be essential for gastric colonization (discussed in an earlier section), not much is known about the role of flagella or the role of spiral shape of *H. hepaticus* in colonization. A recent single study has shown that a mutant lacking the *fliA* gene (σ^{28}, the late flagella gene regulator) or a double mutant lacking both copies of the *flaA* genes did not produce detectable flagella (or produced truncated flagella) and were non-motile or not able to colonize mice, suggesting that motility and flagella are important for hepatic colonization of mice by *H. hepaticus* (278).

Genome properties- Much of the knowledge about the physiology of *H. hepaticus* is from the work done during initial identification and characterization of this bacterium and from speculation based on genome analysis. A complete genome sequence for one of the strains *H. hepaticus* ATCC strain 51449 is available (280). The size of the completed genome is 1.7 Mbp, which is slightly larger than the 1.6 Mbp genome of *H. pylori*. *H. hepaticus* genome is predicted to encode 1,875 genes. Around 950 genes show strong sequence similarities to genes in *H. pylori* and *C. jejuni* respectively while around 800 genes are common to all three organisms. *H. hepaticus* has about 489 species specific unique genes. The genome sequence shows the presence of only 1 copy of 5 S, 16 S and 23 S rRNA genes. The genome shows a G+C content of approximately 35.9%. This bacterium produces catalase and urease. It is able to reduce nitrate (an important feature used for differentiation between *H. pylori* and *H. hepaticus*).

O_2 requirements and respiration- *H. hepaticus* can grow microaerobically and anaerobically. Unlike *H. pylori*, *H. hepaticus* has stringent oxygen requirements and cannot grow under higher pO_2. Growth experiments with wild-type *H. hepaticus* strain 51449 have shown that the organism grows best under 1% pO_2 while showing reduced growth at 3% and 6% pO_2 (129). Anaerobic growth has been reported in a couple of studies (84, 178). Analysis of the respiratory chain indicates the presence of NDH-1 or NDH-2 dehydrogenase and a cytochrome *bd* or a cytochrome *cbb* $_3$ terminal oxidase, suggesting that *H. hepaticus* has a more versatile respiratory chain than *H. pylori* (has only NDH-1 dehydrogenase and cytochrome *cbb_3* terminal oxidase). The citric acid cycle is quite similar to that of *H. pylori* except that *H. hepaticus* lacks the succinyl-CoA acetoacetyl-CoA transferase but contains the malate dehydrogenase (280).

Virulence and pathogenesis, disease, implications for humans

H. hepaticus seems to lack many virulence factors like the VacA, all but three genes of the CagPAI of *H. pylori,* and most of the outer membrane proteins such as BabA, SabA, OipA, AlpAB, and HopZ that are found in *H. pylori*. However, *H. hepaticus* does contain a cytolethal distending toxin (CDT) that is very similar to the multi-subunit CDT produced by *Campylobacter jejuni* and many other bacteria. *H. hepaticusu* also possesses three proteins that are similar to proteins shown to be involved in adhesion in *C. jejuni.* CDT induces cell cycle arrest, cell distention, chromatin fragmentation and eventually apoptosis in host cells. One subunit of the CDT, CdtB, is thought to induce type I DNAse activity, also contributing to the cellular damage (161). It is speculated that due to the DNA and chromatin damage caused by CDT, CDT could play a role in the development of carcinomas in a long term, persistent infection. *H. hepaticus* possesses 11 genes that show faint homology to genes belonging to a family of *H. pylori* OMPs (described earlier), some of which are important adhesins required for attachment *H. pylori* to gastric epithelial cells. Homology studies do not allow speculations about their roles in *H. hepaticus*, although phylogenetic analysis has indicated that five of these outer membrane proteins cluster with *E. coli* porins, suggesting that these OMPs could function as porins in *H. hepaticus.*

Genome analysis had predicted the presence of a candidate pathogenicity island (HHGI1), characterized by a G+C content that differed from the surrounding regions, and a prophage 4-like integrase gene. This genetic island had three genes that showed minimal homology and identity to genes involved in virulence/genes of unknown functions in *Vibrio cholerae, Legionella pneumophila, Agrobacterium tumefaciens and Yersinia pestis.*

Liver cancers and tumors of the lower bowel are the third and fourth causes of human cancer related deaths after lung and stomach cancers. *H. hepaticus* causes hepatitis, liver tumors, cholesterol gallstones, inflammatory bowel disease, and colon cancer in susceptible strains of mice (31, 216, 279, 286). In *H hepaticus*-infected A/JCr mice, two putative tumor markers were increasingly upregulated over time in mice with progressive hepatocellular dysplasia. One factor, H19 is known to activate and regulate insulin-like growth factor 2, an anti-apoptotic factor associated with hepatocarcinogenesis. Secondly, acute-phase proteins, such as lipocalin 2, were upregulated in the liver as a result of *H hepaticus* infection. An increased proliferation of liver cells associated with *H hepaticus* infection has been noted in mice, and is consistent with the observation of an increased risk of HCC. As in the case of *H. pylori*, multiple factors such as- the strain causing infection, the strain of mice used for studies, age and sex of the mice, and host immunological factors, determine the outcome of the infection.

Helicobacter spp. DNA has been detected in many patients with liver disorders such as patients with chronic cholecystitis, liver metastasis, cholelthiasis, liver cirrhosis with and without hepatocellular carcinomas, and other disorders (reviewed in (240). Of the ten studies reviewed, nine studies indicated that they were able to detect 16 S rDNA gene sequences that were specific to *H. pylori*, *H. pullorum* or *H. pylori*-like organisms. While DNA has been isolated, no organism has been cultivated from liver tissues as yet (240). Also, the segments of DNA recovered from liver tissues exhibit polymorphisms at specific locations (at positions 92 and 130), suggesting that the DNA isolated could belong to a new species of the genus *Helicobacter* that is very closely related to *H. pylori* yet possesses different properties such as bile resistance, and ability to colonize the liver. Other studies have identified 16 S rDNA of *H. pylori* in liver tissues while the patient is serologically negative for *H. pylori* antigens (40). Such results are

confounding and more studies are necessary to confirm the association of *Helicobacter* with human liver disorders.

Should a positive association between *Helicobacter sp.* and liver disease and hepatocellular carcinomas be established, it would revolutionize the treatment choices made by physicians to treat such conditions. Viable and cultivable *Helicobacters* have not been isolated from liver specimens and there are currently no studies to indicate if eradication of *Helicobacter* will lead to a better prognosis in patients with *Helicobacter* associated liver disorders. Such studies, along with an understanding of the mechanisms by which a chronic infection progresses to a malignancy will be invaluable in managing and treating *Helicobacter* associated liver conditions in humans. Such studies might lead to early detection, cost effective treatment options and reduction in number of major surgeries such as liver transplants.

NICKEL METABOLISM IN *HELICOBACTER PYLORI* AND *HELICOBACTER HEPATICUS*

Role of Urease and Hydrogenase

As mentioned earlier, the enzyme urease is required for *H. pylori* to colonize the human stomach as it is important for enduring the acidic pH. The ammonia that results from the urease activity is thought to create a region of a higher (more alkaline) pH immediately around the bacterium allowing *H. pylori* to survive the acidity. In fact, urease negative mutants of *H. pylori* are unable to colonize the gastric mucosa of Cynomolgus monkeys or nude mice in various studies (80, 288, 289). *H. hepaticus* is an enterohepatic pathogen and the role of urease in *H. hepaticus* was unclear for a long time. It was speculated that urease was required for survival while passing through the stomach as in the case of urease positive *Yersinia enterocolitica* (54). Urease was thought to be essential for production of ammonia as ammonia could be used as a

potential nitrogen source for protein biosynthesis (91). Ammonia produced damages host cells and urease by itself could trigger host immune response (stimulates phagocyte chemotaxis, activates immune cells, and induces cytokine production**)** (34, 68). A recent study has indicated that *H. hepaticus* urease is essential for liver colonization but not for cecal colonization. Urease negative *H. hepaticus* mutant DNA was not recovered from mice livers while the wild-type parent's DNA was recovered from liver tissues. Absence of *H. hepaticus* DNA correlated with less severe hepatitis and lower levels of pro-inflammatory cytokines such as INF-gamma and TNF-alpha. The wild-type parent also developed higher levels of total IgG compared to the urease mutant suggesting urease of *H. hepaticus* does play an important role in colonizing the liver and in hepatic disease (93).

Research in our lab has shown that *H. pylori* strain SS1 lacking the up-take type hydrogenase (*hyd)* is deficient in colonizing mice (233). The intestinal microorganisms of both humans and rodents produce H_2 as a byproduct of colonic fermentation. It was theorized that this H_2 could be used as a respiratory substrate to obtain energy for persistence in the host. *H. pylori* hydrogenase expression and activity is constitutive and was found to increase as a function of externally supplied hydrogen (27, 167). Since the mice stomach contain 20-fold higher hydrogen than the affinity of hydrogenase for hydrogen (233), it is thought that hydrogen would make an ideal, available source of energy for *H. pylori* living in a nutrient deficient gastric mucosal layer.

A hydrogenase mutant and the wild-type strains of *H. hepaticus* were similar in colonizing the mice but mice livers infected with *H. hepaticus* hydrogenase mutant (hyaB) did not exhibit any histology (lobular inflammation or necrosis) even after prolonged colonization (21 weeks) while the livers of mice infected with the corresponding wild-type parent strain did (197). The wild-type strain had increased rates of H_2 dependent ^{14}C- labeled amino acid uptake

than the mutant, though they had (197). The results suggest that hydrogenase might play a role in causing a disease in the host and long term colonization and development of cancers.

Urease and hydrogenase accessory proteins

The accessory system of proteins involved in the maturation of urease and hydrogenase have been well characterized in both *H. pylori* and *H. hepaticus*. Briefly, *H. pylori* possesses an operon of *ureIEFHG* downstream of the *ureAB* genes. Each of the gene products of the operon is essential for urease activity as loss any of those components results in diminished or no urease activity when expressed in *E. coli* (300). *ureI* encodes a proton-gated urea channel that interacts with urease and was shown to be essential for colonization in a mouse or a gerbil model (251, 252, 269). UreEFGH are similar to the *K. aerogenes* urease accessory proteins discussed earlier except that UreH is homologous to the UreD of *K. aerogenes.* UreE is the Ni binding protein that binds 1 Ni ion per dimer and lacks the his-rich C-terminal tail (22), unlike the *K. aerogenes* UreE with a C-terminal that can bind up to 6 atoms of Ni . UreH is thought to be a chaperone that is required for causing conformational changes to the apo-urease complex that facilitates future steps, based on the function of its homolog in *K. aerogenes* (237). UreG is the GTPase whose activity is essential for carbamylation for the conserved lysine residue and Ni- insertion (195). Recent studies have also shown interactions between UreE and UreG, between UreF and UreH. Of the four accessory proteins, UreE and UreG seem to play critical roles as even addition of supplemental Ni cannot restore urease activities to wild-type level urease activity levels (300). The *H. hepaticus* genes involved in urease maturation are the *ureIEFGH* present downstream of the *ureAB* in the same operon (24). UreEFGH seem identical in their roles and functions to the *H. pylori* UreEFGH system (24). As in *H. pylori*, urease activity could not be restored in the *ureE* or *ureG* mutants even upon addition of excess Ni (24).

The hydrogenase accessory system of proteins HypABCDEF has been very well characterized in *E. coli* and has been discussed in an earlier section. Some differences to be noted are that unlike the *E. coli hypABCDEF* operon, the genes encoding the *hyp* genes are scattered though the genome (285). The most interesting feature of the hydrogenase accessory protein system in *H. pylori* is the involvement of HypA and HypB in urease maturation (234). *H. pylori* HypA and HypB mutants were deficient in urease activities that could be restored by addition of supplemental Ni. Urease expression was not affected in these strains and urease from the HypA and HypB strains had lesser Ni associated with urease (234). *H. hepaticus* hydrogenase accessory protein system is quite similar to that of *H. pylori*. Again, HypA and HypB are required for full urease activity in *H. hepaticus.* While addition of excess Ni (5μM) could restore urease activities in *H. pylori hypA* and *hypB* mutants (235), addition of even 50μM Ni could not restore *H. hepaticus* urease activities and could restore only 2% of the hydrogenase activity (24). These results suggest that HypA and HypB of *H. hepaticus* likely play slightly different roles than in *H. pylori (24).*

Nickel import and efflux systems

H. pylori has a well characterized high affinity inner membrane Ni-specific permease. NixA belongs to the NiCo family of transporters discussed earlier and is similar to HoxN of *Cupriavidus necator* H16. NixA when expressed in *E. coli* increased Ni accumulation and was essential for urease activity in *E. coli* in the absence of supplemental Ni (207). However, mutation of *nixA* in *H. pylori* reduced urease activity only 42% suggesting that *H. pylori* had other redundant systems for Ni import (17). As expected, a *nixA* mutant showed lower levels of *in vivo* colonization in certain strains of mice and was outcompeted by the wild-type parent during colonization experiments (224). *nixA* transcription is repressed by NikR regulator under

high Ni conditions to ward off the toxic effects of Ni (20, 311). *H. pylori* genome analysis has indicated the presence of some components of another Ni transporter of the ABC type ATP binding cassette Ni transporter. The NikABCDE system has been well studied in *E. coli*. Studies conducted in 1995 indicated the presence of NikD homolog, and a homolog to the abcC component of abc-type transporters. Allelic exchange mutagenesis in these genes reduced urease activities (119). There has been no further characterization or mention of these putative Ni transporters in the literature (119).

Recent studies have identified novel outer membrane receptors for Ni (FecB3 and FrpB4) that are also regulated by NikR (53, 75). Interestingly, active transport of Ni by FrpB4 is energized by the TonB/ExbB/ExbD machinery that usually transports only iron or cobalamine compounds (264). In fact, it was shown that at lower pH's this Ton system in *H. pylori* is committed to importing Ni (264). *H. pylori* has the RND (czc) type CznABC that are involved in Ni efflux from the bacteria. This Ni efflux system was essential for colonization as mutants lacking these genes could not colonize a Mongolian gerbil model system (274).

While Ni metabolism has been extensively studied in *H. pylori*, it remains a relatively unexplored area in *H. hepaticus.* Genome analysis indicates that *H. hepaticus* produces urease and hydrogenase and the accessory proteins required for their maturation (discussed earlier). Conspicuously, *H. hepaticus* lacks any homologues to the outer membrane receptors FecB3 and FrpB4. Although, *H. hepaticus* possesses a TonB/ExbB/ExbD system (HH_0353-0355) that is thought to energize FrpB4 mediated Ni transport in *H. pylori. H. hepaticus* does have a homologue to the Ni efflux pump CznABC (HH_0625–0623) (Table 1.2). The predominant Ni transport system is homologous to the ATP binding cassette (ABC) type transporter NikABCDE system of *E. coli* rather than the NixA of *H. pylori. H. hepaticus* 'Nik' system consists of only

NikABDE (HH_0417-0414), lacking NikC that forms a Ni channel along with NikB in *E. coli*. *H. hepaticus* does posses an outer membrane receptor homologue HH_0418, the role of which has not been studied yet. Whether these proteins are functional and the functions and features are yet to be studied.

Nickel dependent regulator, NikR

H. pylori possesses a Ni dependent regulator NikR, that is 30% identical and 68% similar to the *E. coli* NikR (Table 1.2). NikR is a key player in maintaining Ni homeostasis in *H. pylori.* NikR of *H. pylori* has been extensively studied and its structure, DNA binding motifs, and Ni binding properties have been determined (2, 65, 77, 323, 324). Currently, there is no consensus related to the number of Ni ions that are bound by *H. pylori* NikR or the number of sites that are involved in Ni^{2+} binding in NikR. Crystal structures show up to six Ni^{2+} ions bound by NikR while isothermal titrations and electronic absorption spectroscopy reveal only four Ni^{2+} bound by NikR.(2, 65, 323). One study finds that four Ni ions bind in a sequential manner, two at a time to two sets of high affinity sites. They find that unlike in *E. coli*, Ni binding to the lower affinity site, does not specifically increase or change the affinity or the specificity of NikR to DNA (324). Another interesting feature is that *H. pylori* NikR has an additional N-terminal arm composed of some charged amino acids that is absent in *E. coli* NikR (20).

While *E. coli* NikR only regulates the Ni transport operon *nikABCDE*, *H. pylori* NikR has been shown to activate and represses numerous genes involved in Ni metabolism as well as in other functions. NikR regulated genes include FecA3, FrpB4, Hpn and Hpn-like proteins (discussed in the next section), urease, NixA, Fur, the TonB/ExbB/ExbD system, to name a few (45, 53, 74, 75). Hence, in *H. pylori*, NikR is thought to be a global regulator. Evidence for NikR

induction of urease in acidic conditions further increases the number of metabolic functions of NikR involvement (295).

H. hepaticus has a NikR homologue, HH_0352 (280). Nickel metabolism has not been investigated much in *H. hepaticus.* Interestingly, while urease expression is acid induced and induced by NikR in *H. pylori* in the presence of excess Ni, either acid dependent or Ni dependent increase in urease apo-protein or transcript was not observed in *H. hepaticus* (18). Futhermore, iron restriction in the growth medium, increases urease activity and expression of urease (19). Also by DNA protection assays and gel retardation assays it has been shown that Fur binds upstream of urease in *H. hepaticus* and represses urease in a metal dependent manner (19). These results suggest that regulation of Ni metabolism including the function of NikR could be unique in *H. hepaticus.*

Other nickel binding proteins

Other than these outer membrane proteins, *H. pylori* possesses a Ni binding protein HspA (Table 1.2). The N-terminal domain of HspA is homologous to GroES protein chaperone homologues of other bacteria while the C-terminal region is rich in histidine residues and binds Ni. When expressed in *E. coli*, HspA increased plasmid based urease activity four- fold (143, 144)

Other than the Ni binding proteins described earlier, *H. pylori* also possesses two other unique proteins named Hpn and Hpn-like (Table 1.2). These are two small proteins that are extremely rich in histidine residues, and homology searches and sequence analysis find hits only with varied proteins that have short stretches rich in histidine residues, but those proteins have diverse functions and do not allow speculation of the roles of these proteins other than their probable involvement in Ni metabolism (Table 1.1). The TIGR website annotates the Hpn

protein (HP1427, JHP1320) and the Hpn-like protein (HP1432, JHP1321) as the "histidine rich, metal binding polypeptide" and "the histidine and glutamine rich protein", respectively. As their annotations imply, Hpn is rich in histidine residues (28 of 61 amino acids, 46%) and the Hpn-like is rich in histidine (18 of 73 amino acids, 25%) and glutamine residues (30 of 73, 41%). Homology searches indicate that the Hpn protein is unique to *H. pylori* and a few other species in the *Helicobacter* genus (97, 210).

Hpn and Hpn-like proteins both possess 6 to 7 consecutive histidine residues. The Hpn protein is similar to the HypB protein from *Bradyrhizobium japonicum* which is a metal binding GTPase that binds Ni at its histidine residues and is required for the Ni dependent regulation of hydrogenase (210, 231) and UreE, a urease accessory protein rich in histidine residues has Ni sequestering ability that is key to urease maturation (22). Histidine residues have high affinity for Ni ions and are also capable of binding other divalent cations. It has been shown that Hpn protein binds Ni strongly and a *H. pylori* strain lacking Hpn is more susceptible than the wild type to Ni toxicity (97, 210). The Hpn-like protein, named so due to its similarity to the Hpn, is also thought to bind Ni ions (45, 83). Yet, the Hpn-like protein is also rich in glutamine residues (41% of the amino acids are glutamines) and therefore maybe involved in binding of other divalent metal ions or might have a different function too. NikR mediated transcription of *hpn* and *hpn-like* genes is upregulated in the presence of excess Ni (45). Recent genetic profiling under acid stress conditions (pH 5 vs. pH 7) revealed that the Hpn-like is upregulated at pH 5 (36). Transcription of *hpn-like* is controlled by a histidine kinase HP165, which perceives a pH stimulus (83, 245).

Biophysical data characterizing Hpn and Hpn-like in *E. coli* were published in 2006 and 2008, respectively (92, 328). Hpn and Hpn-like were shown to bind Ni with dissociation

constants of 7.1 μM and 3.8 μM respectively. Both proteins exist in multimeric states and Hpn can bind 5 Ni per monomer and Hpn-like 2 Ni ions per monomer. These studies also showed that when expressed in *E. coli*, these proteins conferred a protective effect against higher Ni concentrations. Most interestingly, both Hpn and Hpn-like were able to release Ni in the presence of lower pH or in the presence of chelators such as EDTA.

Hpn and Hpn-like or homologues of the same are absent in *H. hepaticus* (Table 1.2). In fact, Hpn and Hpn-like are present only in a few other gastric helicobacter species. This again indicates that they likely play vital roles, tailored specifically to suit the niche of colonization in gastric helicobacters.

SCOPE OF THIS STUDY

A: Determination of the roles of two unique histidine rich proteins in *H. pylori*

Analysis of the studies described earlier suggested that both Hpn and Hpn-like could play roles in detoxifying excess Ni and could serve as Ni storage reservoirs. But there were no physiological studies that proved the Ni storage or Ni detoxification functions for these proteins in *H. pylori*. My research, discussed in Chapter 2, aimed at the characterization of the roles of Hpn and Hpn-like in *H. pylori.* By studying the phenotype of strains lacking one or both of these genes encoding Hpn or Hpn-like, I was able to determine the dual roles that could be played by these proteins- in excess metal detoxification and Ni storage roles for both Hpn and Hpn-like in *H. pylori. H. pylori* has numerous and often times redundant systems for Ni scavenging, Ni allocation to urease and for maintaining Ni homeostasis (FecB3 and FrpB4 outer membrane Ni receptors; the Ton system utilized for Ni transport, NixA and a putative ABC transport system for inner membrane Ni transport; involvement of HypA and HypB in urease maturation; UreE for Ni binding, presence of a Ni binding chaperone HspA, NikR Ni regulator, CznABC Ni efflux

system). This suggests that maintenance of Ni homeostasis is vital for the survival of this formidable pathogen in its niche. *H. pylori* colonizes the gastric mucus layer (with a pH around 7) of the human host. During initial host colonization and at times when the bacterium enters the gastric lumen from the mucus gel layer, Ni would be required immediately and in large amounts for urease activation and for protection against acidity. The results from my research described in Chapter 2, along with the knowledge of reversible binding of Ni by Hpn and Hpn-like described earlier, strongly prove the functions of both Hpn and Hpn-like as Ni storage reservoirs. My research has also indicated that these proteins could play vital roles in resisting excess metal concentrations by binding and sequestering excess metal ions.

B: Determination of the functions of a NikR homologue in *H. hepaticus*

As discussed in an earlier section, Ni dependent regulators play vital functions in maintaining Ni homeostasis. Genome analysis indicated that *H. hepaticus* lacked some genes involved in Ni metabolism in *H. pylori,* while others such as urease hydrogenase, NikABDE, CznABC and NikR homologs were present.My aim was to determine the role of NikR in *H. hepaticus* by studying the phenotype of a strain lacking the *nikR* homolog (presented in Chapter 3). My specific interest was to analyze the role of NikR in regulating the Ni-enzymes urease and hydrogenase, and the Ni transporter. I have determined that NikR modulates the two Ni enzymes urease and hydrogenase via the Ni transporter NikA. During the characterization of a *nikR* strain, I obtained data that indicate novel functions for NikR in regulating the periplasmic nitrate reductase (NapA) and therefore, possible novel roles in anaerobic respiration. This is the first study characterizing the NikR of *H. hepaticus* and my data indicates functions for NikR other than in maintenance of Ni homeostasis.

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Table 1.1 Nickel binding properties of histidine-rich proteins (328)

Protein	Bacterium	No. of histidines per histidine-rich domain	No. of Ni^{2+} ions per monomer	K_d (μM)	References
HypB	*Bradyrhizobium japonicum*	24/39	9	2.3	[30]
	Rhizobium leguminosarum	17/32	4	2.5	[31]
UreE	*Klebsiella aerogenes*	10/15	3	9.6	[32]
CooJ	*Rhodospirillum rubrum*	16/34	4	4.3	[33]
SlyD	*Escherichia coli*	15/50	3	–	[49]
HspA	*Helicobacter pylori*	8/27	2	2.0	[50, 51]
Hpn	*Helicobacter pylori*	28/60	5	7.1	[18]
HpnI	*Helicobacter pylori*	14/23	2	3.8	This work

Table 1.2: Genes involved in nickel metabolism in sequenced *Helicobacter* spp. (23)

Gene	*Helicobacter* species						Proposed function
	H. pylori			*H. hepaticus*	*H. mustelae*	*H. acinonychis*	
Strain	26695	J99	AG1	51449	43772	Sheeba	
fecA3	1400	1426	1469	-	-	1303	Outer membrane receptor
frpB4	1512	1405	1400	-	-	0072	Outer membrane receptor
hh0418	-	-	-	0418	?	-	Outer membrane receptor
tonB/exbD /exbB	1341–1339	1260–1258	1288–1286	0355–0353	Present	0271–0269	Inner membrane complex
hpn	1427	1320	1352	-	Present	-	Nickel-storage/detoxification
hpn-like	1432	1321	1357	-	-	-	Nickel-storage/detoxification
hspA	0011	0009	0011	-	Present	1697	Nickel-binding chaperone
nikR	1338	1257	1285	0352	Present	0267	Nickel-responsive regulator
nixA	1077	0348	0370	-	Present	1190	Inner membrane Ni transporter
nikABDE	-	-	-	0417–0414	-	-	Nickel ABC transporter
cznABC	0969–0971	0903–0905	0950–0952	0625–0623	Present	1054–1056	Nickel efflux system

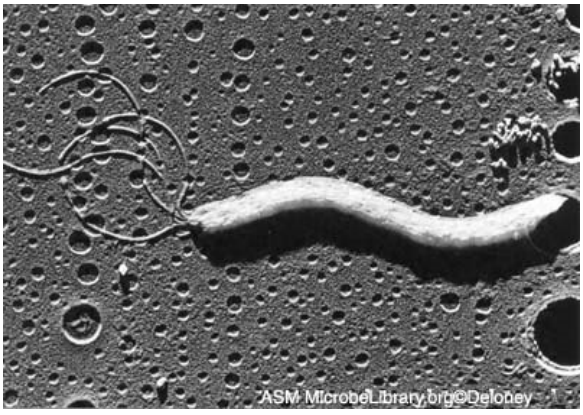

Figure 1.1- Electron Micrograph of *H. pylori*: This electron micrograph of *H. pylori* shows the spiral shape and the presence of five lophotricous flagella

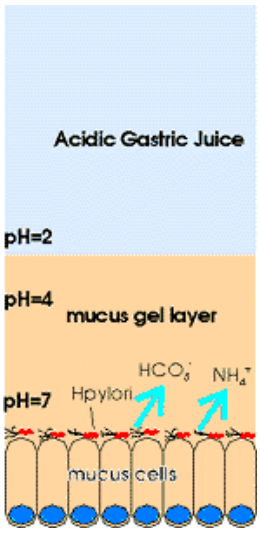

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Figure 1.2 Niche of colonization of *H. pylori*: This is the niche preferred by *H. pylori* for colonization. The stomach is coated with a thick mucus lining that protects the stomach from the gastric juices. *H. pylori* inhabits this mucus gel layer successfully and establishes an infection with the aid of many virulence and colonization factors.

CHAPTER 2

ROLES OF HIS-RICH HPN AND HPN-LIKE PROTEINS IN *HELICOBACTER PYLORI* NICKEL PHYSIOLOGY[1]

[1] **Seshadri, S., S. L. Benoit, and R. J. Maier**. 2007. J Bacteriol. **189**:4120-6
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CHAPTER 2

ROLES OF HIS-RICH HPN AND HPN-LIKE PROTEINS IN *HELICOBACTER PYLORI* NICKEL PHYSIOLOGY

ABSTRACT

Individual gene targeted mutants in *hpn, hpn-like*, and double mutants in both *hpn* genes were more sensitive to nickel, cobalt and cadmium toxicity than the parent strain, with the *hpn-like* strain showing the most metal sensitivity of the two individual his-rich protein mutants. The mutant strains contained up to 8-fold more urease activity than the parent in nickel-deficient conditions, and the parent strain could achieve mutant strain activity levels by nickel supplementation. The mutants contained 3-4 fold more and the double mutant about 10 fold more Ni associated with their total urease pools, even though all the strains expressed similar levels of total urease protein. Hydrogenase activities in the mutants were like those in the parent strain, so hydrogenase is fully activated in the nickel-deficient conditions. The histidine-rich proteins appear to compete with the Ni-dependent urease maturation machinery in low nickel conditions. Upon lowering the pH of the growth medium from 7.3 to 5, the wild type urease activity increased 3-fold but the activity in the three mutant strains was relatively unaffected. This pH affect was attributed to a nickel storage role for the His-rich proteins. In low nickel conditions, addition of a nickel chelator did not significantly affect urease activity of the wild type but decreased activity of all the mutants, supporting a role for the His-rich proteins as Ni reservoirs. These nickel-reservoirs significantly impact active urease activities achieved. The

His-rich proteins play dual roles, as Ni-storage or as metal detoxification proteins, depending on the exogenous nickel levels.

INTRODUCTION

H. pylori is a well studied gram negative, motile, microaerophilic pathogen that colonizes the human gastric mucosa and causes chronic gastritis, duodenal ulcers and certain kinds of gastric cancers (4, 18, 22, 23). *H. pylori* produces the Ni-enzymes urease and hydrogenase (10, 17). Urease accounts for up to 10% of the total cellular protein made and is essential for colonization and virulence (1, 13, 32, 33). The hydrogen utilizing hydrogenase provides the bacterium an alternative respiratory based energy generation mechanism independent of carbon substrates (17). These two enzymes represent the major final sinks for nickel within the cell. Other than the nickel enzymes, *H. pylori* also possesses a NixA nickel specific permease (2), accessory proteins UreEFGH and HypABCDEF (some of which bind nickel) required for proper maturation of the two nickel enzymes (19, 30), a nickel dependent regulator, NikR (6, 8, 35, 36), a recently identified nickel efflux system (CznABC) (28), and a histidine-rich heat shock protein HspA which is a GroES homologue (14).

A protein rich in histidine residues (47% histidine residues, TIGR annotation HP1427) was named 'Hpn' because it was first identified in *H. pylori* and had affinity for nickel ions (12). The presence of Hpn was also noted in the ferret and cat gastric pathogens, *Helicobacter mustelae* and *Helicobacter felis* respectively (12). *H. pylori* mutants lacking *hpn* were less tolerant to nickel and bismuth than the wild type (20). Recent biophysical characterizations showed that Hpn exists mainly as a multimer in solution, with each monomer of 7 kDa reversibly binding 5 nickel ions at pH of 7.4. Nickel is released by a decrease in pH or in the presence of nickel chelating agents such as EDTA (11). The level of nickel in the *H. pylori* strain 26695

cytoplasm was also found to be slightly higher than in an *hpn* deletion mutant (11) and nickel levels in *E. coli* cells expressing *H. pylori* Hpn from an inducible plasmid was higher than in cells lacking the plasmid (11). No difference in the urease activity in the *hpn* mutant compared to the wild type was noted (11, 12).

The *H. pylori* sequence reveals the presence of anotherHis-rich protein termed Hpn-like that is annotated as the histidine and glutamine rich protein (HP1432) (30). Sequence analysis indicates that 25% of the amino acids are histidine residues including a stretch of 6 consecutive histidine residues while 30 of the 72 amino acid residues are glutamine residues (42%). The N-terminal sequence (46 residues) of HP1432 shows 56% identity to Hpn and the protein was thus termed Hpn-like. *H. pylori* 43504 Hpn-like is slightly different from HP1432 and JHP1321. It was sequenced and the GenBank accession number is EF203427.

Both Hpn and Hpn-like are transcriptionally activated in the presence of nickel by the nickel sensor NikR (6). Additionally, Hpn-like was shown to be upregulated at pH 5.0 in comparison to pH 7.0 by the two-component system ArsRS (HP165-HP166) (5, 24, 25). The studies thus far suggest that Hpn may be involved in nickel detoxification and/or nickel storage for its use in nickel deficient times. Our results extend a role for detoxification to Hpn-like protein, and indicate that both proteins play a role in storing nickel that significantly affects urease expression when nickel supplies are highly limited. In low nickel conditions, chelation of extracellular nickel significantly decreased urease activities in the mutant strains but not in the wild type, supporting a nickel storage role for these proteins. Also, the results herein imply that *H. pylori* synthesizes much more (apo)urease than its nickel reserves can satisfy.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Strains used in this study are also indicated in Table 2.1. *H. pylori* ATCC strain 43504 was used as the wild type strain and was also used for mutant strain construction. This strain was used as the parent strain as many nickel metabolism mutant strains are in this genetic background (3, 21). *H. pylori* cells were grown on Brucella agar plates supplemented with 10% defibrinated sheep blood (BA plates) at 37˚ C. The plates were supplemented with either 30 μg ml^{-1} of chloramphenicol or 30 μg ml^{-1} of kanamycin when needed. Cultures were grown in 5% CO_2 incubator with 4% O_2 or were grown in anaerobic jars with anaerobic mix (5% CO_2, 10% H_2, balance N_2).

For liquid cultures, cells were inoculated at an OD_{600} of 0.07 into serum vials containing 20 ml of Brain-Heart infusion broth (BHI broth) supplemented with 5% fetal bovine serum. The bottles were sparged with anaerobic mix for 15 minutes and 5% oxygen was added to the sealed bottles via a sterile needle and syringe. The cultures were grown at 37˚C with shaking at 200 rpm. The pH of the culture medium was set to either 7.3 or 5.0 as desired; after growth the pH was about 6.8 and 5.3, respectively. A nickel free medium was obtained as described earlier by the addition of dimethyl glyoxime (22).

Escherichia coli strain DH5α was grown at 37˚C in Luria Bertani (LB) plates supplemented with no antibiotic, 100 μg ml^{-1} ampicillin, 30 μg ml^{-1} of chloramphenicol, 30 μg ml^{-1} of kanamycin or a combination of these antibiotics as required.

Mutant Construction

The sequence of *hpn* from *H. pylori* strain 43504 is identical to the *hpn* from genome sequenced strain *H. pylori* 26695. For *hpn-like,* the sequence differs slightly as indicated in Table

2.1. An approximately 1.2 kb region containing the *hpn* (183 bp) or the *hpn-like* gene (214 bp) and 500 bp flanking region on either side was amplified by PCR using the primer pairs described in Table 2.1 and cloned into the pGEM-T vector to give plasmid pGEM-*hpn* or pGEM-*hpn-like* respectively. A kanamycin resistance *aphA3* cassette was introduced into the *Xcm*I site of the *hpn* gene and a chloramphenicol acetyl transferase (*cat)* cassette was inserted into the *Blp*I site within the *hpn-like* gene. The plasmids containing the disrupted genes were introduced into *H. pylori* by natural transformation to promote allelic exchange via homologous recombination and antibiotic resistance was used to select colonies that contained the disrupted genes. The insertion of the cassette in each gene was confirmed by PCR using DNA from each mutant as template (data not shown). The double mutant was obtained by introducing the pGEM-*hpn-like: cm*^{R} plasmid into the *H. pylori hpn* mutant background; colonies resistant to both the antibiotic markers were isolated and the presence of the antibiotic cassette in both the loci was confirmed by PCR (data not shown).

Metal toxicity

A starting cell suspension of ~ 5 x 10^8 cells/ml of each strain was suspended in sterile PBS and was incubated with either1 mM nickel chloride ($NiCl_2$), 20 μM cobalt chloride ($CoCl_2$), 10 μM cadmium chloride ($CdCl_2$) or without any metal. The tubes were sealed with stoppers and sparged with anaerobic mix (5% CO_2, 10% H_2, balance N_2) by use of inflow and outflow needles; oxygen was then added from a 100% O_2 tank via syringe to a final 4% partial pressure. The sealed test tubes were incubated at 37˚C and dilutions were plated from the suspensions at regular intervals over a 12 h period onto BA agar plates.

Urease activities

Cell extracts from two day old cultures grown on BA agar plates or on BA agar plates with the indicated nickel supplementation were used. If urease activities of liquid cultures were assessed, 24 hour old cultures were centrifuged at 7,000 x *g* and urease assays were carried out as described. Briefly, cells were washed and resuspended in 50mM HEPES buffer, pH 7.5. Supernatants from cells broken by two passages through a French pressure cell (12,000 lb/in^2) were incubated for 20 minutes with the same buffer containing 25 mM urea, and the ammonia evolved was measured via a phenol hypochlorite assay as described (38). A standard ammonium chloride concentration curve was used to convert the absorbance at 625 nm to nmoles of ammonia.

Urease immunoblots

Crude cell extracts were treated with SDS buffer, boiled for 5 min and subjected to SDS-PAGE according to the method of Laemmli (16). Separated proteins were transferred electrophoretically onto a nitrocellulose membrane as described previously (31). Immunoblotting was carried out as described earlier with the only difference being that Anti-UreA antibody was used as the primary antibody at a dilution of 1: 1000 (19).

Nickel content in urease fractions

Urease was purified as described earlier (21) with the exception that YM10 centricons (Millipore, Bedford, MA) were used to concentrate the urease fractions and to exchange the high salt in the elution buffer for a lower salt content. Purification was carried out using identical protocols for each strain and the elution profiles obtained for each strain were identical (data not shown). The peak fractions were run on SDS-PAGE gels; the UreA and UreB bands were the most prominent bands from all strains and were judged to represent at least 95% of the total

protein (data not shown). Equal protein amounts of each fraction were subjected to graphite furnace atomic absorption spectrophotometry and the nickel content of the various samples calculated as described earlier (21). Briefly, this involved creating a standard curve using various known nickel concentrations (0-0.5 μM nickel) and measuring the nickel content of the various urease containing samples within this standard curve. Where nickel was added in the growth medium, relatively high but not toxic levels (500 μM) were used, to attempt to obtain the greatest amount of Ni- activated enzyme. Levels as high as 2 mM have been previously used to study *H. pylori* Ni physiology (20).

RESULTS

Mutant Construction

To explore the roles of the *hpn* and *hpn-like* genes, we created individual gene-disruption mutants in *H. pylori* ATCC strain 43504. Since *hpn* and *hpn-like* may have overlapping roles in the cell, and the presence of one of the genes might mask the effect of disrupting the other, we also created a double mutant deficient in both the *hpn* genes. The growth rate of the three mutants were determined in BHI medium supplemented with 5% FBS by monitoring the absorbance at 600nm over a 24 hour period. No difference in the growth rates were observed between the mutants and the parent, indicating that the disruption of the *hpn* genes did not affect products that are essential for growth (Figure 2.5).

Reduced metal tolerance in the mutant strains

In order to examine the possible roles of *hpn* and *hpn-like* in nickel detoxification, we determined the survival ability of the *hpn, hpn-like* and *hpn/hpn-like* double mutant strains in the presence of added nickel in comparison to the wild type *H. pylori*. This was accomplished by suspending equal number of cells in non-growing conditions with excess nickel (phosphate

buffered saline (PBS) with 1 mM $NiCl_2$). Samples were taken at regular intervals, serially diluted, and plated onto BA agar plates. The colony counts were then determined and the results are expressed as Log_{10} cfu ml^{-1} (see Figure 2.2). The results show that the nickel tolerance of the *hpn* mutant is less than wild type. Decreased tolerance of the *hpn* mutant to nickel was also reported by Mobley *et al.* (20). We observed a greater Ni-dependent viability loss for the *hpn-like* mutant and the double mutant. The effects could be connected to nickel as all four strains showed similar survival rates (in PBS) in the absence of nickel stress (see Figure 2.1). Also, a chloride ion effect was ruled out as $CaCl_2$ (1 mM) did not cause a viability loss of the strains (data not shown). The nickel stress experiment suggests that along with Hpn, Hpn-like also has a role in nickel detoxification in *H. pylori.*

To determine if Hpn and Hpn-like proteins play a role in other divalent metal ion tolerance, similar experiments were carried out with zinc (data not shown), cadmium and cobalt (see Figures 2.3 and 2.4, respectively). As for nickel, loss of viability was observed for the Hpn, Hpn-like and the double mutant in the presence of cobalt and cadmium but not when exposed to excess zinc. Hpn-like appears to play the larger role in metal resistance at least for cadmium and nickel. Nevertheless, both Hpn and Hpn-like play a role in nickel, cobalt and cadmium detoxification.

Urease activities are higher in the mutant strains

To assess whether mutations in the putative nickel detoxification/nickel storage proteins have an effect on the major nickel sink in the cell (each UreB subunit can bind 2 Ni^{+} ions), urease activity of the mutant strains was compared to the wild type (see Figure 2.6). After growth in Ni-unsupplemented medium, urease activity was increased over wild type levels by at least eight fold in the *hpn* mutant, three fold in the *hpn-like* mutant, and more than eight fold in the

hpn/hpn-like double mutant strain. Upon 1 μM nickel supplementation, urease activities of the mutants increased slightly, whereas the urease activities of the wild type strain increased about four fold by 1 μM or 5 μM nickel supplementation (compared to unsupplemented conditions, see Figure 2.7). Supplemental nickel commonly induces *H. pylori* urease expression (35, 36). The decreased activity in the *hpn* mutant at 5 μM compared to the 1 μM is likely due to the increased susceptibility of this strain to nickel toxicity.

As a nickel storage role is ascribed to these His-rich proteins, we presumed the gene disruption may display a urease deficient phenotype. Thus, the observed urease activity results were not expected. The reason for higher urease activities in mutants could be due to the availability of a larger cytoplasmic nickel pool for the urease maturation enzymes; this would create a higher amount of active urease species in comparison to the (nickel deficient) wild type strain. Alternatively, the increase in urease activities could be due to Hpn and Hpn-like affecting (increasing) urease transcription. These possibilities were explored by comparing urease protein levels and nickel levels in the urease fractions from various strains.

Hydrogenase activity is not affected in the mutant strains

Hydrogenase is the other nickel containing enzyme in *H. pylori* (17). To assess whether mutations in *hpn* and *hpn-like* genes were affecting the hydrogenase activities in the cell, hydrogenase assays were performed amperometrically by detecting the hydrogen uptake activities in whole cells as previously described (17). Significant differences in hydrogenase activities were not observed in the various mutant strains as compared to the wild type strain (data not shown). Hydrogenase is a minor Ni- sink compared to urease as it is made in much lesser amounts and it also contains much less nickel per enzyme molecule than urease. Also, the two Ni-enzyme sinks (hydrogenase and urease) contain different accessory maturation proteins;

these would be expected to affect nickel allocation between the two Ni-enzymes based on their individual affinities for the metal. The absence of a hydrogenase phenotype affect by loss of the nickel sequestering proteins (i.e. in the mutants) likely means that hydrogenase is fully nickel activated even in low nickel conditions in all the strains.

Urease protein levels are similar in all the strains

To explore whether the increase in urease activities in the mutant strains was due to a corresponding increase in the amount of urease produced, immunoblotting was done as previously described (19). Anti-UreA antiserum was used to immunoblot crude extracts from wild type, *hpn*, *hpn-like* and the double mutant strain (see Figure 2.8). From the results, it was determined that the amount of urease protein made by the mutants is not greater than that made by the parent strain. This suggested that the differences in urease activities were not due to differences in urease protein synthesis.

Nickel in urease fractions is higher in mutant strains

Urease activities in the mutant strains were higher than for the wild type, yet there was not an increase in the amount of urease protein made in the cell. It was possible the urease activity differences could be accounted for by the amount of nickel associated with the urease pool in the strains. If this was true, the amount of nickel bound per unit of urease protein would be higher in the mutants in comparison to the wild type. Indeed, the urease fractions from the two single mutant strains showed a three-fold to almost four-fold greater nickel content per mg urease protein. The amount of nickel measured in our studies for the wild type *H. pylori* is 32 ng /mg protein (see Table 2.2), which is very close to the nickel content reported for wild type *H. pylori* in a previous study from our lab (21). In a separate experiment (see Table 2.2) the double mutant contained almost 10-fold more Ni per mg urease protein than the parent. A similar result

(higher nickel content in the urease of mutant strains) was observed from experiments performed by adding radioactive ^{63}Ni to the liquid growth medium (Muller Hinton Broth with 5% fetal bovine serum in sealed serum vials); the vials were incubated in an atmosphere composed of 4% O_2, 5% CO_2, 10% H_2 (and balance N_2) and the counts per minute compared among the strains by scintillation spectrometry of the partially purified urease fractions (data not shown).

Nickel content in urease fractions from wild type *H. pylori* cells grown with 500 μM nickel supplementation (see materials and method) was found to be about three fold greater than the nickel content of urease from cells grown on the regular (not Ni-supplemented) growth medium. This indicates that without supplementation the wild type synthesizes more apo-urease than its nickel pools can satisfy. It has been proposed previously that in Ni-deficient times, only a minor percentage of *H. pylori* urease is Ni-activated (29) and that nickel can be a factor sometimes limiting urease activity rather than activity being limited by the amount of urease protein (35). It was previously observed that addition of 1 μM nickel to the growth medium increased the urease activities but did not cause an increase in urease expression levels (36) and disrupting *H. pylori* genes encoding a metal efflux pump (*cznABC)* led to an increase in urease activity (28), presumably by making cytosolic nickel available.

Effect of His-rich proteins on urease activities as a function of pH

It is known that Hpn releases nickel upon lowering the pH (11). Therefore, upon lowering the pH of the medium, one may expect to see an increase in urease activities in the wild type strain (due to availability of nickel) but no or a lesser affect on urease in the *hpn* or *hpn-like* strains. Figure 2.9 shows the wild type urease was 3-fold greater at pH 5 than at pH 7.3, whereas the strains lacking *hpn*, *hpn-like* or both genes did not have increased activity at the preferred pH for Ni accessibility. The greater absolute activities here (compared to the Figure 2.6 data) are

attributed to the use of broth cultures. The pH experiment shown (Fig 2.9) was also conducted in BHI medium with 50 mM 3-(N-Morpholino]propanesulfonic acid and 50 mM potassium acetate to closely maintain the desired pH. The pH of this well buffered medium after growth had changed only slightly (pH 5 to 5.08 ± 0.08 and pH 7.3 to 7.18 ± 0.12, based on 8 measurements at each pH). The results were similar to those in (Fig 3) but all activities were lower than the activities from the regular BHI medium.

Chelator effects

Dimethyl glyoxime is a chelator with high affinity for nickel. The chelator had little effect on urease activity achieved in the wild type (Figure 2.9). This was also true for the wild type when the chelator was added at pH 5.0. However, the mutant strains activities were significantly affected by the chelator (Figure 2.9). These results support a role for Hpn and Hpn-like in nickel storage, as the mutant strains Ni-urease activities were much more dependent on exogenous (available) nickel than the wild type.

DISCUSSION

Previous studies on *H. pylori hpn* deletion mutants reported there was no change in the urease activities compared to the parent strains (11, 12). However, even slightly higher amounts of available nickel in the complex growth medium (that also contain Ni-sequestering serum components) could abolish phenotypic differences observed in the urease activities. The parent strain in which the *hpn* mutants were obtained previously (various clinical strains (12) and *H. pylori* ATCC strain 26695 (11)) are also different from ours and variation in results with regard to urease activities with different strains is well known in the *H. pylori* field. Due to an efficient high affinity Ni-permease, Ni levels could reach toxic levels, and the His-rich proteins are proposed to then render nickel tolerance to *H. pylori*. Even in low Ni conditions stringent

sequestering of (limiting) nickel rather than allowing activation of all available apo-urease may have nickel-storage advantages.

In some conditions, *H. pylori* apparently synthesizes more urease apo-enzyme than it can (Ni) activate (24). For example, a three to four fold increase in urease activity upon decreasing the pH for 2-3 h was demonstrated previously (27). Also, the Hpn protein releases nickel under low pH conditions and *E. coli* cells expressing Hpn contain 4 fold higher nickel levels than *E. coli* containing vector alone (11). These results suggest that Hpn proteins may play an important role in sequestering nickel for the cell. Besides, the studies herein demonstrate that both Hpn and Hpn-like play a role in cobalt and cadmium tolerance as well. The largest metal tolerance was attributed to Hpn-like. Also, we observed a striking increase in the urease activities in the *hpn, hpn-like* and the *hpn/hpn-like* double mutant when compared to the wild type, but there was no corresponding increase in the amount of urease protein in the different mutant strains. The urease activity differences could be attributed to the nickel level associated with purified urease, so that these Hpn proteins appear to compete with Ni-dependent urease maturation in nickel deficient conditions.

We found that at a lower pH, the urease activity of the wild type strain increased, probably due to release of nickel from the intact His-rich proteins. Urease activity did not increase in the single mutants or the double mutant. This means the His-rich nickel proteins serve as stores for the metal, probably in a pH dependent manner. A pH dependence is expected if the His-rich proteins bind Ni at neutral pH and release Ni at pH 5.0. For Hpn, the nickel was reported to be less than half saturated at pH below 6.3 (11). Although the pH experiments can be explained from the perspective of the nickel binding properties of pure Hpn (11), these physiology experiments must be subject to other interpretations. For example, our experiments

involve whole cell physiology in which many other Ni-metabolism parameters (NixA, intracellular pH regulation affecting Ni availability, urease transcription rates) could change during growth. Such physiological changes could be affected by the lack of Hpn or Hpn-like proteins. Also, each Hpn or Hpn-like protein is subject to independent regulation and one of these two are of course still present in each single mutant. Certainly other Ni-level influencing models and mechanisms need to be considered to obtain an integrated picture of nickel metabolism and urease activity in the gastric pathogen.

In the presence of a nickel chelator the wild type urease activity (in contrast to the mutants) was not significantly reduced compared to the no-chelator condition, indicating internal Ni-reservoirs supply nickel to urease in the parent strain. Chelation had a significant inhibitory effect on urease activities of the mutants, indicating they are dependent on exogenous nickel for their urease activation. Therefore the Hpn and Hpn-like are important for supplying nickel to urease. The results provide physiological evidence that the His-rich proteins are acting as nickel stores for *Helicobacter pylori*.

The work presented here answers some questions about Ni- homeostasis and detoxification while raising others. For instance, while we have shown that Hpn and Hpn-like proteins play a role in nickel (and other metal) detoxification, the role of these proteins in concert with other metal toxicity prevention mechanisms is not understood. High nickel levels repress expression of the NixA transporter and the other Ni responsive outer membrane protein studied (7, 9, 39), presumably to aid in preventing nickel toxicity; perhaps the His-rich proteins provide a more rapid (detoxification) response to transient nickel fluctuations before the (transporter) expression changes can have an affect. Also, Ni-efflux pumps (CznABC) have been identified that are proposed to pump out excess nickel (28); this adds another feature to the overall Ni

homeostasis/detoxification picture. Why multiple mechanisms are needed is not clear. Also whether intracellular Ni (or other metal) concentrations become high enough *in vivo* such that Hpn and Hpn-like proteins are crucial for survival is not yet known.

Strains that are deficient in functional Hpn, Hpn-like or both proteins have higher urease activity. This can be explained if Ni binds with a higher affinity to the His-rich proteins than to Ni-binding urease maturation enzymes at neutral pH. This could represent a mechanism to limit, via nickel levels, the amount of active urease inside the cell at physiological pH. Upon encountering acidic pH, like in the gastric mucosa during initial stages of colonization, an increase in the amount of apo-urease is observed (26, 27). Yet NixA and the nickel responsive outer membrane protein HP1512 are both down regulated at pH 5 in comparison to pH 7 (5, 34). This is where the His-rich proteins would be expected to play a key role as nickel storage reservoirs. Our data that support such roles for Hpn and Hpn-like are shown in Fig. 3 where urease activity is significantly greater in the wild type at low pH than at neutrality, while the activity of mutants is decreased or remains the same by low pH incubation compared to neutrality. Still, the mechanism of Ni release from Hpn and Hpn-like, or whether the Ni-affinity is regulated by factors other than pH need to be addressed. It would be interesting to determine if Ni is directly released from the His-rich proteins freely into the cytoplasm or if other proteins are involved in (directed) nickel transfer/sequestering processes.

The His-rich proteins play dual roles depending on nickel levels; these are nickel storage and nickel detoxification. Nevertheless, these His-rich proteins are just two of the many Ni-binding proteins *H. pylori* uses to maintain Ni-homeostasis and Ni-enzyme function.

ACKNOWLEDGMENTS

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Table 2.1 Strains, Plasmids, Primers

Strains/Plasmids/Primers	Genotype/Description/Sequence	Source/Reference
Helicobacter pylori strains		
H.p. 43504 or wild type	*H. pylori* ATCC strain 43504	ATCC
hpn mutant	Kan^R; H.p. 43504 with *hpn*: kan^R	This study
hpn-like mutant	Cm^R; H.p. 43504 with *hpn-like*: cm^R	This study
double mutant	Kan^R, Cm^R; H.p. 43504 with *hpn*: kan^R, *hpn-like*: cm^R	This study
Escherichia coli strains		
E. coli DH5α	Cloning strain	Invitrogen
Plasmids		
pHP1	Plasmid containing the kanamycin resistance gene *aph*A3	(15)
pUC20cat	Plasmid containing the chloramphenicol acetyl transferase gene *cat*	(37)
pGEM-T	Amp^R; Cloning vector	Promega
pGEM-*hpn*	pGEM-T vector with the *hpn* gene from H.p. 43504	This study
pGEM-*hpn-like*	pGEM-T vector with the *hpn-like* gene from H.p. 43504	This study
pGEM-*hpn*: kan^R	pGEM-*hpn* with the Kan^R cassette inserted into the *Xcm*I site of *hpn*	This study
pGEM-*hpn-like*: cm^R	pGEM-*hpn-like* with the Cm^R cassette inserted into the *Blp*I site of *hpn-like*	This study
Primers		
HPN- F	5'- TGTTGCGGTTGGATTGG-3'	IDT*
HPN-R	5'- CAAGTGGGTTGCTCGTTTTGTTTC-3'	IDT*
1432- F	5'- AGCCAACGCCTTTTCTTTCAG-3'	IDT*
1432- R	5'- TTTTACACCCCATTACGACCACTC-3'	IDT*

* Primers were purchased from Integrated DNA Technologies, Inc. Coralville, IA

Table 2.2 Nickel content in the urease fraction

Strain	ng Ni/ mg protein
Wild type[a], Wild type[c]	32 ± 4[a], 11 ± 2[c]
Wild type (with nickel supplementation)[b]	69 ± 6
hpn mutant[a]	139 ± 11
hpn-like mutant[a]	104 ± 11
Double mutant[c]	109 ± 12

[a] Nickel content in the urease fractions from cells grown under nickel-non supplemented conditions. [b] *H. pylori* wild type cells grown with 500 μM $NiCl_2$ supplemented in the growth medium. The results are the mean and standard deviation from at least three measurements. Both mutant strain results (*hpn* and *hpn-like*) are significantly greater than for the wild type at $P < 0.05$ (students t-test). [c] The nickel urease content of the double mutant was determined in comparison to the wild type in an independent experiment. Variability in the two wild type values is likely due to variation in the nickel levels in the different sources of (blood) media. The double mutant value is significantly greater ($p < 0.05$) than the wild type.

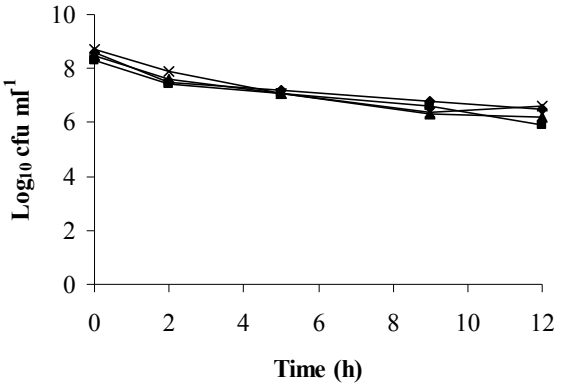

Figure 2.1 Survival in PBS with no added metals: Strains: *H.pylori* strain 43504 (♦), *hpn* (■), *hpn-like* (▲) or double mutant strain (×). The experiment was performed in triplicate and the data shown is the mean from all three data sets for each strain. No significant differences among strains were observed.

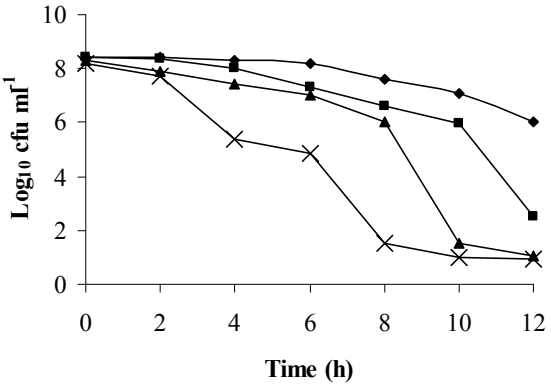

Figure 2.2 Survival in the presence of 1 mM $NiCl_2$: Strains: *H.pylori* strain 43504 (♦), *hpn* (■), *hpn-like* (▲) or double mutant strain (×).The data is from four independent experiments, each one sampled three times, so each data point is the mean of 12 readings. The standard deviation ranged between 3-20% of the mean values, so that all the *hpn* mutant strain results are significantly different ($P < 0.05$) than the wild type at 6 hours and all later time points while the

other two mutant strains are significantly different from wild type ($P < 0.05$) at 4 hours and all later plating points.

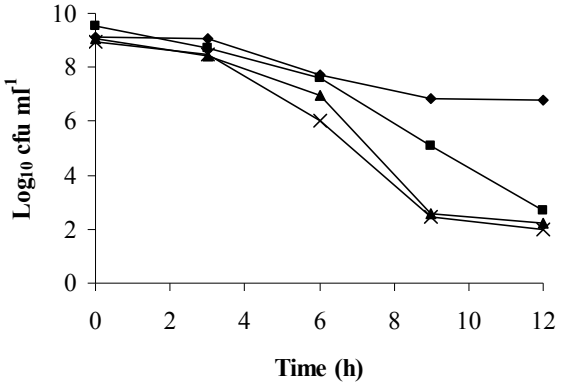

Figure 2.3 Survival in the presence of 10 µM $CdCl_2$: Strains: *H.pylori* strain 43504 (♦), *hpn* (■), *hpn-like* (▲) or double mutant strain (×). The data is from two independent experiments, each one sampled twice, so each point is the mean of 4 values. The values for the *hpn-like* and the double mutant are significantly different ($p < 0.05$) than the wild type at 6 h and later, whereas the *hpn* is different at 9 and 12 h time points ($p < 0.05$)

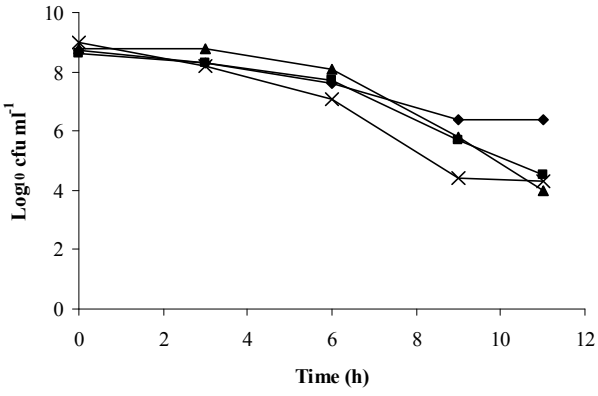

Figure 2.4 Survival in the presence of 20 µM $CoCl_2$: Strains: *H.pylori* strain 43504 (♦), *hpn* (■), *hpn-like* (▲) or double mutant strain (×). The data is from two independent experiments,

each one sampled twice, so each point is the mean of 4 values. The His-rich protein mutants are significantly less tolerant than the wild type at 9 and 11 h points ($p < 0.05$), and the double mutant significantly less than wild type at 6 h and later sampling points.

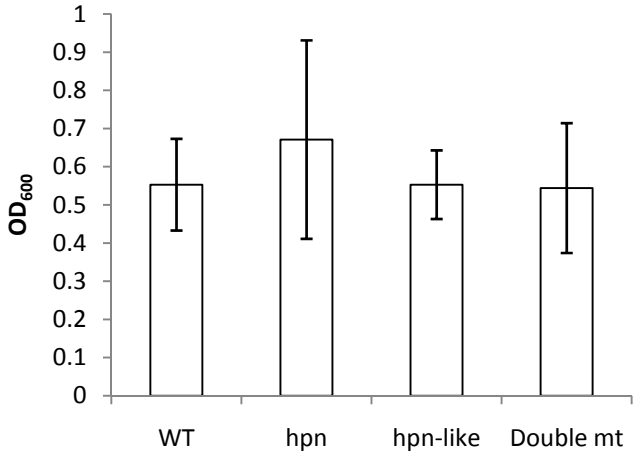

Figure 2.5 Growth Rate: Growth rate of all the four strains were measured by measuring the OD_{600} after 24 hours of growth in BHI medium supplemented with 5% FBS.

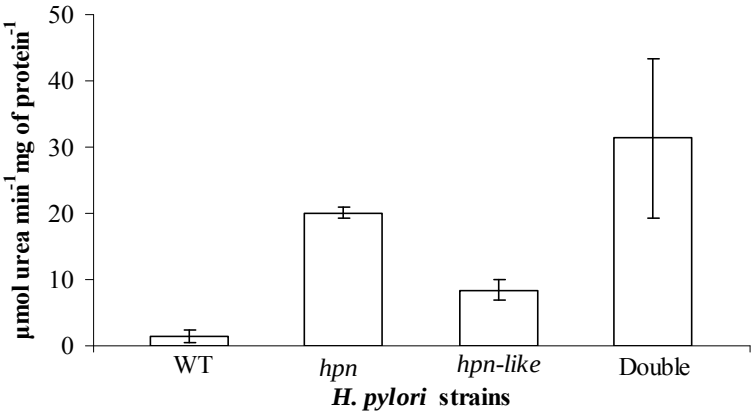

Figure 2.6 Urease activities without nickel supplementation: Urease activities of cells grown without nickel supplementation on BA plates. The results shown are the means and standard deviations of 6 replicate samples (these six represent 3 measurements from each of two

independent cultures); the experiment was performed four additional times with similar results. Among the five experiments the double mutant strain mean exceeded the Hpn strain in all cases, by a total range of 5 to 65%. All three mutant strains have a significantly greater urease activity than wild type ($P < 0.01$) based on students t-test analysis.

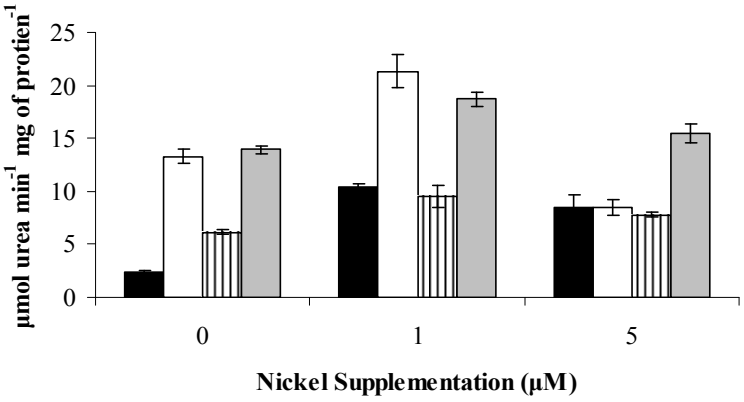

Figure 2.7 Urease activity after growth with nickel supplementation: Urease activities of cells grown with (1 or 5 µM $NiCl_2$) or without nickel supplementation. Cells were grown on BA plates. The strains are represented as follows: Wild type *H. pylori* (black), *hpn* strain (white), *hpn-like* strain (stripes), and the double mutant bar (gray). The measurements were performed in triplicate (one culture assayed three times); the wild type urease activities are greater in Ni-supplementation conditions than without Ni-supplementation ($P < 0.01$) based on student's t-test analysis and the mutant strains exceed the wild type activities in the zero nickel condition ($P < 0.05$). The entire experiment was done two other times with results similar to those shown.

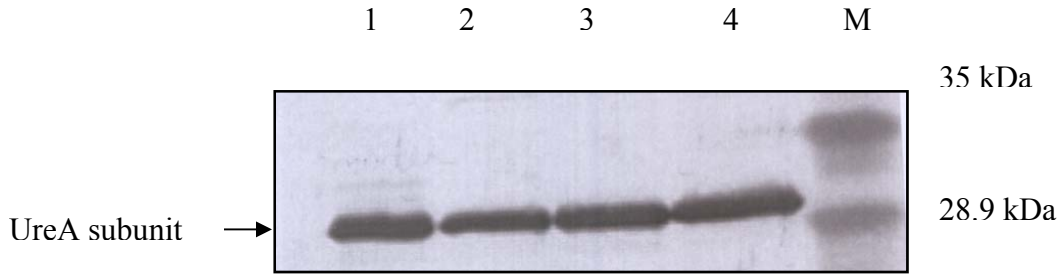

Figure 2.8 Urease protein in the different mutant strains: Anti-UreA immunoblots performed on gel resolved peptides of crude cell extracts from cells grown in non Ni-supplemented medium. Crude extracts were loaded (5 μg protein) of double mutant (lane 1), *hpn-like* (lane 2), *hpn* (lane 3) or wild type strain (lane 4). Lane M is the low range pre-stained molecular weight marker, with sizes indicated on the picture. Densitometric scanning of each lane revealed no significant differences in (UreA) protein amounts between strains.

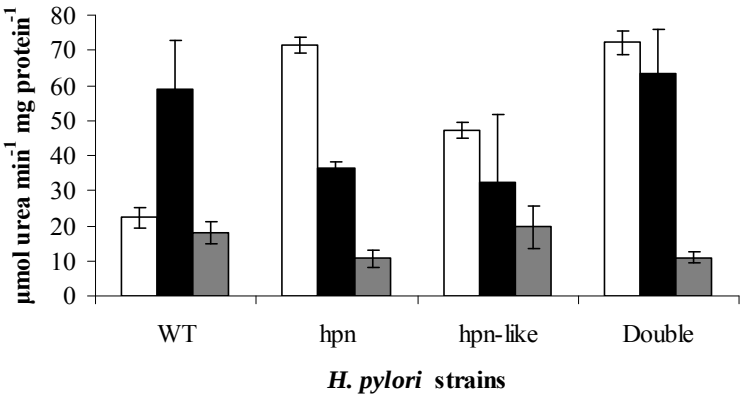

Figure 2.9 Effect of pH and dimethyl glyoxime on urease activities: Urease activities of strains grown in BHI medium with 5% FBS at pH 7.3 (white), pH 5 (black) or with nickel chelator dimethyl glyoxime at pH 7.3 (gray) are shown. The results shown are the means and standard deviations of 6 replicate samples; these 6 replicates are from two independent cultures, each sampled three times. The entire experiment was performed 2 additional times (again using 2 cultures for each experiment) with similar results to those shown. At pH 5, the urease activities of the wild type are significantly higher ($p < 0.05$) than at pH 7.3, and the chelator added condition was not different than without chelator (pH 7.3). The activities of the chelator added condition (for mutants) are significantly less than without chelator ($P<0.01$).

CHAPTER 3

H. HEPATICUS NIKR MODULATES UREASE AND HYDROGENASE ACTIVITIES VIA THE NIKA NICKEL TRANSPORTER[1]

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[1] To be submitted to Microbiology

My contribution to this work is all figures and tables except figures 3.3 and 3.7

CHAPTER 3

H. HEPATICUS NIKR MODULATES UREASE AND HYDROGENASE ACTIVITIES VIA THE NIKA NICKEL TRANSPORTER

ABSTRACT

Helicobacter hepaticus open reading frame HH0352 was identified as a nickel responsive regulator NikR. The gene was disrupted by insertion of an erythromycin resistance cassette. The *H. hepaticus nikR* mutant had a 5- to 6-fold higher urease activity and a 2-fold greater hydrogenase activity in comparison to the wild-type strain. However the urease gene expression levels were similar in both the wild-type and the mutant. No binding of NikR to either the urease or the hydrogenase promoter was revealed by electrophoretic mobility shift assay. This result is in contrast to what was observed for *H. pylori* NikR-dependent urease regulation, where NikR recogniszed *ureA* promoter. Instead, purified NikR was shown to bind to its own promoter, as well as to the *nikA* (encoding for one component of the NikABDE putative nickel transport system) promoter, and this binding occurred only upon addition of nickel. In agreement with this result, the *nikR* strain had increased cytoplasmic nickel levels and about 4.5-fold more *nikA* specific transcript as compared to the wild-type. Taken together, these results indicate that *nikR* plays a role in modulating nickel-enzyme activities post-translationally via repression of the nickel transport system/nickel internalization in *H. hepaticus.* The *nikR* strain had a 2 to 4-fold lower growth yield than the parent, suggesting that the regulatory protein might play additional

roles in the mouse pathogen. A periplasmic nitrate reductase of *H. hepaticus* was upregulated in a *nikR* strain and the *nikR* strain had increased levels of hydrogenase activity with nitrate as the electron acceptor. Also, nitrate disappearance rates were greater than for the wild-type. Hydrogenase activity of the wild-type *H. hepaticus* strain was higher with nitrate as electron acceptor rather than with oxygen. This suggests niche dependent adaptation by *H. hepaticus* to obtain energy via H_2 oxidation in the absence of oxygen. This type of respiration pertains to the organisms natural environment- the enterohepatic and colonic regions.

INTRODUCTION

Maintenance of nickel homeostasis is crucial for many bacteria because high nickel levels can be toxic, whereas limiting levels can compromise important cellular functions. The proper balance is oftentimes achieved *via* the nickel responsive regulator NikR, which modulates the expression of key genes involved in diverse functions, such as nickel import or export systems, nickel binding and storage proteins or nickel-enzymes (20, 22, 35). NikR is a ribbon-helix-helix protein belonging to the CopG/ MetJ/ ArcA family of DNA binding, transcriptional activators (12). Like most proteins of this family, NikR contains an N-terminal region involved in DNA binding and a C-terminus involved in metal binding. NikR was first described in *Escherichia coli* (EcNikR) as repressor of the nickel uptake operon *nikABCDE* (17); it is the most well characterized ortholog of this family followed by the *Helicobacter pylori* NikR (HpNikR). It is known that both HpNikR and EcNikR bind nickel with very high affinities and that the *E. coli* NikR also possesses a second lower affinity nickel binding site (1, 7, 10, 13, 14).

In *H. pylori*, NikR has been shown to regulate directly or indirectly the expression and maturation of two important Ni-containing enzymes, urease and hydrogenase, which are required for early colonization and persistence in the stomach (21, 37). Indeed, NikR induces urease

expression by binding to a loosely conserved operator sequence upstream of the *ure*A gene (1, 18, 25, 46). Microarray-based studies showed that the hydrogenase genes *hydABC* are repressed in the presence of excess nickel in wild-type cells, but not in a *nikR* mutant (15); however in this case, this repression is thought to be indirect, as it is likely due to regulation of *hydABC* genes by the ferric uptake regulator (Fur) (24, 26), which itself is under the control of NikR (15). Besides this transcriptional control, NikR also affects urease and hydrogenase activities by controlling intracellular Ni levels; this is achieved through regulation of Ni transport through the outer and inner membrane (*exbBD*, *fecA3*, *frpB4*, *nixA*) or modulation of Ni-storage (*hpn*, *hpn-like*). All this makes HpNikR a global regulator, which does not seem to be the case for EcNikR, so far known to regulate only the various components of the ABC type Ni transporter (14).

While Ni homeostasis begins to be well understood in *H. pylori* and *E. coli*, not much is known about it in the murine pathogen *Helicobacter hepaticus*. *H. hepaticus* also possesses both the hydrogenase and urease enzymes (3, 33). *H. hepaticus* hydrogenase mutants are deficient in hydrogen-supported amino acid uptake and cannot cause liver lesions in mice (33), while the role of urease in *H. hepaticus* survival in the host remains to be demonstrated. Genome sequence analysis suggest that nickel transport and urease expression differ significantly among the two *Helicobacter* (*pylori* and *hepaticus*) species. Indeed, *H. hepaticus* urease gene expression does not respond to nickel concentration fluctuations (4), and Fur, not NikR, is proposed to be the major transcriptional regulator of *H. hepaticus* urease (5). The nickel transport systems also seem to be of different types among the two Helicobacters, with *H. pylori* having a high affinity nickel specific permease (NixA) (34) and *H. hepaticus* having the *E. coli*-like ATP dependent ABC type nickel transporter (NikABDE)(3, 4, 8). *H. hepaticus* also lacks the nickel storage proteins termed Hpn and Hpn-like, shown to affect urease in *H. pylori* (41). These findings suggest that

H. hepaticus, though closely related to *H. pylori*, may use different mechanisms to maintain nickel homeostasis.

To determine the functions of NikR in *H. hepaticus,* I started by studying the phenotype of a *nikR* mutant strain. The *nikR* mutant strain is viable, though it has a significantly slower growth rate than the wild-type. Our studies indicate that NikR modulates urease and hydrogenase activities by controlling nickel transport (*nikA* transcription) and therefore internal nickel levels in the cell, rather than by regulating the expression of *ureAB* or *hyaABC* genes. This indirect regulation of urease activity by NikR in *H. hepaticus* is in sharp contrast to the direct activation of urease genes by NikR in *H. pylori.* I also obtained results suggesting a role for NikR in functions other than nickel metabolism, particularly in anaerobic respiration. Considering the dearth of regulatory proteins in *H. hepaticus,* it is not surprising that NikR plays roles other than in nickel metabolism. This is another example of niche dependent difference in regulation among two closely related organisms.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions.

H. hepaticus ATCC strain 51449 was used as the wild type strain and was also used for mutant strain construction. *H. hepaticus* cells were grown on Brucella agar (Difco) plates supplemented with 10% defibrinated sheep blood (BA plates) at 37˚ C. The plates were supplemented with 5 μg ml^{-1} of erythromycin when needed. Cultures were grown under micro-aerobic conditions with 5% CO_2, 2% O_2 and 93% N_2.

Escherichia coli strain DH5α or BL21DE3 was grown aerobically at 37˚C on Luria-Bertani (LB) medium supplemented with ampicillin (100 μg ml^{-1}) or on Brain Heart Infusion (BHI) agar plates supplemented with 150 μg ml^{-1} erythromycin, when required. Isopropyl β-D-

1-thiogalactopyranoside (IPTG) (0.5 mM) and nickel chloride (Sigma) (up to 50 μM) were added when needed.

Construction of *H. hepaticus hh0352* (*nikR*) and *hh0351* mutants

Genomic DNA from *H. hepaticus* strain 51449 and primers nikR-Mut1 and nikR-RMut2 (Table 2) were used to PCR amplify the 432-bp long *nikR* gene (HH0352; J. Craig Venter Institute, www.tigr.org) along with some flanking sequence. The resulting 917-bp long PCR product was cloned into pGEM-T vector to yield pGEM-*nikR*. A 1140-bp long erythromycin resistance cassette (*ery*) was inserted into a unique *Hind*III restriction site within *nikR* in the pGEM-*nikR* vector to yield pGEM-*nikR*::*ery*. A similar strategy was followed to construct the *hh0351::ery* mutant (control for polar effect): primers hh0351-Mut1 and hh0352-Mut2 (Table 2) were used to amplify the 657-bp open reading frame *hh0351* along with 500 bp flanking each side and the resulting PCR product was cloned into pGEM-T to yield plasmid pGEM-*hh0351*. The *ery* cassette was then inserted into a unique *Nde*I restriction site (within *hh0351*) in the pGEM-*hh0351* vector to generate plasmid pGEM-*hh0351*::*ery*. Each plasmid (pGEM-*nikR*::*ery* or pGEM-*hh0351*::*ery*) was introduced into *H. hepaticus* by electroporation to promote allelic exchange via homologous recombination. Erythromycin resistance was used to select colonies that contained the disrupted gene. Dozens of clones appeared for the *nikR::ery* mutant construction, whereas none could be obtained for the *hh0351::ery* mutation despite several attempts. The insertion of the *ery* cassette within the *H. hepaticus nikR* gene was confirmed by PCR using DNA from the mutant as template and primers nikR-Mut1 and nikR-RMut2.

Growth yield analysis

Cells were grown on BA agar plates supplemented with either no nickel or 50 μM $NiCl_2$, harvested after 18 or 36 h and washed in phosphate buffer saline (PBS) buffer. Then cells were

broken by sonication and centrifuged at 15,000 X *g* for 10 minutes to remove cell debris and unbroken cells. The supernatant was used to determine the protein concentration (BCA assay, Thermo Fisher Pierce). The experiment was done with cells harvested from 3 plates for each time point and each condition.

Overproduction and purification of recombinant *H. hepaticus* NikR

The *nikR* open reading frame was PCR amplified from *H. hepaticus* 51449 chromosomal DNA using primers NikR-OP1 and NikR-OP2 (Table 2). These primers were designed to incorporate a 5' *Nde*I and a 3' *Xho*I restriction site, respectively. The purified PCR product was digested with *Nde*I and *Xho*I restriction enzymes and ligated into similarly-cut pET21b vector to generate plasmid pET-NikR. This plasmid was then transformed into *E. coli* BL21-DE3 Gold strain. *E. coli* cells were grown at 37˚C in 1 l of LB until OD_{600} of the culture reached 0.5. Expression of the 16 kDa HhNikR was induced by addition of 0.5 mM IPTG for 4 h at 37˚C. Cells were then harvested by centrifugation (15,000 X *g*, 20 min, 4˚C), washed twice with phosphate buffer saline (PBS) pH 7.2 and broken by two passages through a cold French pressure cell at 18,000 lb/in^2. Cell debris was removed by centrifugation at 15,000 X *g* and the supernatant was subjected to ultracentrifugation at 100,000 X *g* for 2 h. The membrane-free supernatant was subjected to stepwise ammonium sulfate precipitation. *H. hepaticus* NikR protein (free of major contaminant proteins) was present in 60% and 70% ammonium sulfate saturated pellets (resuspended in PBS buffer) as shown by sodium dodecyl sulfate-12.5% polyacrylamide gel electrophoresis (SDS-PAGE). Both fractions were pooled and concentrated to a final volume of 2 mL using a 3K centricon device (Millipore). This 2 ml fraction was then subjected to size exclusion chromatography (HiLoad Superdex 75 prep grade column, GE Healthcare). Fractions were analyzed by SDS-PAGE, and those containing pure *H. hepaticus*

NikR were pooled and concentrated using a 3K centricon device. Protein concentration was determined using the BCA protein assay kit. The purified HhNikR was used for electrophoretic mobility shift assays. To obtain Ni-free NikR, the protein was dialyzed twice at 4°C against one liter of 20 mM Tris-HCl, pH 7.5, KCl 0.1 M, 1 mM DTT, with 1 mM of each of the chelating agents EDTA and DMG (Dimethylglyoxime).

Immunoblotting and 1-dimentional SDS-PAGE analysis

Whole cell extracts (10 μg of total protein) from cells grown without added Ni to the medium were subjected to SDS-12.5%PAGE with a mini-protean II apparatus (Bio-Rad), according to the method of Laemmli and proteins were separated in a single dimension (30). The gel was stained with Coomassie G-250 (Pierce) and was destained with 40% methanol and 7% acetic acid. Bands which differed in their expression levels among the two strains were cut and sent to the Proteomics Core Laboratory at the Medical College of Georgia for by identification by mass spectrophotometry.

Cells extracts were subjected to SDS-PAGE analysis as described above, only instead of staining and destaining, they were used for immunoblots. Where such gels were used for immunoblotting, prestained SDS-PAGE standards purchased from Bio-Rad were used as markers. Proteins were transferred to nitrocellulose as described by Towbin *et al.* using a Bio-Rad Trans Blotter (45). Immunoblotting was performed using anti-*H. pylori* UreB rabbit antisera (a gift from HL Mobley, University of Michigan Medical School), dilution 1:2,000, and goat anti-rabbit immunoglobulin G-alkaline phosphatase (Bio-Rad), dilution 1:2000. Bound antibodies were 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).

Electrophoretic mobility shift assays.

Tris-borate running buffers, 7% native polyacrylamide gels and binding buffer were prepared and used as described by Bloom and Zamble(10). Nickel chloride (100 μM final concentration) or EDTA (1mM) was added to the binding buffer, running buffer and the polyacrylamide gel before polymerization.

Primers nikA-EMSA1 and nikA-EMSA2 (Table 2) were used to amplify a 120-bp PCR product containing the *nikA (hh0417)* promoter; primers nikR-EMSA1 and nikR-EMSA 2 were used to amplify a 131-bp PCR product containing the *nikR* (*hh0352*) promoter; primers hyaA-EMSA1 and hyaA-EMSA2 were used to amplify a 400-bp PCR product containing the *hyaA* (hh0056) promoter; and primers ureA-EMSA1 and ureA-EMSA2 were used to amplify a 173-bp PCR product containing the *ureA* (*hh0406*) promoter (Table). Each PCR product was gel purified using the QIAquick gel extraction kit (Qiagen) and end-labeled with [γ-^{32}P] ATP and T4 polynucleotide kinase, and unincorporated nucleotides were removed using the QIAquick kit.

Each PCR product with DNA concentration ranging from 30 to 45 pM (6,000 to 9,000 cpm/sample) was incubated with purified NikR for at least 30 min at room temperature in a final volume of 50 μl before loading on a 7% polyacrylamide gel running at 200 V, 4°C, that had been prerun for 20 min. The gels were vacuum-dried and exposed to a phosphor screen, then scanned using a Typhoon phosphorimager (GE Healthcare) and analyzed with ImageQuant 5.0 software.

Urease activities

Cell extracts from 2-day old cultures grown on unsupplemented BA plates or on BA plates with the indicated nickel supplementation were used. Briefly, cells were washed and suspended in 50 mM HEPES buffer, pH 7.5. Cells were broken by sonication and the lysate was centrifuged at 15,000 X *g* for 20 min to remove the cell debris and unbroken cells. The cell-free

supernatant (CFS) was incubated for 20 min with the same buffer containing 25 mM urea, and the ammonia evolved was measured via a phenol hypochlorite assay as previously described (47) A standard ammonium chloride concentration curve was used to convert the absorbance at 625 nm to nmoles of ammonia. The release of 2 micromoles of ammonia was considered to be equivalent to the hydrolysis of 1 micromole of urea. Urease activity is expressed in micromoles of urea hydrolysed per min per mg of total protein.

Hydrogen uptake with O_2 as acceptor

Hydrogenase assays were carried out on intact whole cells (2-day old cultures) using a previously described amperometric method (32). Hydrogen uptake coupled to oxygen as electron acceptor is reported as nanomoles of H_2 gas used per min per 10^9 cells.

Hydrogen uptake with NO_3^- as electron acceptor and nitrate disappearance assays

Hydrogenase assays were carried out as described above except that potassium nitrate was added to a final concentration of 1 mM to the electrode chamber, after ensuring the removal of oxygen. To remove oxygen from the chamber, wild-type or *nikR* mutant strain cells were suspended in PBS to obtain a thick suspension (OD_{600} in various experiments ranging between 3 and 5). This cell suspension was sparged with N_2 gas for 15 minutes. When the hydrogenase activity was measured, there was no uptake of H_2 unless O_2 saturated solution (100 μL) of was added. Then the oxygen dependent H_2 rate was assessed until no further H_2 uptake occurred even upon addition of excess hydrogen. Upon addition of small amounts of O_2, H_2 uptake would resume, so it was concluded (when no H_2 uptake occurred) O_2 was depleted. To doubly ensure the absence of oxygen, (~0.1 units of) Oxyrase® was added to the chamber (oxyrase is an enzyme system that naturally, selectively, and efficiently removes oxygen (Oxyrase Inc, Manfield, OH)). After waiting for a few minutes to allow oxyrase dependent removal of

remaining O_2, KNO_3 solution, (also previously sparged with N_2) was added to the amperometric electrode chamber. Then the nitrate dependent rates of H_2 oxidation were measured. A reaction with just Oxyrase® and nitrate was performed as a control to ensure that no nitrate reduction occurred due to the activity of the Oxyrase® enzyme system. The presumed anaerobic conditions were also verified by adding 1 μM sodium dithionite to 2 mL samples removed from the chamber (via a syringe) and observing the ability of the dithionite containing sample to reduce benzyl viologen compared to 2 mL N_2 sparged buffer also containing 1 μM sodium dithionite.

To measure the activity of the periplasmic nitrate reductase in the two *H. hepaticus* strains, a similar experiment to that described above was performed, except that nitrate levels were determined after incubation of cells with H_2 and nitrate. A thick cell suspension was made in PBS to which 1 unit of Oxyrase® was added and the entire suspension was sparged with N_2 gas mix for 15 min. Then N_2 sparged 2.5 mM potassium nitrate was added. Cell suspensions were continually sparged with anaerobic mix and 1 mL samples were removed at various time intervals. The cells were spun down and the resulting supernatant was filtered with a 0.2 μ Millipore filter. This sample was analyzed at Dr. Samantha Joye's lab at the University of Georgia using the Antek model 745 (vanadium reduction) with model #7050 chemiluminescent detector.

Determination of nickel levels in *H. hepaticus* cells

H. hepaticus wild-type and *nikR* mutant cells were grown for 2 days on BA plates with the indicated nickel supplementation (0-50 μM). Cells were washed twice in 10 mM Tris-HCl. pH 7.4, resuspended in the same buffer, broken by sonication and the lysate was centrifuged at 15,000 X *g* for 20 min to remove the cell debris and unbroken cells. The protein concentration of the CFS was determined (BCA assay) and the amount of nickel in 1 mL of the CFS was assayed

by an inductively coupled plasma emission mass spectrometer (ICP-MS). The amount of nickel in each strain and for each condition is expressed in ng of nickel per mg of total protein.

Quantitative real time PCR (qRT-PCR)

RNA was extracted and purified from 36-h old cultures (grown on unsupplemented BA plates) using the PureLink Micro-to-Midi Total RNA Purification system (Invitrogen). To remove genomic DNA from the RNA samples, the RNA preparation was incubated with DNAseI (BioRad) for 30 min at room temperature and re-purified using the same PureLink Total RNA purification kit. The quality and quantity of the RNA was monitored using the *Bio*Photometer (Eppendorf). cDNA was synthesized from 5 µg total RNA using the iScript Select cDNA synthesis kit (BioRad) following manufacturer's instructions. Random hexamers provided with the kit were used to generate the cDNA. A control reaction lacking only the reverse transcriptase was performed on every sample, to rule out genomic DNA contamination in cDNA samples. The cDNA obtained was used in qRT-PCR with the primers listed in Table 2. First, primer concentrations and annealing temperatures were standardized by performing a gradient and melt curve analysis on the iCycler (BioRad) using iQ SYBR green supermix (BioRad). This also checked for the presence of non-specific PCR products as well as the presence of primer-dimers. A dilution series with cDNA as the template was performed to determine and standardize PCR efficiencies. Finally, reaction mixes contained 100 µM of each primer, 1000-fold diluted cDNA, SYBR green supermix, nuclease free water in a final volume of 20 µL. Each reaction was performed in triplicates and the whole experiment repeated with three independent RNA samples. The transcript levels were normalized to the levels of 23 S rRNA gene and differences in RNA transcript levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

RESULTS

H. hepaticus possesses a NikR homolog

Analysis of the sequenced genome of *H. hepaticus* strain 51449 (43) revealed the presence of an open reading frame (HH0352) that shows 66% identity and 78% similarity with the well studied *H. pylori* nickel-responsive regulator NikR; *H. hepaticus* shares only 33% identity and 60% similarity with the other well studied NikR ortholog, the *E. coli* NikR. Amino-acid sequence comparison between all 3 NikR sequences suggests a putative role for N-terminal residues of HhNikR in DNA binding (Figure 3.1), as previously shown for HpNikR and EcNikR (6). The 9-residue long N-terminal arm of HpNikR (MDTPNKDDS) has been shown to be required to avoid non-specific/low affinity DNA binding and for binding to the *nixA* promoter (6); however HhNikR possesses only 4 of the 9 residues and only one of which is a charged residue. The C-terminus has conserved residues that are involved in Ni binding, similar to both EcNikR and HpNikR (13, 19).

The genomic region containing *H. hepaticus hh0352* (herein referred to as *nikR*) is organized in a similar way as in other sequenced species of the genus *Helicobacter* (8) (Figure 3.2). The gene convergently transcribed downstream of *nikR*, *hh0351*, is homologous to a conserved gene downstream of *nikR* in *H. pylori* or *H. acinonychis* species (2, 23, 36, 44); likewise, the genes divergently transcribed with respect to *H. hepaticus nikR* (*hh0355/354/353*) share significant homology with the genes located upstream of *nikR* in *H. pylori*; those genes encode for the ExbB-ExbD-TonB complex which provides energy for Ni transport through the outer membrane (39).

Construction of *nikR* mutant of *H. hepaticus*

A *nikR* mutant strain was constructed by inserting an erythromycin resistance (*ery*) cassette in the *hh0352* locus. The disruption of the *nikR* gene was confirmed by PCR as an increased size of the PCR product on an agarose gel (917 bp for the wild type versus 2,057 bp for the *nikR* mutant) (Figure 3.1). Since *H. hepaticus nikR* is part of a bicistronic operon *hh0352-hh0351*, this prompted us to construct an *hh0351::ery* mutant as a control for polar effect. Attempts to disrupt this region always resulted in loss of viability, as we were unable to recover any transformant. Homology searches revealed that *H. hepaticus hh0351* encodes a putative nicotinic acid mononucleotide adenylyltransferase known to be essential in several bacteria, including *Salmonella typhimurium* (28). We concluded that the disruption of *nikR* gene with the erythromycin cassette does not cause any polar effect as the *nikR* mutant is viable. This is in good agreement with previous results suggesting that the *ery* cassette is a safe tool to construct targeted mutants in *H. hepaticus* (9).

Urease activity, but not urease expression, is increased in the *nikR* mutant

To determine whether the NikR regulator controls urease activity in *H. hepaticus*, the urease activity was measured in the cell free extract from both the wild-type and the *nikR* mutant strains grown in media with or without supplemental Ni. When no Ni was added to the medium, the urease activity was 5- to 6-fold higher in the *nikR* mutant strain in comparison to the wild-type (Table 3.3). This result suggested either that NikR acts as a transcriptional repressor of urease or/and that NikR affects urease activity via its (Ni-) maturation. To address whether the increased urease activity was due to an increased amount of urease apoenzyme, immunobloting was performed on cell-free extracts from the wild-type and the mutant grown on unsupplemented medium. The significant degree of identity between *H. pylori* and *H. hepaticus* UreB (69%)

allowed us to use anti *H. pylori* UreB to probe *H. hepaticus* UreB, as previously shown (9). There was no difference in the amount of urease apoprotein made among the wild-type and the *nikR* mutant (Figure 3.4), indicating that (i) *nikR* does not regulate transcription of urease and (ii) the increase in urease activity in the mutant is probably due to enhanced Ni-maturation of the urease.

Addition of $NiCl_2$ to the medium dramatically increased the urease activity in the wild-type, as previously reported (4, 9) (Table 3.3). Interestingly, the same activation trend was observed in the *nikR* mutant, although levels of urease activity were always higher as compared to the parental strain. These results suggest a role for HhNikR in modulating the urease activity, regardless of the amount of Ni present in the medium.

***H. hepaticus nikR* mutant strain shows increased hydrogen uptake with O_2 as electron acceptor**

Hydrogen uptake abilities of the *nikR* mutant and the wild-type strains were measured by whole cell amperometry and compared: the hydrogenase activity was consistently at least 2-fold higher in the *nikR* mutant strain as compared to of the wild-type strain (Table 3. 4). When the growth medium was supplemented with increasing concentrations of Ni, hydrogenase activity in the wild-type increased, as previously shown (9, 33). Likewise, Ni-supplementation resulted in increasing hydrogenase activity in the *nikR* mutant, but levels were constantly 2- to 4-fold higher in the mutant as compared to the wild-type, for any given Ni concentration (Table 3. 4). This suggested that HhNikR either directly (transcriptional repression) or indirectly (maturation) affects the hydrogenase of *H. hepaticus*. Since anti-hydrogenase antiserum is not available at this time, it is not known whether the increase of hydrogenase activity can be linked to an increase in hydrogenase synthesis in the mutant.

Increased intracellular nickel pools in a *nikR* strain

As stated above, the fact that the *nikR* mutant had increased urease activities with similar levels of urease apoenzyme suggested a difference in Ni-maturation, linked to an increase in Ni import into the mutant strain cell. To test this hypothesis, the amount of intracellular nickel of wild-type and mutant strains grown under different Ni concentrations was determined by ICP-MS. As expected, the intracellular nickel levels detected in the wild-type cells increased when Ni was added to the medium (Figure 3.5). Interestingly, there was a consistent increase in the nickel levels in the *nikR* mutant strain compared to the wild-type strain, with a significant difference in nickel levels at 5- 50 μM nickel supplementation. This result suggested that Hh NikR could play a role with respect to Ni transport in the cell.

Higher *nikA* transcript levels in the *nikR* strain

Analysis of the genome sequence suggests that *H. hepaticus* relies on the NikABDE transport system (*hh0417-0414*), which displays homologies to the *E. coli* nickel-specific ABC NikABCDE transport system(3, 4, 8). In *E. coli*, NikR has been shown to repress the transcription of the *nikABCDE* genes in presence of nickel (14, 17). To determine whether NikR would play a similar role in *H. hepaticus*, we compared the *nikA* transcript levels in the wild-type and the *nikR* mutant strains. Total RNA was extracted from each strain, and then used in a two-step quantitative reverse transcription PCR analysis. Specific primers (Table 3. 2) were used to detect *nikA* transcript levels; in each experiment, the *nikA* transcript levels were normalized to the levels of the 23 S rRNA transcript levels, our internal control. Among the different experiments, there was a range of 2- to 7-fold more *nikA* transcript in the *nikR* mutant than in the wild-type. This result indicates that NikR represses *nikA* and by extension the *nikABDE* operon involved in Ni import system in *H. hepaticus.*

NikR binds to P_{nikA} or P_{nikR}, but not to P_{hyaA} or P_{ureA} promoters

To determine if NikR represses the transcription of the *nikABDE* operon *via* direct binding to the P_{nikA} promoter region, electrophoretic mobility shift assays (EMSA) were carried out with pure Hh NikR. The Hh *nikR* gene was cloned under the control of the *T7* promoter and expressed in *E. coli* upon addition of IPTG in the growth medium. The soluble protein was purified using ammonium sulfate precipitation followed by size exclusion chromatography. After this last step, the protein was considered 95-99% pure (data not shown) and was suitable for gel shift assays. The protein was incubated with four different ^{32}P-labeled DNA sequences: a 120-bp long PCR product containing (P_{nikA}), a 131-bp long DNA sequence containing P_{nikR}, a 173-bp long fragment containing bases -115 to +57 relative to the transcription start site of the *ureA* urease gene as determined by Belzer *et al*. (5), or a PCR product containing 400 bases upstream of the *hyaA* (hydrogenase) translation start site ("P_{hyaA}"). When purified NikR (0, 16 or 64 nM) was incubated in presence of 100 μM NiCl2 with each of the four promoters, a shift was observed with ^{32}P-labeled P_{nikR} (Figure 3.7, panel A) with 64 nM NikR, while there was no binding with either P_{nikA}, P_{ureA} *or* P_{hyaA} (data not shown). Higher concentrations of purified NikR were tested (up to 1 μM) with each of the promoters; while no retardation could ever be seen for P_{ureA} *or* P_{hyaA} under any condition, a NikR- P_{nikA} complex was observed with concentrations of NikR starting at 375 nM and higher (Figure 3.7, panel B). When HhNikR was dialyzed against a buffer that contained chelation agents to remove NikR-bound Ni, EMSA studies revealed that Apo-NikR was unable to bind to the P_{nikA} anymore (Figure 3.7, panel C). Upon addition of $NiCl_2$ the NikR-P_{nikA} complex was observed again (data not shown). Taken together, results from these EMSA studies indicate that in presence of Ni, HhNikR is able to bind to the *nikA* promoter and with a greater affinity to its own promoter. In contrast, HhNikR does not seem to bind to either

P_{ureA} *or* P_{hyaA} promoters. Therefore, the increased urease and hydrogenase activities observed in the *H. hepaticus nikR* mutant are thought to be the consequence of de-repression of the NikABDE transporter, leading to better Ni-maturation.

Growth effect

Both the wild-type and *nikR* mutant were grown on BA plates with either no nickel or 50 μM $NiCl_2$, and the growth was estimated for each strain after 18h or 36h by harvesting the cells, breaking them and estimating the protein concentration. At 18h or 36h time points, the growth yield of the *nikR* mutant was 2-to 3-fold lower than the growth yield of the wild-type, with or without Ni added to the medium (Figure 3.9). This suggests that NikR plays other roles in *H. hepaticus* besides its predicted involvement in nickel metabolism.

Up-regulation of periplasmic nitrate reductase (NapA) and increased H_2/NO_3 respiration in the NikR strain

To determine if NikR perhaps plays other roles in *H. hepaticus* physiology by regulating other proteins, the crude cell extracts of a wild-type and *nikR* mutant was subjected to 1-dimensional SDS-PAGE analysis as described in the methods section. An immediate observation was the upregulation of a band slightly >100kDa in the *nikR* mutant strain cell free extract (Figure 3.9). Mass spectrophotometry (Score 570, peptides matching-32, data not shown) identified the band as the periplasmic nitrate reductase A-subunit (NapA) of *H. hepaticus. H. hepaticus* possesses a periplasmic nitrate reductase system (NapAGHBLD) similar to the NapAGHBFLD of another *Epsilonproteobacterium*, *Wolinella succinogens* (29, 43). It is well known that in *W. succinogens*, the electron transport from formate (by formate dehydrogenase) or hydrogen (by NiFe hydrogenase) can be coupled to nitrate, nitrite or nitrous oxide reduction

due to the positive redox potentials for the nitrate/nitrite, nitrite/ammonium, nitrous oxide/dinitrogen redox pairs as any of these reactions are highly exergonic (29).

Since hydrogenase activity (with O_2 as electron acceptor, Table 3.4) was higher in the NikR mutant and the periplasmic nitrate reductase A-subunit was upregulated in the NikR mutant (Figure 3.9), hydrogen oxidation coupled to nitrate reduction was determined as described in the methods section (Figure 3.10). Surprisingly, wild-type *H. hepaticus* hydrogenase activities were consistently higher with nitrate rather than oxygen as electron acceptor. Hydrogenase activities of the NikR strain were higher than the wild-type irrespective of the electron acceptor. No significant difference was observed in the hydrogen oxidation rates with O_2 versus NO_3^- in the NikR mutant. These assays were performed three independent times with numerous replicates each time (see table).

To confirm our results, we wished to determine if there was a difference in periplasmic nitrate reductase activities by comparing the nitrate disappearance rates for wild-type and *nikR* mutant, where whole cells were incubated anaerobically with H_2 plus nitrate. When nitrate levels remaining in the wild-type and nikR mutant were measured. We observed a 3-4 fold decrease in the nitrate remaining in *nikR* mutant strain compared to the wild-type, after a 10 minute incubation time (Figure 3.11).

DISCUSSION

In this study, we report the construction and characterization of the first *nikR* mutant generated in the mouse pathogen *H. hepaticus*. Disruption of the gene by insertion of an erythromycin resistance cassette led to increased activities of two Ni-requiring enzymes, urease and hydrogenase. Since there was no change in urease levels between the mutant and the wild-type (as shown by immunoblotting against UreB, Figure 3.4), this suggests that the difference in

urease activity is solely due to a difference in post-translational Ni-maturation, in agreement with (i) increased transcription of the putative *nikABDE* Ni import operon in the mutant, as seen by qRT-PCR and (ii) higher levels of Ni detected in the *nikR* mutant, as shown by ICP-MS. These results are in agreement with those reported by Belzer and coworkers, who showed that the increase in urease activity of *H. hepaticus* was not associated with elevation of urease mRNA or protein levels (4). This kind of urease regulation (only at the post-translational level) has already been reported for *Streptococcus salivarius* (11). Although at this time it is not possible to detect and compare hydrogenase enzyme levels in the *nikR* mutant and the wild-type, it is tempting to speculate that the increase in hydrogenase activity observed in the *H. hepaticus nikR* mutant is solely due to increased maturation of the hydrogenase, rather than transcriptional regulation. Indeed, electrophoretic mobility shift assays revealed that purified *H. hepaticus* NikR failed to bind to promoters P_{ureA} *or* P_{hyaA} even when high amounts of protein (up to 1 μM) were used in the assay. In contrast, HhNikR-P_{nikR} and HhNikR-P_{nikA} complexes were isolated in the presence of $NiCl_2$, confirming that NikR can regulate both its own synthesis as well as synthesis of the ATP dependent nickel transporter NikABDE by direct binding to their respective promoters. Under the conditions tested, the binding of NikR to P_{nikR} seemed to happen at lower NikR concentrations (64 nM) than the binding to P_{nikA} (375 nM). These results indicate that HhNikR binds to P_{nikR} or P_{nikA} promoter with a different affinity, therefore suggesting that both promoters differ in their NikR-recognition and -binding DNA motifs.

Previous studies with *H. pylori* or *E. coli* NikR have led to identification of DNA binding sites for NikR (15). While it is understood that the location of these binding motifs are different depending on whether NikR acts as a repressor (for instance EcNikR for $P_{nikABCDE}$ or HpNikR for P_{nixA} or P_{nikR}) or as an activator (HpNikR for P_{ureA}), it appears that the DNA sequences involved

in NikR binding are not well conserved between species. Moreover, even when NikR acts as a repressor for two different genes within the same species (as it appears to be the case in our study with P_{nikA} and P_{nikR}) it is hard to define a consensus motif. Based on our own analysis of the intergenic region upstream of either gene of interest, one possible HhNikR binding site is proposed for P_{nikA}: a 5'-TAATAT-N9-ATATTA-3' inverted DNA motif was identified upstream of the *nikABDE* operon (Figure 2); alternatively, a second 5'-ATATTA-3' sequence is found in the vicinity and could be involved in the binding to HhNikR. Likewise, another inversed repeat sequence located within the P_{nikR} promoter (5'-TTTTAA-N5-TTAAAA-3') is proposed as a putative HhNikR binding site (Figure 2). Future site-directed mutagenesis and DNA footprinting assays will be needed to define more precisely which sequences are involved in recognition and binding to HhNikR.

In addition, the genes located upstream and divergently transcribed with respect to *H. hepaticus nikR* (*hh0355/354/353*) are homologs of *exbB*/*exbD*/*tonB* which have been shown to provide energy for Ni import through the outer membrane (Table 1.2) (39). Since these genes have been shown to be repressed by NikR in *H. pylori*, it is tempting to speculate that HhNikR regulates (represses) the transcription of *hh0355/0354/0355* by binding to another motif located on the same intergenic region. Likewise, the product of the *hh0418* gene (Table 1.2), divergently transcribed with respect to *nikA*, shares homology with FecA3 and FrpB4 which are NikR regulated outer membrane proteins involved in Ni import in the cell (16, 25). Therefore, in *H. hepaticus*, the *hh0418* gene might be regulated (repressed) by NikR as well. Reporter gene fusion or qRT-PCR studies done with the wild-type and the *nikR* mutant strains will be needed to confirm these hypotheses.

Besides their difference in urease regulation and Ni transport, *H. hepaticus* and *H. pylori* also differ with respect to accessory proteins and Ni-storage. Insertion of Ni into the catalytic site of urease or hydrogenase is a complex process that requires accessory proteins. In *H. pylori* as well as in *H. hepaticus*, hydrogenase accessory proteins such as HypA, HypB, HypC, HypD or HypE are needed for hydrogenase maturation (7, 9, 38). In addition, HypA and HypB have been shown to be needed for urease maturation as well in both species (38); other urease specific accessory proteins such as UreE, UreF, UreG or UreH are also required for full urease activation in both *Helicobacter* species (31). Upon addition of $NiCl_2$ in the medium, urease activity can be restored to wild-type levels in *H. pylori hypA* or *hypB* mutants; it is not the case in *H. hepaticus hypA* or *hypB* mutants. These results suggest different urease maturation pathways for *H. hepaticus* and *H. pylori*; the stringent requirement for HypA or HypB proteins in *H. hepaticus* might reflect the inability of the mouse pathogen to modulate the urease expression in response to Ni levels: because *H. hepaticus* urease activity can only be post-translationally affected, its dependance on accessory proteins is probably higher as compared to *H. pylori*. Besides, *H. pylori* possesses two histidine-rich proteins termed Hpn and Hpn-like, which have been shown to play dual roles as Ni-storage and metal-detoxification, depending on the exogenous nickel levels in the microorganism (27, 41). Both proteins have been shown to be transcriptionally activated by NikR (15). Analysis of the genome sequence of *H. hepaticus* indicated that these proteins are not present in the mouse pathogen. Unless other mechanisms are involved, it means that *H. hepaticus* is not capable of storing any intracellular Ni pool and can only rely on its Ni-transport system for rapid supplies of Ni when the metal is needed, in a similar fashion as for *E. coli*. Likewise, excretion of excess Ni out of the cell might be more critical for *H. hepaticus* than it is for *H. pylori*. *H. hepaticus* HH0623/0624/0625 gene products are homologs of the *H. pylori*

CznABC Ni export system (42). For the reason stated above, this operon is likely to be regulated by NikR in *H. hepaticus*, although it remains to be demonstrated.

The NikR mutant has a lower growth rate than the wild-type strain (Figure 3.8). Also, H_2 oxidation rates (the hydrogenase activity) of *H. hepaticus nikR* mutant strain was higher than for the wild-type irrespective of nitrate/O_2 being used as electron acceptor (Figure 3.10). The wild-type strain also had lower nitrate depletion rates than the *nikR* strain (Figure 3.11). One possible reason for the increase in hydrogenase activities with nitrate as the electron acceptor in *nikR* strain could be due the fact that increase in hydrogenase activities produces electrons that need a terminal substrate. Interestingly, supporting this theory, the periplasmic nitrate reductase was upregulated in the *nikR* strain (Figure 3.9). Hence, when external nitrate was supplied in the presence of hydrogen, H_2 oxidation coupled to nitrate reduction was higher in the *nikR* mutant. NikR may coordinately repress both enzymes

Surprisingly, the H_2 oxidation rate coupled to nitrate reduction in *H. hepaticus* wild-type strain was higher than with oxygen as the electron acceptor (Figure 3.10). Nitrate is oftentimes used as an alternative electron acceptor in organisms closely related to *H. hepaticus* such as *W. succinogens.* The *nikR* strain results taken together suggests that *in vivo* NikR plays a role in repressing (directly/indirectly) NapA transcription resulting in the use of O_2 as the preferred electron acceptor, which obviously would yield maximum energy. But, it also seems that *H. hepaticus* is very well adapted to growth in anaerobic conditions (like in its natural niche), and is thus equipped to utilize nitrate that could be present in the liver and intestinal tract for hydrogenase dependent energy production. Nevertheless, my results suggest novel roles for *H. hepaticus* NikR and these roles likely aid the bacteriums adaptation to colonize its specific niche.

Further studies of *H. hepaticus* NikR, specifically with respect to identification of its regulon, DNA recognition motif, nickel binding affinities, factors providing specificity in DNA binding and structure elucidation would enrich our knowledge on the evolution and adaptation of a regulator that responds to the immediate environment.

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Table 1. **Bacterial strains and plasmids**

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
H. hepaticus		
51449	Parent strain	ATCC[b]
nikR mutant	*nikR::ery*; Er^r	This study
E. coli		
TOP10	Cloning strain	Invitrogen
BL21DE3 Gold	Host for protein overproduction of Hh NikR	Novagen
Plasmids		
pGEM-T	Cloning vector; Ap^r	Promega
pGEM-*nikR*	pGEM-T with a 917-bp sequence containing *H. hepaticus nikR*	This study
pGEM-*nikR::ery*	pGEM-*nikR* with *ery* gene inserted within *Hin*dIII of *nikR;* Ap^r Er^r	This study
pGEM-*hh0351*	pGEM-T with a 1600-bp sequence containing *H. hepaticus hh0351*	This study
pGEM-*hh0351::ery*	pGEM-*hh0351* with *ery* gene inserted within *Nde*I of *hh0351;* Ap^r Er^r	This study
pET21b	Expression vector; Ap^r	Invitrogen
pET-NikR	*H. hepaticus nikR* cloned into *Nde*I-*Xho*I sites of pET21b	This study
pSLB167	pBC-KS with *ery* (*Eco*RV); Cm^r Er^r; source of *ery* cassette	Mehta et al (2007)

[a] Ap^r, Ampicillin resistance; Er^r, erythromycin resistance
[b] ATCC, American Type Culture Collection, Manassas, VA, USA.

Table 2. **Primers used in this study**

Name	Sequence (5'-3')[a]	Usage
nikR-Mut1	TTGGCATAAGGATAATCCGC	Construction of *nikR* mutant
nikR-Mut2	ACTGTGCCTAAAAGCCCCAC	Construction of *nikR* mutant
hh0351-Mut1	CGATTTAGTGTATCTCTCCCGCAG	Construction of *hh0351* mutant
hh0351-Mut2	AAGCACAAACAAAAAAAACGC	Construction of *hh0351* mutant
nikR-OP1	GAAAATCATATGAAGTCTAGTATTATCCG	Overproduction of NikR
nikR-OP2	GAAAATCTCGAGTTACTCAAATCGGCTTGC	Overproduction of NikR
nikA-EMSA1	GTTATTATGACATAGGAT	Amplification of *nikA* promoter for EMSA
nikA-EMSA2	CATTTTCTCCTTCCTC	Amplification of *nikA* promoter for EMSA
nikR-EMSA1	CTGCATATTATAAAAATCATTCT	Amplification of *nikR* promoter for EMSA
nikR-EMSA2	AATACTAGACTTCATTATGTCTCC	Amplification of *nikR* promoter for EMSA
hyaA-EMSA1	CTCTGTATCAAAGTTATCATTAG	Amplification of *hyaA* promoter for EMSA
hyaA-EMSA2	CCCTTTCCTTATGATTTTTG	Amplification of *hyaA* promoter for EMSA
ureA-GS1	GATTTTACTTATTTTCTTTTGG	Amplification of *ureA* promoter for EMSA
ureA-GS2	GTTAATCTCATACTCTCTCC	Amplification of *ureA* promoter for EMSA
nikA-RT1	TGTATGGGTGTATTGTGGG	qRT-PCR (*nikA* mRNA transcripts)
nikA-RT2	AACCATCTTGACTTGTATTCC	qRT-PCR (*nikA* mRNA transcripts)
23S-RT1	GCAGTATCCAGAGTAGGTCAGG	qRT-PCR (23S rRNA transcripts)
23S-RT2	GGTTCTATTTCACTCCGCTCA	qRT-PCR (23S rRNA transcripts)

[a] Restriction sites are underlined.

Table 3. Urease activity of wild-type and *nikR* mutant as a function of nickel added to the growth media.

Strain	Urease activity (μmoles urea min^{-1}mg total $protein^{-1}$)[a]				
	Nickel added (μM)				
	0	1	5	10	50
WT 51449	0.05 ± 0.02	0.35 ± 0.17	1.56 ± 0.23	3.27 ± 0.96	11.27 ± 2.22
nikR	0.31 ± 0.08	1.11 ± 0.54	3.14 ± 0.83	9.14 ± 3.34	23.56 ± 7.76

[a] Results are an average of three independent experiments, each performed in duplicate. Results were statistically different between the two strains at all nickel levels: $P<0.005$ as determined by Student's t-test.

Table 4. Hydrogenase activity of wild-type and *nikR* mutant as a function of nickel added to the growth media.

Strain	Hydrogenase activity (nanomoles $H_2.min^{-1}10^9$ $cells^{-1}$)[a]				
	Nickel added (μM)				
	0	1	5	10	50
WT 51449	0.28 ± 0.18	1.26 ± 0.30	1.32 ± 0.44	2.26 ± 0.98	2.42 ± 0.60
nikR	0.40 ± 0.13	1.68 ± 0.18	4.10 ± 0.66	6.91 ± 1.56	17.86 ± 5.47

[a] Results are an average of three independent experiments, each performed in duplicate. Results were statistically different between the two strains at all nickel levels: $P< 0.005$ as verified by Student's t-test for every nickel supplementation condition tested, except for 1 μM nickel supplementation, where $P< 0.01$.

```
Hh NikR   MKS-----SIIRFSVSLPQNLLETLDERLTHKGYSSRSEIVRDMIREKLNEEIWSSGAEN 55
Hp NikR   MDTPNKDDSIIRFSVSLQQNLLDELDNRIIKNGYSSRSELVRDMIREKLVEDNWAEDNPN 60
Ec NikR   ---------MQRVTITLDDDLLETLDSLSQRRGYNNRSEAIRDILRSALAQEATQQHG-- 49

Hh NikR   TQG-VAVLTIIYDHHQRELNQRMIDIQHTSTHKGNVEILCNTHVHLDQHNCLETIILRGN 114
Hp NikR   DESKIAVLVVIYDHHQRELNQRMIDIQHAS----GTHVLCTTHIHMDEHNCLETIILQGN 116
Ec NikR   TQG-FAVLSYVYEHEKRDLASRIVSTQHHH----HDLSVATLHVHINHDDCLEIAVLKGD 104

Hh NikR   GVHIEDLSIEIGGLKGVKFSKLTRASRFE--- 143
Hp NikR   SFEIQRLQLEIGGLRGVKFAKLTKASSFEYNE 148
Ec NikR   MGDVQHFADDVIAQRGVRHGHLQCLPKED--- 133
```

Figure 3.1: NikR protein sequences of *H. hepaticus, H. pylori* and *E. coli* NikR. The residues shaded in grey and located toward the N-terminal region are conserved residues previously identified to be involved in DNA binding in H. pylori or E. coli. The H and C residues shaded in grey and located towards the C-terminus have been shown to be involved in Ni binding in *E. coli* (40).

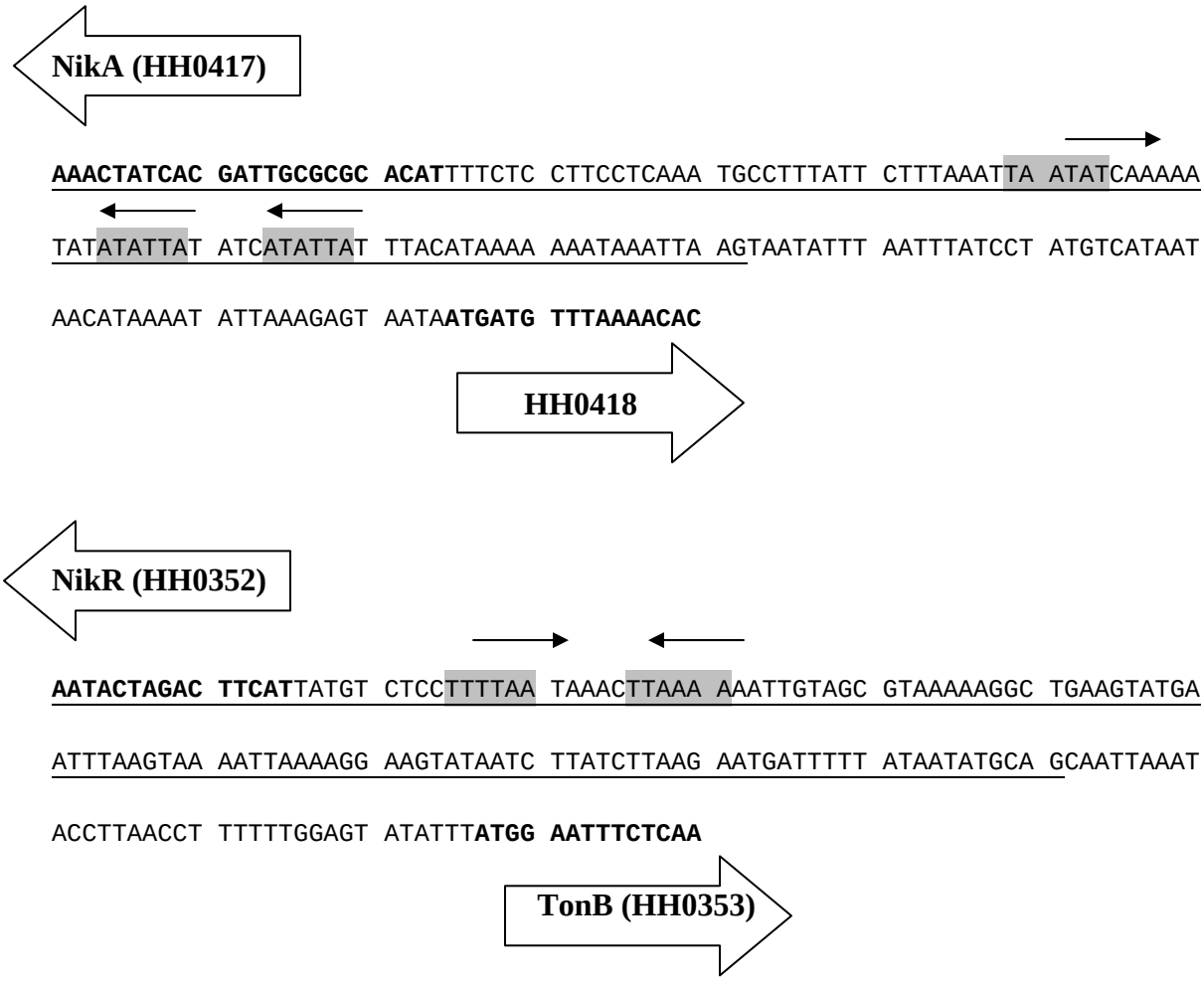

Figure 3.2 FIG. 2 Nucleotide sequence of the *nikA-hh0418* and *nikR-tonB* intergenic regions. Putative open reading frames are shown in bold. PCR products used for electrophoretic mobility shift assays are underlined. Putative NikR binding boxes are highlighted in grey.

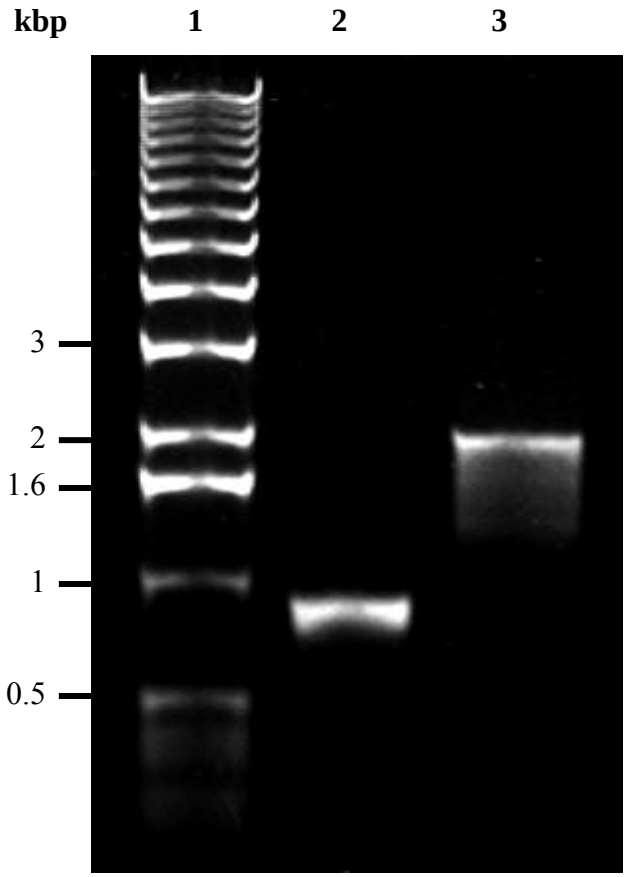

Figure 3. 3 Agarose gel of PCR products used to verify cassette insertion: Lane 1 contains a 1-kb DNA ladder, with sizes indicated on the left. Lane 2: PCR product obtained with genomic DNA from *H. hepaticus* parent strain 51449. Lane 3: PCR product obtained with genomic DNA from *nikR::ery* mutant. Primers nikR-Mut1 and nikR-mut2 were used.

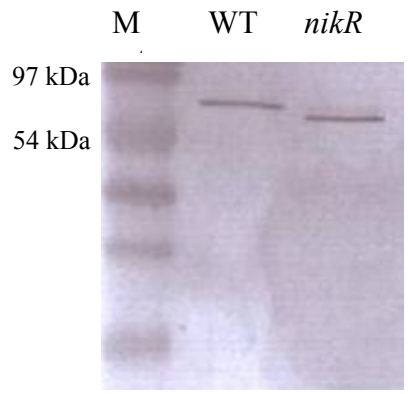

Figure 3.4 Immunoblot with anti-UreB antibody: Immunoblots with anti-UreB antibodies from *H. pylori* was performed to detect expression of urease protein in *H. hepaticus* wild-type strain and the *nikR* mutant strain.

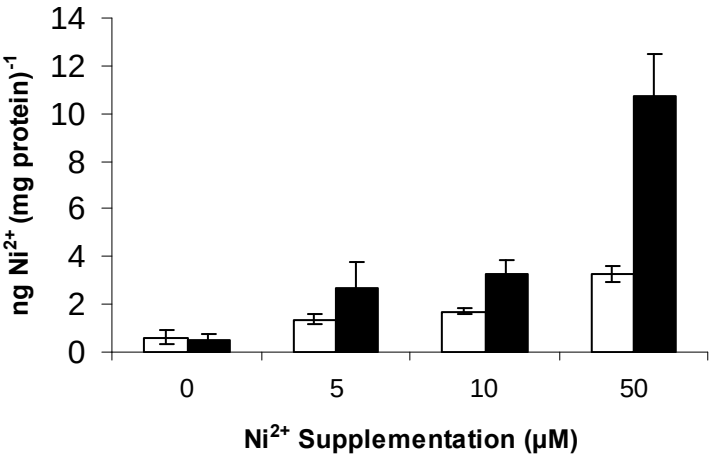

Figure 3.5 Amount of Nickel detected in cells by ICP-MS: White box- Wild-type, Dark box- *nikR* mutant strain. The results are from averages of six measurements. The experiment was performed three other times wherein the same trend was observed. The mutant values were greater than the parent for all nickel supplementation levels: $P<0.005$ as determined by student's t-test.

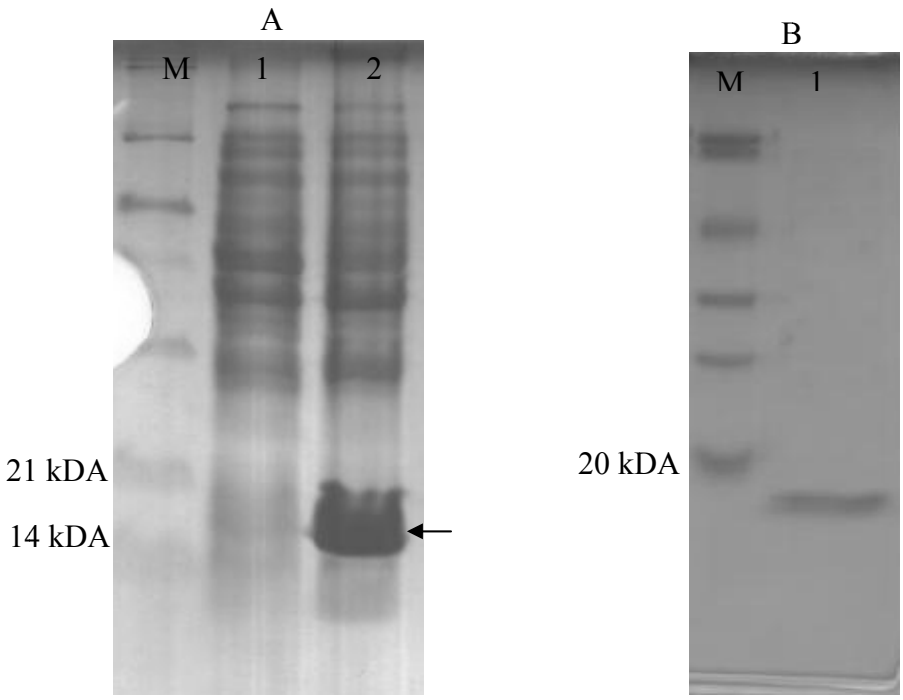

Figure 3.6 NikR overproduction and purification: Panel (A) Showing NikR being overproduced in *E. coli* in lane 2. Panel (B) Showing purified NikR in lane 1

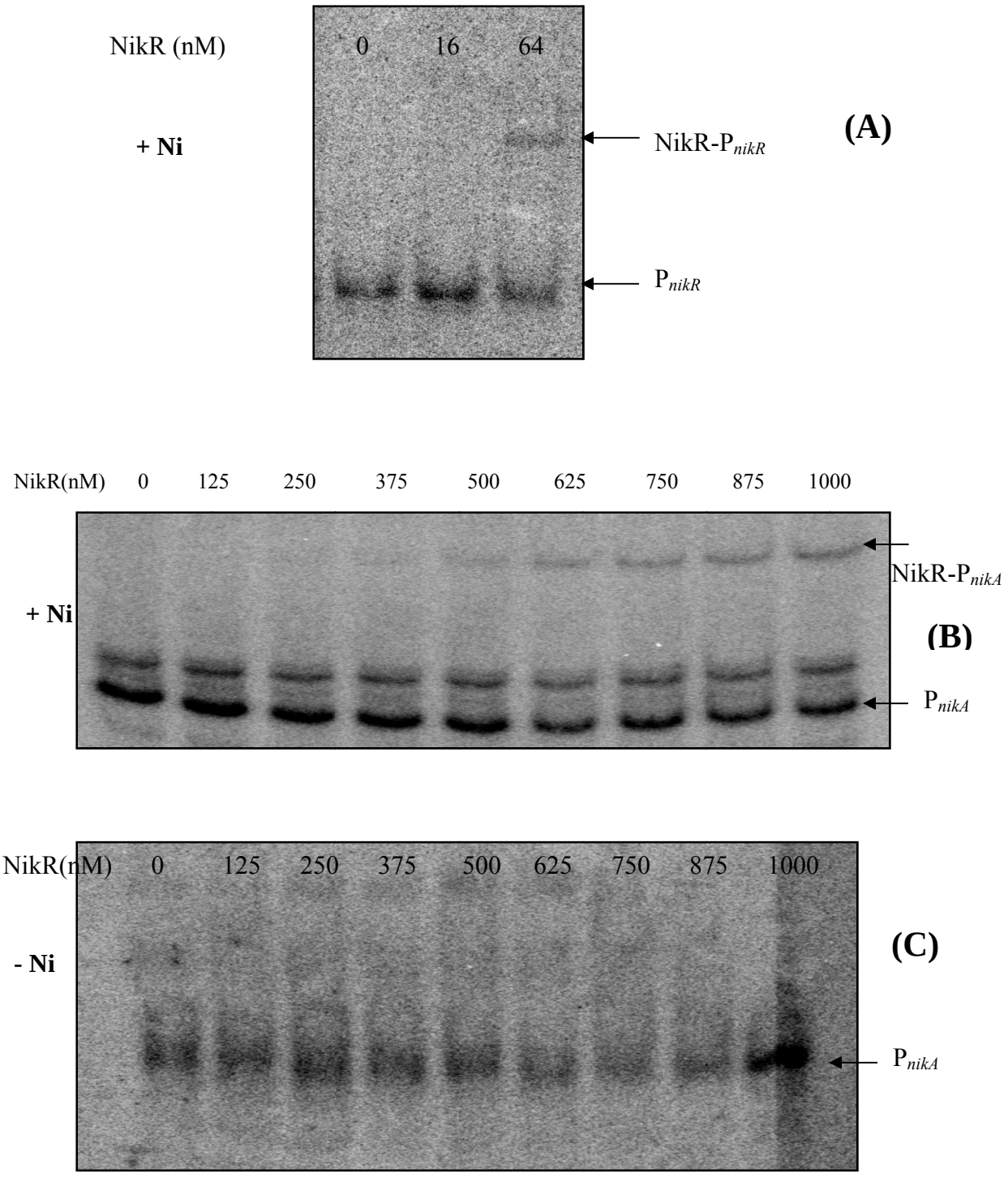

Figure 3.7 Electrophoretic mobility shift assays: (A) Electrophoretic mobility shift assay done with 44 pM of radiolabeled P_{nikR} fragment (131 bp) and increasing concentrations of purified *H.*

hepaticus NikR in presence of $NiCl_2$. The NikR-P_{nikA} interaction was studied with 36 pM of radiolabeled P_{nikA} fragment (120 bp) and increasing concentrations of purified *H. hepaticus* NikR in presence (B) or absence (C) of $NiCl_2$. Concentrations of purified NikR used in these studies are shown above the lane.

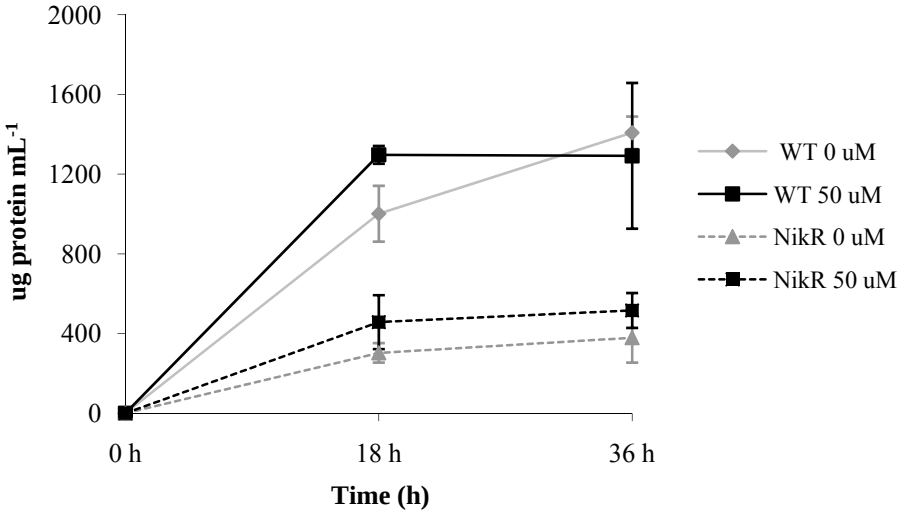

Figure 3.8 Protein Yield: Protein yield of the *H. hepaticus* wild-type and a *nikR* mutant strain was detected at various time points in the presence or absence of the nickel to be an indicator of growth in these two strains. The results are the averages and standard deviations from three independent experiments.

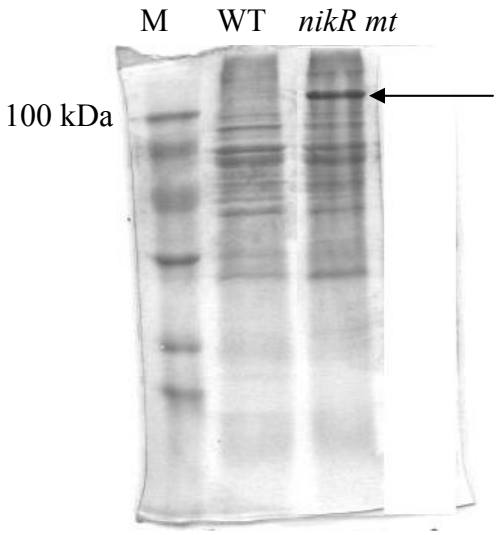

Figure 3.9 Single dimension SDS-PAGE analysis of *H. hepaticus* strains cell free extracts: One dimensional SDS-PAGE analysis of the *H. hepaticus* wild-type and *nikR* mutant strains cell extracts showing the upregulation of a band, slightly greater that 100 kDa in size, which was identified as the periplasmic nitrate reductase NapA of *H. hepaticus.*

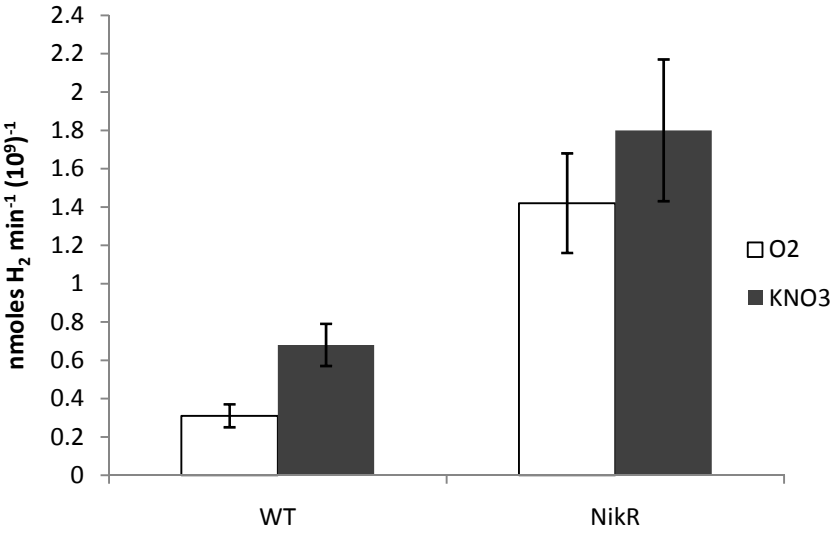

Figure 3.10 Hydrogen oxidation coupled to nitrate reduction: The results are from 6 to 7 repeats for each condition and the whole experiment was repeated three independent times with

the same trend. Results were statistically different between the two strains $P < 0.005$ as determined by Student's t-test. Also, the O_2 and NO_3 dependent H_2 oxidation rates for the wild-type were significantly different.

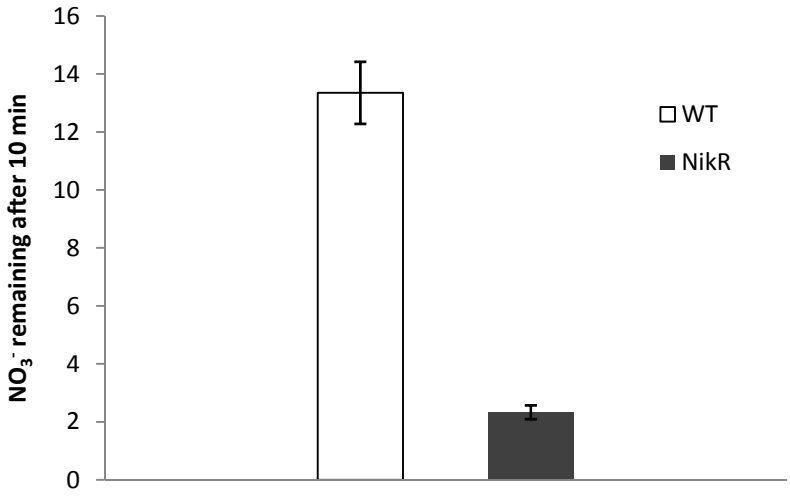

Figure 3.11 Nitrate disappearance in wild-type and *nikR* strains of *H. hepaticus*: Nitrate levels were determined after incubation of whole cells with nitrate plus hydrogen for 10 mins. Figure presented here is the averages and standard deviations from four repeats.

CHAPTER 4

DISSERTATION SUMMARY AND FUTURE DIRECTIONS

ROLES OF THE HIS-RICH HPN AND AND HPN-LIKE IN *H. PYLORI* NICKEL PHYSIOLOGY

Initial identification of Hpn and Hpn-like protein led to the speculation of their involvement in nickel metabolism, due to their histidine rich nature (7, 10). Later, studies with pure protein showed that these two proteins bind nickel reversibly and are able to release the bound nickel in the presence of a chelator such as EDTA or in acidic pH (6, 15). Also, expression of these proteins in *E. coli* afforded protection against high, toxic concentrations of nickel (6, 15). This further led them to theorize that Hpn and Hpn-like could be nickel storage proteins and could play roles in excess nickel detoxification by sequestration of Ni^{2+}.

My research presented in chapter 2 of this dissertation provides physiological evidence in *H. pylori* that Hpn and Hpn-like play dual roles in nickel detoxification as well as in nickel storage. Furthermore, I was able to determine that both these his-rich proteins protect *H. pylori* against toxic concentrations of other metal ions such as Co^{2+} and Cd^{2+}. Urease activities and Ni associated with urease is higher in the *hpn, hpn-like* or a double mutant deficient in both these genes. These proteins seem to compete with urease for nickel under low nickel conditions and at physiological pH. Also, under acidic conditions urease activity of wild-type *H. pylori* increases when no supplemental nickel was added but this increase was negligible in the three mutant strains. Furthermore, after growth with a nickel specific chelator added to the growth medium,

urease activities of the three mutant strains decreases considerably while there is no significant change in the wild-type urease activity levels. It is already known that nickel and pH can affect urease activities post-translationally (2, 12-14). Taken together, my results suggest that Hpn and Hpn-like permit storage of the metal and this affects urease activities.

H. pylori colonizes nearly 50% of the world population and causes gastritis, peptic and duodenal ulcer diseases and is associated with gastric cancers (1, 4, 5, 8, 9). Urease plays an important role in *H. pylori*. Mutants lacking *ureAB* are unable to colonize gnotobiotic piglets (3).

My results have shown that both Hpn and Hpn-like can affect urease activities and hence might be important for *H. pylori in vivo* under low nickel conditions. *H. pylori* is a human gastric mucosal pathogen and hence might encounter vastly variable concentrations of metal ions, depending on the host diet or nutrition. Again, Hpn and Hpn-like probably bind excess metal ions and protect the bacterium against the harmful effects of free metal ions. My results show that Hpn and Hpn-like play important roles in nickel metabolism in *H. pylori* and they probably contribute in making this bacterium a successful pathogen.

It would be interesting to study if *hpn, hpn-like* or a double mutant strain is deficient in mouse colonization and if this is in turn affected by nickel levels. Since the levels of nickel, along with other metals probably fluctuates in the gastric region, if either Hpn or Hpn-like affects host colonization, it would be evident only in a nickel deficient environment. The strains used for the host colonization could be grown in nickel deficient conditions (defined media without nickel or with nickel chelators) before mouse inoculation and the mouse nickel intake could be varied.

Purified Hpn and Hpn-like proteins release nickel in the presence of acidic pH or a chelator. It wouldn’t be surprising if nickel release from these proteins is mediated by other proteins. In fact, it is possible that Hpn and Hpn-like interact with the Ni-binding accessory

proteins such as UreE or HypB before nickel is finally transferred to urease. This can be determined by doing co-immunoprecipitation assays using anti-Hpn or anti-Hpn-like as baits to capture Hpn and Hpn-like and other proteins that are interacting with Hpn or Hpn-like.

H. HEPATICUS **NIKR MODULATES UREASE AND HYDROGENASE ACTIVITIES** ***VIA*** **THE** ***NIKA*** **NICKEL TRANSPORTER**

NikR plays important roles in maintaining nickel homeostasis by regulating genes involved in nickel metabolism in many bacteria. *H. hepaticus* produces the Ni-enzymes urease, hydrogenase, has a NikR, a NikABCDE and a CznABC homologue (11). But *H. hepaticus* lacks the other Ni-binding proteins such as FecA3, FrpB4, Hpn, Hpn-like and HspA. Also *H. hepaticus* is not a gastric pathogen (11). Therefore, need for nickel-activated urease might be different that for *H. pylori*. Collectively this led us to speculate that nickel metabolism/regulation might be unique or different in *H. hepaticus.*

A deletion mutant in *H. hepaticus* NikR was made and I have determined the some of the phenotypes of this strain (described in chapter 3). I determined that NikR modulates Ni-enzyme activities but not expression via regulating the nickel transporter NikABDE. We also determined that NikR is self-regulated and represses NikA in a nickel dependent manner by binding upstream of NikA. Interestingly, we also determined that NikR plays other roles in *H. hepaticus* as growth is affected in a NikR strain. Furthermore, the periplasmic nitrate reductase is negatively regulated by NikR. The NikR mutant strain has higher activities than the wild-type strain with nitrate or oxygen as electron acceptor. This gives novel evidence for the involvement of *H. hepaticus* NikR in anaerobic respiration.

NikRs have been extensively studied with respect to their nickel binding affinities, nickel binding residues, structure, DNA binding domains, etc in other bacteria. My work on NikR has

only explored the tip of the iceberg with the preliminary characterization of the phenotype of a NikR mutant. It will of interest to map the NikR regulon in *H. hepaticus* which will give a better indication of the role of NikR other than in nickel metabolism using *in silico* techniques. Also, it will be of interest to identify NikR binding sites and elucidate the structure of NikR.

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